Functional characterization of Angptl4 protein

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Thesis

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To my mother

Contents

Chapter 1

Introduction

Nutrition and genes importantly contribute to the development of numerous diseases, including diabetes, dyslipidemia and coronary artery disease. Recently, a new field emerged aimed at investigating the interaction between nutrition and genes: Nutrigenomics. Nutrigenomics research studies the link between nutrition and health using genomics tools. Within our group we have embraced nutrigenomics as an important experimental approach to better understand the effects of dietary fatty acids. Past and current research within the group has illustrated the vast effects of dietary fatty acids on gene expression and has demonstrated a major role for the Peroxisome Proliferator Activated Receptors (PPARs) in mediating the effects of fatty acids on gene expression. One of the genes that emerged from these studies encodes the Angiopoietin like protein 4. We hypothesize that induction of Angptl4 may account for some of the molecular effects of dietary fatty acids.(Figure 1.1). PPARs are nuclear receptors that bind to DNA as a heterodimer with the

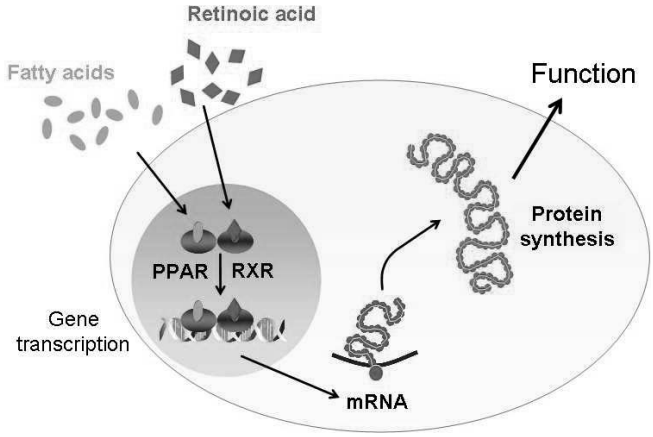

Figure 1.1: *Activation of PPAR target genes. Fatty acids enter the cell and the nucleus to bind PPAR. 9 cis retinoic acid binds RXR. The heterodimer binds to response elements (PPRE) in PPAR target genes. Induction of target gene transcription is followed by enhanced protein synthesis. Adapted from Arner P. Diabetes Obes Metab 2001; 3 (Suppl 1):S11-S19.*

retinoid X receptor (RXR). They are able to respond to both synthetic and endogenous ligands, including fatty acids and various fatty acid-derived compounds. Three different PPARs can be distinguished: PPARα, PPARβ/δ and PPARγ. In response to binding of ligand, PPARs activate the transcription of numerous genes involved in various pathways of lipid metabolism. As indicated above, one of the genes that is highly induced by fatty acids and by PPARs in a variety of tissues encodes the secreted protein Angptl4. In this thesis we pursued a variety of approaches to better characterize the biological role and regulation of Angptl4.

1.1 Angiopoietin like protein 4.

Angptl4 is a secreted protein of about 50 KDa, which belongs to the family of fibrinogen/angiopoietin like protein. Angptl4 is closely related to Angptl3 (Figure 1.2).

Similar to other angiopoietins and Angiopoietin like proteins, Angptl4 can be divided into distinct regions including a N-terminal signal sequence, a unique sequence, a coiled-coil domain and a large fibrinogen/angiopoietin-like domain. Angptl4 is cleaved, most likely via specific proprotein convertases, to release a N-terminal and C-terminal fragment. Full length Angptl4 can thus be considered a prohormone that serves as precursor for two distinct biological molecules. Recent studies suggest that the C-terminal Angptl4 fragment is important for governing angiogenic function (Yang et al. 2008). Indeed, other members of the angipoietin family such as Angiopoietin 1 and 2 have been described to have angiogenic properties mediated by binding to the Tie2 receptor. However, Angptl4 does not bind to Tie1 or Tie2 receptors. In analogy with Angptl3, which bind to αVβ3 integrin, Angptl4 may be hypothesized to bind certain integrins. However, this thesis is focused on the lipid related function of Angptl4.

1.2 LPL and lipoproteins

Fatty acids can circulate in the bloodstream as free fatty acid bound to albumin or as triglycerides packaged within lipoproteins. Lipoproteins are particles that contain both proteins and lipids and display variable densities depending on their lipid composition. Lipoproteins are classified from larger and less dense, to smaller and more dense lipoproteins: chylomicrons (CM), very-low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), and high density lipoprotein (HDL). The larger lipoproteins have higher TG content and a higher ratio of TG/proteins compared to the smaller lipoproteins. CMs are synthesized in the small intestine and contain dietary triglycerides. They are secreted into intestinal lymph vessels which eventually drain into the blood circulation. Once the CM enters into plasma, the triglycerides are hydrolyzed to release fatty acids, which are taken up by adipose tissue, heart and skeletal muscle, while the remnants of CM particles are taken up by the liver. In contrast to CM, VLDL particles contain endogenous triglycerides and are synthesized in liver. Hydrolysis of its TG and phospho-

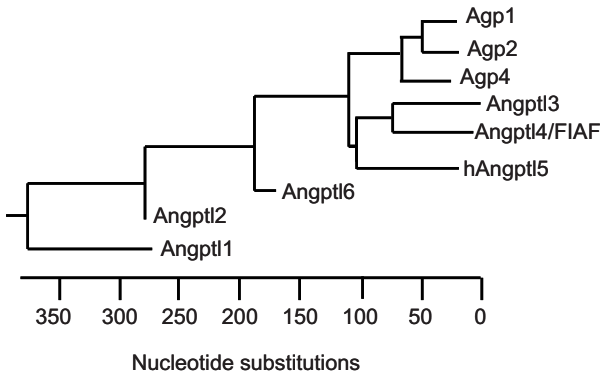

Figure 1.2: *Phylogenetic tree of angiopoietin and angiopoietin-like family of proteins. Adapted from Ge et al. JLR 46: 1484-1490, 2005.*

lipids allow the successive formation of IDL, LDL and indirectly HDL (Figure 1.3). Lipoprotein lipase (LPL) is a member of triglyceride lipase gene family, which also includes hepatic lipase (HL), pancreatic lipase (PL) and the recently discovered endothelial lipase (EL). LPL is a key regulator enzyme in the clearance of the TG rich chylomicrons (CM) and very-low density lipoprotein (VLDL). In Chapter 2 we review regulation of LPL activity by the recently discovered proteins Angptl3, Angptl4 and GPIHBP1. Beside the LPL protein, several other proteins play an important function in lipoprotein metabolism. Enterocytes in the small intestine synthesize apoB48, which undergoes lipidation by the microsomal TG transfer protein (MTP) within the endoplasmic reticulum. Via transport through the Golgi the small lipidated apoB48 particle undergoes further modifications to become a prechylomicron and subsequently a chylomicon, followed by secretion via exocytosis. In the lymph and bloodstream, CM contains apoB48, apoAI and apoAIV. Through a series of apolipoprotein exchanges with HDL, chylomicrons acquire apoCI, apoCII, apoCIII and apoE. Moreover, CM can exchange TG and cholesteryl ester (CE) via the cholesteryl ester transfer protein (CETP), and phospholipids via the phospholipid transfer protein (PLTP). The liver is a key organ in lipoprotein metabolism. ApoE recognition by LDL receptor (LDLr) and LDLr-related protein (LRP) mediate uptake of chylomicron remnants. To allow lipid transport from liver to the periphery, hepatocytes synthesize VLDL via a mechanism comparable to intestinal CM synthesis, with apoB100 replacing apoB48. Once secreted into the blood circulation, VLDL is enriched with apolipoprotein analogous to CM. Within the capillaries of skeletal muscle, heart and adipose tissue, the TG-rich lipoproteins are hydrolyzed via the action of lipoprotein lipase, which is attached to the vascular endothelium. LPL mediates peripheral tissues FA uptake and promotes VLDL remnant formation, leading to formation of IDL and ultimately LDL. LDL is taken up by the liver via the LDL receptor, which is subject to regulation via the SREBP pathways. High plasma LDL

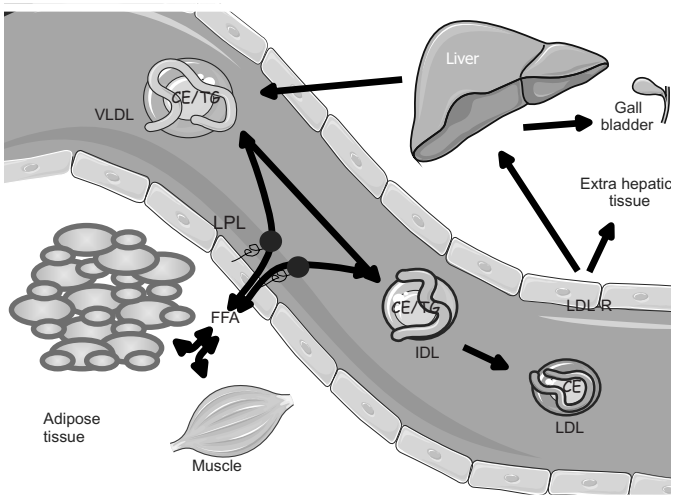

Figure 1.3: *LPL role in lipoprotein clearance. Represents the clearance of TG-rich lipoprotein by LPL, leading to fatty acid uptake and cholesterol elimination.*

levels have been shown to promote coronary heart disease as LDL undergoes oxidative modifications that render it a better ligand for uptake into macrophages, which is mediated by scavenger receptors. In contrast, high plasma HDL levels have been shown to be protective against coronary heart disease. Synthesis of HDL occurs via the lipidation of the apolipoprotein apoAI by ATP binding cassette transporter A1 (ABCA1). Esterification of incoming cholesterol is catalyzed by lecithin cholesterol acyltransferase (LCAT) to yield a mature HDL particle. As indicated above, numerous exhanges occur between HDL and other lipoproteins, which are partially mediated by CETP. HDL-cholesterol finally ends up in the liver, partially via exchange with LDL and partially via SR-B1. By returning cholesterol from macrophages and other peripheral cells to the liver, HDL is believed to exert an anti-atherogenic effect.

1.3 Small intestine, key organ in fatty acid absorption

The first tissue to be exposed to dietary constituents is the small intestine. The gastrointestinal track is the key organ in charge of proper nutrient digestion and fat absorption. Its central role in food absorption and nutrient distribution requires an elaborate vascular network that includes lymph and blood vessels. Within the small intestine another organ has been described to be crucial for nutrient absorption, which is the gut microbiota. The gut microbiota is composed of trillions

of bacteria that colonize the infant's gut throughout birth. Without microbiota, the human body would be unable to utilize the undigested carbohydrates. Recent research has implicated the gut microbiota in body weight control and energy homeostasis (Ley et al. 2006, Turnbaugh et al. 2006, Backhed, Manchester, Semenkovich and Gordon 2007, Backhed et al. 2004, Bäckhed et al. 2005, Ley et al. 2005). Germ-free mice, uncolonized by bacteria, fed a normal chow diet showed a 40% decrease in weight-gain (Backhed et al. 2004), compared to germ-free mice colonized with gut microbiota from conventional mice. Obesity has been described to be associated with a state of chronic low grade inflammation which has been suggested to be related to macrophage infiltration into adipose tissue (Weisberg et al. 2003). Especially increased visceral fat has been associated with elevated circulating inflammatory cytokines, such as C reactive protein (CRP) and serum amyloid A (SAA) (van Dielen et al. 2001). Alternatively, it has been suggested that low grade inflammation associated with obesity may be related to changes in intestinal microbiota. Evidence has been provided that gut permeability is increased in mice fed high fat diet (Cani et al. 2008), leading to enhanced entry of LPS into the circulation. Angptl4 is expressed in both adipose tissue and small intestine, and expression of small intestine in subject to regulation by the intestinal microbiota. Accordingly, a modulating role for Angptl4 in obesity-induced chronic inflammation could be hypothesized. The role of Angptl4 in high fat diet-induced inflammation is explored in this thesis.

1.4 Outline of this thesis

This thesis aims to better characterize the role of Angptl4 in the regulation of lipid metabolism. The data collected thus far in mice indicate that Angptl4 inhibits clearance of plasma triglycerides and stimulates adipose tissue lipolysis. In chapter 2 we provide an overview of the current knowledge on Angptl4 and the related proteins Angptl3 and GPIHBP-1. In chapter 3 we took advantage of the sensitivity of Angptl4-transgenic mice to fasting to better characterize the metabolic function of Angptl4. The data underscore the important role of Angptl4 in governing lipid metabolism, especially during fasting. Furthermore we report the development of an ELISA for quantitative measurement of Angptl4 in human plasma (Chapter 4). We show that plasma Angptl4 levels are increased by fasting, caloric restriction, and exercise, which is likely mediated by elevated plasma FFAs. Chapter 5 is aimed at studying the functional involvement of Angptl4 in a feedback loop that minimizes large deviations in cardiac fatty acid uptake and thereby protects against lipid overload. Finally, former studies in our lab have pointed towards a possible role of Angptl4 in the small intestine during high fat feeding. Accordingly, we have studied the effect of high fat diet in mice lacking Angptl4, revealing that Angptl4 protects against the pro-inflammatory effects of high fat feeding (Chapter 6).

Chapter 2

LiPoLysis revisited

Changes in the plasma level of lipoproteins are known to impact atherosclerosis and associated coronary heart disease (CHD). While elevated plasma low-density lipoprotein (LDL) levels increase the risk for CHD, high levels of high-density lipoprotein (HDL) levels are considered to be protective. In addition to high LDL, high plasma triglycerides (TG) are recognized as an important, independent risk factor for CHD. TG are present in blood plasma mainly in the form of chylomicrons and very low density lipoproteins (VLDL). Levels of TG in the plasma are thus determined by the balance between the rate of production of chylomicrons and VLDL in intestine and liver, respectively, and their rate of clearance in skeletal muscle, heart and adipose tissue. Clearance of TG-rich lipoproteins is mediated by the enzyme lipoprotein lipase (LPL), which is tethered to the capillary endothelium via heparin sulphate proteoglycans. LPL is a triglyceride lipase that belongs to the same lipase family as hepatic lipase (HL), pancreatic lipase (PL), and endothelial lipase (EL). It contains a number of distinct domains important for its lipolytic function including a 27-amino-acid signal peptide, a catalytic center and a C-terminal heparin binding domain (Merkel et al. 2002, Sendak and Bensadoun 1998). LPL is produced by a variety of cell types including adipocytes, (cardio)myocytes and macrophages, and is secreted towards the vascular endothelium. Maturation of nascent LPL occurs the endoplasmic reticulum and is dependent of the lipase maturation factor 1 (Pterfy et al. 2007). The enzyme is catalytically active as a dimer composed of two glycosylated 55 kDa subunits connected in a head-to-tail fashion by noncovalent interactions (Zhang et al. 2005). Consistent with the fat storage function of adipose tissue, expression of LPL in adipose tissue is highest after a meal, whereas skeletal muscle LPL expression is increased by fasting and exercise training. Expression of LPL is under control of a number of ligand-activated transcription factors. In adipose tissue and macrophages, LPL is upregulated by the peroxisome proliferators activated receptor (PPAR)γ (Schoonjans et al. 1996, Li et al. 2002), whereas liver LPL is stimulated by Liver X receptor (LXR)α and PPARα (Zhang et al. 2001, Schoonjans et al. 1996). Besides at the level of gene transcription, LPL is extensively regulated at the level of enzyme activity (Merkel et al. 2002). Several modulators of LPL activity are known, including apolipoproteins APOC3, APOC2, APOC1, and APOA5. APOC2 stimulates LPL activity by serving as a cofactor via interaction with the N-terminal part of LPL. Similarly, APOA5 stimulates LPL activity, whereas APOC3 inhibits LPL. In the past few years two proteins belonging to the family of angiopoietin-like proteins (ANGPTLs), ANGPTL3 and ANGPTL4,

have emerged as novel modulators of LPL activity. Combined with the discovery of GPIHBP1 as likely LPL anchor, these findings have led to a readjustment of the mechanism of LPL function. This review provides an overview of our current understanding of the role and regulation of ANGPTL3, ANGPTL4 and GPIHBP1, and places the newly acquired knowledge in the context of the established function and mechanism of LPL-mediated lipolysis.

2.1 Angiopoietin Like Protein 4

ANGPTL4 was independently identified as a new member of the angiopoietin family (HFARP: hepatic fibrinogen/angiopoietin-related protein) (Kim et al. 2000), as a novel target of PPARγ in adipose tissue (PGAR: PPARγ angiopoietin related) (Yoon et al. 2000), and as a novel PPARα target in liver (FIAF: fasting induced adipose factor) (Kersten et al. 2000, Kim et al. 2000). Similar to other Angiopoietins and Angiopoietin-like proteins, ANGPTL4 can be divided into distinct regions including a N-terminal signal sequence, a unique sequence, a coiled-coil domain and a large fibrinogen/angiopoietin-like domain. Upon secretion from HEK293 cells, ANGPTL4 is proteolytically cleaved into a N-terminal portion (nANGPTL4) and a C-terminal portion containing the fibrinogen like domain (cANGPTL4) (Figure 2.1) (Ge, Yang, Huang, Motola, Pourbahrami and Li 2004, Yang et al. 2008). Cleavage occurs in a serum-dependent manner and appears to be mediated by proprotein convertases including PCSK3 via recognition of the amino acid sequence $_{161}$RRKR$_{164}$ (Yang et al. 2008, Chomel et al. 2009). There is some uncertainty as to whether the same cleavage site is used in vivo as the N and C-terminal ANGPTL4 fragments observed in human plasma are consistent with cleavage further downstream (Mandard, Zandbergen, Tan, Escher, Patsouris, Koenig, Kleemann, Bakker, Veenman, Wahli, Muller and Kersten 2004, Xu et al. 2005). In addition to proteolytic cleavage, ANGPTL4 undergoes oligomerization partially via intermolecular disulfide bonds mediated by cysteine residues Cys^{76} and Cys^{80} and partially via hydrophobic residues (Figure 2.1) (Ge, Yang, Huang, Motola, Pourbahrami and Li 2004, Mandard, Zandbergen, Tan, Escher, Patsouris, Koenig, Kleemann, Bakker, Veenman, Wahli, Muller and Kersten 2004, Yin et al. 2009, Shan et al. 2009). Oligomer formation occurs prior to secretion, in contrast to cleavage. Once secreted, ANGPTL4 interacts with the extra-cellular matrix through heparin sulphate proteoglycans (Cazes et al. 2006). Furthermore, ANGPTL4 may physically associate with plasma lipoproteins (Mandard et al. 2006). In mouse, the highest ANGPTL4 mRNA levels are found in white and brown adipose tissue followed by ovary, liver, lung, heart and intestine (Kersten et al. 2000, Yoon et al. 2000, Zandbergen et al. 2006). In human, ANGPTL4 expression is ubiquitous with high expression in liver, adipose tissue, small intestine and heart (Zandbergen et al. 2006, Kersten et al. 2009). At the functional level, a wealth of data acquired using a variety of animal models of ANGPTL4 overexpression or deletion now support a stimulatory effect of ANGPTL4 on plasma TG levels, which is achieved by inhibiting LPL-dependent clearance of TG-rich lipoproteins (Yu et al. 2005, Koster et al. 2005, Mandard

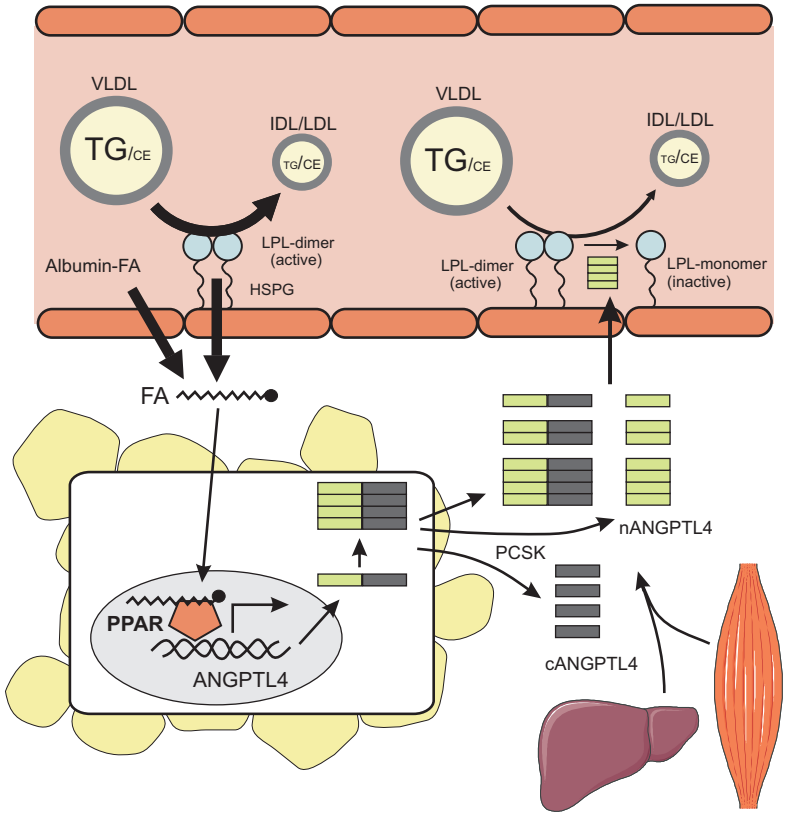

Figure 2.1: *Inhibition of LPL-mediated lipolysis by ANGPTL4. Hydrolysis of TG-rich lipoproteins generates fatty acids, which are taken up in underlying tissues including adipose tissue, skeletal muscle and heart. Fatty acids induce ANGPTL4 gene expression via PPARs. ANGPTL4 forms oligomers inside the cell, and is cleaved upon secretion into nANGPTL4 and cANGPTL4 by proprotein convertases (PCSKs). ANGPTL4 inhibits LPL activity by promoting the conversion of active LPL dimers into inactive LPL monomers.*

et al. 2006, Lichtenstein et al. 2007, Desai et al. 2007). As a consequence, uptake of fatty acids and cholesterol into tissues is decreased (Lichtenstein et al. 2007). Compelling evidence indicates that ANGPTL4 inhibits LPL by promoting the conversion from active LPL dimers to inactive LPL monomers (Figure 2.1) (Sukonina et al. 2006).

In vitro studies have suggested that ANGPTL4 enzymatically catalyzes the dimer to monomer conversion (Sukonina et al. 2006, Yau et al. 2009), whereas in vivo studies suggest that ANGPTL4 disables LPL by binding LPL monomers, thereby driving the LPL dimer-monomer equilibrium to-

ward inactive monomers (Lichtenstein et al. 2007). Inhibition of LPL activity and conversion to monomers is mediated by a short stretch of amino acids close to its N-terminus. Remarkably, a 12 AA peptide containing these 3 amino acids is already able to inhibit LPL, although less efficiently compared to nANGPTL4 and flANGPTL4 (Yau et al. 2009). According to the current view, oligomerization of nANGPTL4 and flANGPTL4 is mainly aimed at stabilizing the protein and does not influence the intrinsic ability of ANGPTL4 to inhibit LPL (Yin et al. 2009, Shan et al. 2009). Consistent with this notion, LPL can interact with several oligomeric forms of nANGPTL4 (Yau et al. 2009). In addition to LPL, there is evidence that ANGPTL4 also inhibits hepatic lipase and possibly endothelial lipase (Lichtenstein et al. 2007)(plus unpublished data). Supporting the role of ANGPTL4 as LPL inhibitor in human, carriers of a rare E40K variant of the ANGPTL4 gene exhibit elevated plasma HDL and decreased TG levels (Talmud et al. 2008, Folsom et al. 2008, Nettleton et al. 2008, Romeo et al. 2007). The E40K mutation leads to a near complete loss of ability of ANGPTL4 to inhibit LPL, which may be partially caused by destabilizing the protein (Yin et al. 2009, Shan et al. 2009). Interestingly, a recent genome-wide association study showed that a common sequence variant at a locus near the ANGPTL4 gene is associated with plasma HDL but not TG concentration (Kathiresan et al. 2009). Together, these data suggest that genetic variation within the ANGPTL4 gene contributes to the variation in plasma lipid levels within specific populations. As its alternative name fasting-induced adipose factor implies, ANGPTL4 is upregulated by fasting in adipose tissue (Kersten et al. 2000). Increased ANGPTL4 mRNA is associated with a decrease in adipose LPL activity and can be hypothesized to disfavour fat storage during fasting (Sukonina et al. 2006). Induction of ANGPTL4 by fasting, however, is not limited to adipose tissue and occurs in a variety of organs including heart and skeletal muscle (Kersten et al. 2000, Wiesner et al. 2004, Yu et al. 2005, Ge et al. 2005, Wiesner et al. 2006, Sukonina et al. 2006, Lichtenstein et al. 2007). In humans, plasma ANGPTL4 levels are increased by fasting and chronic caloric restriction, which is mediated by increased free fatty acid levels (Kersten et al. 2009). Indeed, plasma ANGPTL4 levels in humans are correlated with fasting plasma free fatty acids (Staiger et al. 2009). Since ANGPTL4 is also known to stimulate adipose tissue lipolysis (Mandard et al. 2006), induction of ANGPTL4 during fasting may be aimed at shifting fuel use from lipoprotein-derived fatty acids towards circulating free fatty acids, and thus promote use of stored fat. The fasting- and fatty acid-induced increase in ANGPTL4 is mediated by PPARs, which transcriptionally activate ANGPTL4 expression via a set of PPAR-response elements in intron 3 of the ANGPTL4 gene (Figure 2.1)(Mandard et al. 2004, Heinaniemi et al. 2007). Supporting regulation by PPARs, synthetic agonists for PPARα and PPARγ raise plasma ANGPTL4 levels in human subjects (Xu et al. 2005, Kersten et al. 2009). In mice, agonists for all three PPARs upregulate ANGPTL4 expression in liver, skeletal muscle, heart, WAT and intestine (Kersten et al. 2000, Yoon et al. 2000, Mandard et al. 2004, Yoshida et al. 2004, Ge et al. 2005, Dutton et al. 2008, Rieck et al. 2008, Gealekman et al. 2008, He et al. 2008, Bunger et al. 2007). In addition to fasting, in heart ANGPTL4 mRNA is also induced following

an oral fat load, which is mediated by PPARβ/δ . Induction of cardiac ANGPTL4 by dietary fatty acids is part of a feedback mechanism aimed at protecting the heart against lipid overload and consequently lipotoxicity (Georgiadi et al. Submitted). The dramatic induction of ANGPTL4 by PPARβ/δ agonist and by fatty acids have led to the use of ANGPTL4 as PPARβ/δ and fatty acid marker gene (He et al. 2008, Schug et al. 2007, Staiger et al. 2008),(ANGPTL4/FIAF as marker for PPARδ modulation, European Patent Application EP1586902). Finally, via inhibition of LPL activity ANGPTL4 has been suggested to serve as critical mediator of the effect of the intestinal microbiota on bodyweight (Backhed et al. 2004). Apart from its role in lipid metabolism, several studies have linked ANGPTL4 to processes such as angiogenesis, cell migration, endothelial cell function, vascular leakage and cell adhesion (Zhu et al. 2002, Ito et al. 2003, Ge et al. 2005, Cazes et al. 2006, Murata et al. 2009, Yang et al. 2008, Gealekman et al. 2008, Galaup et al. 2006, Hermann et al. 2005, Padua et al. 2008). Consistent with a role in angiogenesis, expression of ANGPTL4 is highly induced by hypoxia in cardiomyocytes, brain, endothelial cells, and adipocytes (Le Jan et al. 2003, Belanger et al. 2002, Wiesner et al. 2006, Gustavsson et al. 2007, Wang et al. 2007). These functions are likely conferred by cANGPTL4, which functions independently of nANGPTL4. Thus, flANGPTL4 may be considered a prohormone whose pleiotropic actions are mediated by nANGPTL4 and cANGPTL4. Finally, in relation to atherosclerosis, ANGPTL4 deletion was shown to exert a protective influence in the APOE KO model (Adachi et al. 2009). Whether this effect is mediated via changes in lipid metabolism, or via other pleiotropic activities of ANGPTL4 remains to be determined.

2.2 Angiopoietin Like Protein 3

ANGPTL3 was discovered while searching EST databases for signal sequences and amphipathic helices (Conklin et al. 1999). Structurally and functionally, ANGPTL3 is highly similar to ANGPTL4. ANGPTL3 shares the modular structure of ANGPTL4 consisting of a signal sequence, a small unique region, a coiled-coil domain and a large fibrinogen/angiopoietin-like domain. Similar to ANGPTL4, ANGPTL3 is proteolytically cleaved by proprotein convertases via recognition of the amino acid sequence $_{221}$RAPR$_{224}$ to yield a N-terminal and C-terminal ANGPTL3 fragment (Figure 2.2) (Ono et al. 2003, Jin et al. 2007). However, in contrast to ANGPTL4, ANGPTL3 is expressed almost exclusively in the liver, with minor expression found in kidney (Zandbergen et al. 2006).

The plasma lipid modulating properties of ANGPTL3 were discovered via positional cloning using KK/San mice, which represent a mutant strain of KK obese mice with markedly lower plasma free fatty acid and TG levels (Koishi et al. 2002). Induction of plasma TG by ANGPTL3 was corroborated using a variety of animal models of ANGPTL3 overexpression or deletion, and is achieved by inhibiting clearance of TG-rich lipoproteins (Shimizugawa et al. 2002, Koster et al. 2005, Fujimoto et al. 2006). Similar to ANGPTL4, ANGPTL3 inhibits LPL activity, al-

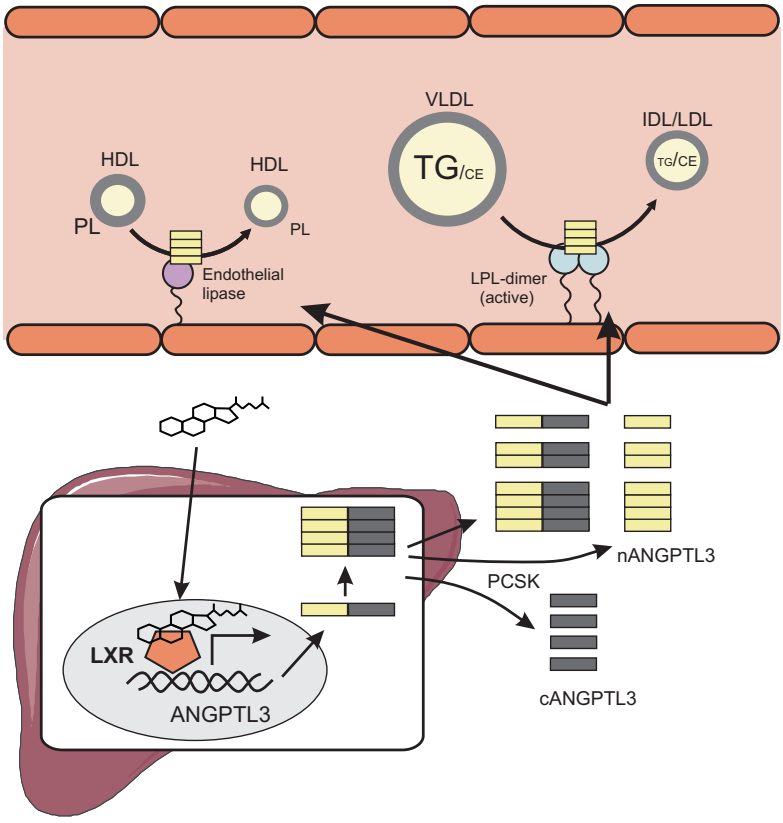

Figure 2.2: *Inhibition of LPL-mediated lipolysis by ANGPTL3. Oxysterols induce ANGPTL3 gene expression in liver via LXRs. ANGPTL3 forms oligomers inside the cell, and is cleaved upon secretion into nANGPTL3 and cANGPTL3 by proprotein convertases (PCSKs). ANGPTL3 inhibits LPL activity via a mechanism that likely differs from ANGPTL4. ANGPTL3 also inhibits endothelial lipase (EL) and possibly hepatic lipase.*

though presumably via different mechanisms. Whereas ANGPTL4 catalyzes LPL inactivation, ANGPTL3 suppresses LPL catalytic activity in the presence of substrates (Figure 2.2) (Shan et al. 2009), which might explain its 100-fold lower potency towards LPL (Sonnenburg et al. 2009). Supporting different mechanisms of action, heparin can rescue LPL from inhibition by ANGPTL3 but not ANGPTL4. Analogous to ANGPTL4, cleavage of ANGPTL3 does not impact the intrinsic ability of ANGPTL3 to inhibit LPL as nANGPTL3 and flANGPTL3 are equally effective towards inhibiting LPL activity in vitro (Ono et al. 2003). However, cleavage appears to be important for

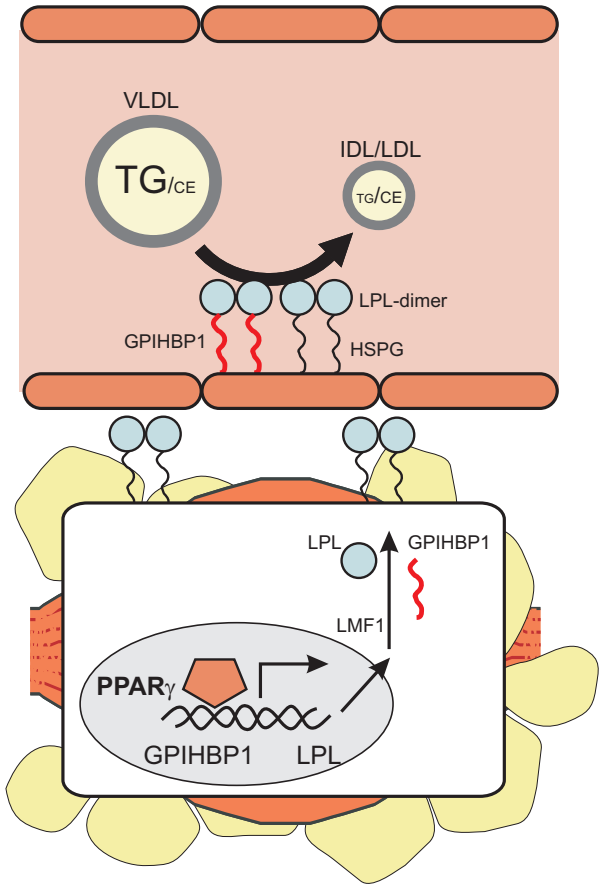

Figure 2.3: *GPIHBP1 represents a high affinity anchor system for LPL. GPIHBP1 is expressed in the same tissues that express LPL and similar to LPL is under control of PPARγ. Processing of LPL in the endoplasmic reticulum requires lipase maturation factor 1 (LMF1). GPIHBP1 is tethered into the capillary endothelium and is capable of binding chylomicrons and LPL. GPIHBP1 may represent a system for high affinity and stable attachment of LPL to the endothelium, as opposed to the lower affinity attachment of LPL via heparin sulphate proteoglycans (HSPG).*

activating ANGPTL3 in vivo (Ono et al. 2003).

Besides LPL, ANGPTL3 also inhibits the activity of EL and HL (Figure 2.2) (Shimizugawa et al. 2002, Fujimoto et al. 2006, Shimamura et al. 2007). Supposedly, nANGPTL3 is a more effective EL inhibitor compared to flANGPTL3 (Jin et al. 2007). ANGPTL3 has been suggested to

mediate the effect of proprotein convertases on EL activity and plasma HDL levels (Jin et al. 2007). As nANGPTL3 also inhibits LPL, this mechanism should impact plasma TG as well, although no data are available. Currently, the relative importance of the various lipases in explaining the effect of ANGPTL3 on plasma lipoprotein levels remains unclear. In analogy with ANGPTL4, ANGPTL3 stimulates adipose tissue lipolysis, thereby raising plasma free fatty acid and glycerol levels (Koishi et al. 2002, Fujimoto et al. 2006, Shimamura et al. 2007).

At the present time, the mechanism behind this effect remains unclear but must be independent of LPL inhibition. Expression of ANGPTL3 is influenced by numerous stimuli. Dramatic induction of ANGPTL3 mRNA is achieved by activation of LXR, which binds to a LXR response element in the ANGPTL3 promoter (Figure 2.2) (Kaplan et al. 2003). Accordingly, it has been suggested that induction of ANGPTL3 accounts for the stimulatory effect of LXR agonists on plasma TG levels (Inaba et al. 2003). Hepatic ANGPTL3 expression has also been shown to be under hormonal control. Both leptin and insulin down regulate ANGPTL3 mRNA expression (Shimamura et al. 2004, Inukai et al. 2004) and a similar effect is exerted by thyroid hormone mediated by the thyroid hormone receptor beta (Fugier et al. 2006). While PPARβ/δ is a potent inducer of ANGPTL4, PPARβ/δ suppresses ANGPTL3 expression, probably by competing with LXRα for RXRα (Matsusue et al. 2006). Recent genome-wide association studies have shown that a common sequence variant at a locus near the ANGPTL3 gene is associated with plasma concentration of TG, but not other lipoprotein parameters (Kathiresan et al. 2009, Willer et al. 2008). Moreover, a ANGPTL3 sequence variant (M259T) that failed to inhibit LPL activity in in vitro studies was associated with lower plasma levels of TG. This sequence variant is common among African Americans but rare among European Americans (Romeo et al. 2009). These data suggest that genetic variation within the ANGPTL3 gene contributes to variation in plasma TG levels and furthermore validate the importance of ANGPTL3 in the regulation of lipoprotein metabolism in humans. A limited number of studies have measured ANGPTL3 levels in human plasma. Plasma ANGPTL3 levels varied between 218 and 764 ng/mL (Hatsuda et al. 2007, Shoji et al. 2009, Yilmaz et al. 2009, Moon et al. 2008, Stejskal et al. 2007), which is markedly higher than values reported for plasma ANGPTL4. In one study, ANGPTL3 was inversely correlated with VLDL- and IDL-cholesterol levels, and positively with HDL-cholesterol (Shoji et al. 2009). Significant correlations have also been reported with systolic blood pressure, plasma LDL, and plasma A-FABP (Stejskal et al. 2007). Furthermore, a positive correlation was found between plasma ANGPTL3 and carotic artery intima-media thickness (Hatsuda et al. 2007). Finally, levels of ANGPTL3 were elevated in patients with non-alcoholic steatohepatitis (Yilmaz et al. 2009). In addition to its role in lipid metabolism, ANGPTL3 has been shown to stimulate endothelial cell adhesion and migration by serving as a ligand for integrin αVβ3 (Camenisch et al. 2002). Furthermore, ANGPTL3 has been shown to stimulate ex vivo expansion of hematopoietic stem cells (Zhang et al. 2006). With respect to atherosclerosis, ANGPTL3 deletion reduced hypertriglyceridemia and atherosclerosis in the apoE KO model (Ando et al. 2003). In human subjects,

plasma ANGPTL3 was positively correlated with femoral artery intima-media thickness (Hatsuda et al. 2007).

2.3 Glycosylphosphatidylinositol-Anchored HDL-Binding Protein 1

GPIHBP1 was initially identified by expression cloning in Chinese hamster ovary (CHO) cells as a GPI-anchored high-density lipoprotein (HDL)-binding protein 1 (Ioka et al. 2003). GPIHBP1 is a protein of 28 kDa that consists of a signal sequence, a highly negatively charged N-terminal domain, a short linker domain, a Ly-6 domain containing 10 cysteine residues, and a hydrophobic carboxyl-terminal region harboring the GPI-anchored motif. GPIHBP1 is secured in the plasma membrane by the GPI anchor, which can be cleaved by phosphatidylinositol-specific phospholipase C. Similar to LPL, GPIHBP1 is expressed in the heart, adipose tissue, and skeletal muscle, and is secreted to the capillary lumen of those tissues (Figure 2.3) (Beigneux et al. 2007). The protein is glycosylated at Asn-76 within the Ly-6 domain, which is important for the proper trafficking of GPIHBP1 to the cell surface (Beigneux et al. 2008). Recently, it was shown that GPIHBP1 modulates LPL-mediated clearance of TG-rich lipoproteins as revealed by the severe hypertriglyceridemia in mice lacking GPIHBP1 (Beigneux et al. 2007). Intriguingly, GPIHBP1 deficient mice exhibit milky plasma already on a low fat diet. In vitro, GPIHBP1 binds to both chylomicrons and LPL with high affinity (Kd =3.6 * 10^{-8} M). Binding is mediated by the acidic amino terminal domain within GPIHBP1 and the positively charged heparin binding domain with LPL. Recently, evidence was provided that membrane-anchored GPIHBP1 also represents an important binding site for LPL in vivo (Figure 2.3) (Weinstein et al. 2008). Binding of LPL to GPIHBP1 may render LPL more stable and diminish inhibition of LPL by ANGPTL3 and ANGPTL4 (Sonnenburg et al. 2009). Limited information is currently available about regulation of GPIHBP1 expression. Fasting was found to increase GPIHBP1 expression level in heart, BAT and WAT, while high fat feeding upregulated GPIHBP1 mRNA expression in WAT, skeletal muscle and liver (Davies et al. 2008). Surprisingly, PPARγ agonist but not PPARα or PPARβ/δ agonists increased GPIHBP1 mRNA in WAT, BAT, heart and skeletal muscle (Figure 2.3).

We observed that a single oral fat load increased GPIHBP1 expression in heart, which was independent of either PPARα or PPARβ/δ (unpublished data). Supporting the importance of GPIHBP1 in plasma triglyceride clearance in humans, two rare mutations within the GPIHBP1 gene were recently found to be associated with severe hypertriglyceridemia and chylomicronemia (Wang and Hegele 2007). The Q115P mutation gives rise to a protein that, despite being present at the cell surface, binds poorly to LPL and chylomicrons (Beigneux et al. 2009). The effect of the G56R mutation on GPIHBP1 function is not clear. Indeed, the ability of GPIHBP1 to reach the cell surface and to bind LPL, chylomicrons, or APOA5 are not affected by the mutation (Gin et al. 2007). Overall, the data suggest that GPIHBP1 serves as a high affinity anchor for LPL which may function additively to the lower affinity attachment of LPL to heparin sulphate

proteoglycans. Currently, the functional and structural distinction between these two systems requires further elaboration.

2.4 Conclusion

The recent elucidation of ANGPTL3, ANGPTL4 and GPIHBP1 as major modulators of LPL function has highlighted the complexity of the process of plasma triglyceride clearance. While ANGPTL3 and ANGPTL4 inhibit LPL, either reversibly or irreversibly, GPIHBP1 appears to function as stabilizer or even anchor of LPL, which may counteract ANGPTL3 and ANGPTL4-mediated LPL inhibition. Additional research is necessary to better define the intricacies of the interaction between LPL, ANGPTLs, and GPIHBP1. Furthermore, more work is required to better establish the overall role of ANGPTLs and GPIHBP1 in the physiological regulation of fuel selection as well as its impact on cellular metabolism. Overall, the discovery and functional characterization of ANGPTL3, ANGPTL4 and GPIHBP1 has greatly improved our understanding of the regulation of LPL function.

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Chapter 3

Angptl4 up-regulates cholesterol synthesis in liver via inhibition of LPL- and HL-dependent hepatic cholesterol uptake

Abstract

Dysregulation of plasma lipoprotein levels may increase the risk for atherosclerosis. Recently, Angiopoietin-like proteins 3 and 4 were uncovered as novel modulators of plasma lipoprotein metabolism. Here we take advantage of the fasting-dependent phenotype of Angptl4-transgenic (Angptl4-Tg) mice to better characterize the metabolic function of Angptl4. In 24h fasted mice, Angptl4 over-expression caused a 24-fold increase in plasma triglycerides (TG), which was attributable to elevated VLDL-, IDL/LDL- and HDL-TG content. Angptl4 over-expression decreased post-heparin LPL activity by stimulating conversion of endothelial-bound LPL dimers to circulating LPL monomers. In fasted but not fed state, Angptl4 over-expression severely impaired LPL-dependent plasma TG and cholesteryl ester clearance and subsequent uptake of fatty acids and cholesterol into tissues. Consequently, hepatic cholesterol content was significantly decreased, leading to universal up-regulation of cholesterol and fatty acid synthesis pathways. The hypertriglyceridemic effect of Angptl4 is attributable to inhibition of LPL-dependent VLDL lipolysis by converting LPL dimers to monomers, and Angptl4 up-regulates cholesterol synthesis in liver secondary to inhibition of LPL- and HL-dependent hepatic cholesterol uptake.

3.1 Introduction

The enzyme lipoprotein lipase (LPL) plays a pivotal role in the regulation of plasma TG levels. By catalyzing the irreversible hydrolysis of plasma TG, LPL promotes the clearance of TG-rich lipoproteins from plasma. Synthesized mainly by adipocytes and (cardio)myocytes, LPL protein is translocated to luminal endothelial cell surfaces where it is attached via heparan sulphate proteoglycans (HSPG) (Merkel et al. 2002). Since LPL is rate-limiting for the clearance of TG-rich lipoproteins, the activity of LPL is carefully regulated via numerous mechanisms. At the level of gene transcription, LPL is upregulated by the transcription factors peroxisome proliferators activated receptor (PPAR)γ in adipose tissue and by liver X receptor (LXR)α and PPARα in liver (Schoonjans et al. 1996). In accordance with the fat storage function of adipose tissue as the major fat storage organ, expression of LPL in adipose tissue peaks in the postprandial state,

whereas in skeletal muscle expression of LPL is highest in the post-absorptive state (Olivecrona et al. 1995). LPL is also extensively regulated at the level of enzyme activity. Indeed, several modulators of LPL activity are known, including Apoc3, Apoc2, Apoc1, and Apoa5, all of which are apolipoproteins associated with specific lipoproteins (Berbée et al. 2005, van Dijk et al. 2004, Jong et al. 1999). In addition to apolipoproteins, recently it became evident that two proteins belonging to the family of angiopoietin-like proteins (Angptls), Angptl3 and Angptl4, can modulate LPL activity (Kersten 2005). In contrast to Angptl4, Angptl3 is produced exclusively in liver and is a target of LXR. It was shown that a mutation in the gene for Angptl3 is responsible for the low plasma TG of KK/Snk mice, a mutant strain of KK obese mice (Koishi et al. 2002). The stimulatory effect of Angptl3 on fasting plasma TG can be attributed to suppression of VLDL clearance via LPL inhibition (Shimizugawa et al. 2002). Angptl4, also known as fasting-induced adipose factor Fiaf, was discovered by screening for genes responsive to the PPARα and γ, which serve as the molecular targets for the hypolipidemic fibrate and the insulin sensitizing thiazolidinedione drugs, respectively (Kersten et al. 2000, Yoon et al. 2000). Angptl4 has a molecular mass of about 50 kDa, is secreted into the blood, and may act in a paracrine and endocrine fashion. In humans, Angptl4 mRNA expression is highest in liver, followed by adipose tissue, whereas in mice the order is reversed (Zandbergen et al. 2006). The abundance of Angptl4 in blood plasma is increased by fasting and decreased by high fat diet, suggesting a potential role in lipid metabolism (Kersten et al. 2000). Evidence linking Angptl4 to regulation of plasma lipoproteins was provided by the demonstration that injecting mice with recombinant Angptl4 protein significantly increased plasma TG concentrations, possibly by inhibiting LPL activity (Yoshida et al. 2002). Subsequent studies using various models of in vivo Angptl4 overexpression or inactivation have confirmed the stimulatory effect of Angptl4 on plasma TG levels via a mechanism independent of VLDL-TG secretion (Ge, Yang, Yu, Pourbahrami and Li 2004, Yu et al. 2005, Xu et al. 2005, Koster et al. 2005, Mandard et al. 2006). The hypertriglyceridemic effect of Angptl4 may be modulated by formation of higher order oligomers as well as by proteolytic processing (Ge, Yang, Yu, Pourbahrami and Li 2004). Recent in vitro studies suggest that 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). Currently, it is unclear whether this mechanism of LPL inhibition also occurs in vivo. It has been suggested that in addition to lipid metabolism, Angptl4 also governs glucose homeostasis. Adenoviral-mediated Angptl4 overexpression markedly reduced plasma glucose and improved glucose tolerance (Xu et al. 2005). However, studies with Angptl4 knock-out mice failed to reveal an effect of Angptl4 on glucose homeostasis (Koster et al. 2005). Thus, the effects of Angptl4 on glucose homeostasis remain ambiguous. A partial analysis of the phenotype of our Angptl4 transgenic mouse model has been previously published (Mandard et al. 2006). The transgenic mice were generated using the full-length mouse genomic Angptl4 sequence which contains the regulatory regions mediating responsiveness to PPARs. As a result, Angptl4 over-expression is amplified by fasting. Here we take advantage

of the sensitivity of Angptl4-Tg mice to fasting to better characterize the metabolic function of Angptl4 and address: (1) the in vivo molecular mechanisms underlying its hypertriglyceridemic effect, (2) the consequence of LPL-inhibition for hepatic cholesterol metabolism and cholesterol-dependent hepatic gene regulation, and (3) the effect of Angptl4 on glucose homeostasis.

3.2 Materials and Methods

Animal experiments. The Angptl4-transgenic mice on FVB background have been previously described (Mandard et al. 2006). Only male mice were used. Animals were kept in standard cages with food and water ad libitum. Animal were fed normal laboratory chow (RMH-B 2181, Arie Blok animal feed, Woerden, the Netherlands), containing 23.5% protein, 5% fat, 38.3% starch, and 4% sugar. Mice in fed state were sacrificed at the beginning of the light cycle. Mice in fasted state were deprived of food for 24 hours starting at the beginning of the light cycle. At the time of sacrifice animals were between 2 and 4 months of age. Blood was collected via orbital puncture into EDTA tubes. After sacrificing the mice by cervical dislocation, tissues were excised and immediately frozen in liquid nitrogen. All animal experiments were approved by the animal experimentation committee of Wageningen University.

Plasma metabolites. Blood was collected into paraoxon-coated capillaries to prevent ongoing in vitro lipolysis. Blood samples were placed on ice and centrifuged at $4°C$ for 10 minutes at 10,000 g. The plasma glucose concentration was determined using a kit from Elitech (Sopachem, Wageningen, Netherlands). Plasma and tissue TG and plasma glycerol concentrations were determined using kits from Instruchemie (Delfzijl, Netherlands). Plasma free fatty acids were determined using a kit from WAKO Chemicals (Sopachem, Wageningen, Netherlands). Plasma ketones (β-hydroxybutyrate) were determined using Precision Xtra (Abbott laboratories, Bedford, MA, USA). Plasma lathosterol was determined by capillary gas chromatography. Plasma insulin was determined using a kit from Linco (St. Charles, MO, USA).

Liver cholesterol. Liver cholesterol content was determined as previously described (Thompson and Merola 1993). Briefly, a ~100 mg liver samples was saponified by heating in ethanol-KOH, and cholesterol was extracted from the saponified solution with hexane. After evaporation of hexane, cholesterol was derivatized to trimethylesters using bis-silyl-tri-fluor-acetamide. Cholesterol was subsequently quantified by gas chromatography.

Lipoprotein profiling. Plasma lipoproteins of wild-type and Angptl4-Tg mice were separated using fast protein liquid chromatography (FPLC). 0.2 mL of pooled plasma was injected into a Superose 6B 10/300 column (GE Healthcare Bio-Sciences AB, Roosendaal, Netherlands) and eluted at a constant flow of 0.5 mL/minute with phosphate buffered saline (pH 7.4). The effluent was collected in 0.5 mL fractions and TG and cholesterol levels were determined (Instruchemie). Separation of lipoproteins by gel permeation HPLC was performed as a lipoprotein analysis service by LipoSEARCH (Tokyo, Japan) using 10μL of pooled plasma.

Modulated plasma LPL and HL activity assay. 24 hour fasted mice were injected via the tail vein with heparin (0.1U/g; Leo Pharmaceutical Products B.V., Weesp, Netherlands) and blood was collected after 10 min. The plasma was snap-frozen and stored at -80°*C* until analysis of LPL and HL activity as previously described (Duivenvoorden et al. 2005).

In vitro LPL activity assay. The effect of recombinant hAngptl4 (residues 26-229, hAngptl4 S2) (Biovendor Laboratory Medicine, Modrice, Czech Republic) on LPL-dependent TG Hydrolysis of [^{3}H]TO-VLDL-like emulsion particles was determined as described (solubilised LPL assay)(Berbée et al. 2005). A HSPG-bound LPL assay was performed by first coating plates with HSPG (250ng/well), followed by coating with LPL (0.2U/well). HSPG-bound LPL was subsequently pre-incubated with recombinant hAngptl4 for 30 min at 37°*C*, washed three times with 100 mM Tris-HCl pH 8.5, followed by addition of [^{3}H]TO-VLDL-like emulsion particles. After 15 minutes incubation, the reaction was terminated and lipids extracted as described (Herrmann et al. 1995). The amount of [^{3}H]FFA released was measured by scintillation counting and expressed as a percentage of [^{3}H]FFA released in the absence of Angptl4.

Heparin-Sepharose Chromatography. Heparin-sepharose chromatography was carried out on a Pharmacia FPLC system. A HiTrap Heparin HP 1 mL column (GE Healthcare Bio-Sciences AB, Roosendaal, Netherlands) was equilibrated with 0.25M NaCl/20% glycerol/1% BSA/10mM sodium pH 6.5. Pre- and post-heparin plasma was adjusted to the concentration of the equilibration buffer before loading. After loading the equivalent of 0.2 ml mouse blood plasma, the column was washed (10ml), followed by elution with a linear gradient of NaCl (from 0.25 to 1.5 M) in equilibration buffer at a flow rate of 0.5 ml/minute. BSA was omitted when fractions were intended for analysis by western blot. Murine LPL in fractions was measured by enzyme-linked immunosorbent assay (van Vlijmen et al. 1999).

Isolation of total RNA and Q-PCR. Extraction of RNA, cDNA synthesis and quantitative PCR were carried out as previously described (Mandard et al. 2006).

Western blot. Fractions collected of the HiTrap HP-column or Superose 6B column were separated by SDS-PAGE and transferred to Immobilon-P membrane (Millipore, Bedford, MA, USA). Western blotting was carried out as described previously (Kersten et al. 2000, Mandard et al. 2004). The primary antibody (anti-mouse LPL CA1787, Cell Applications Inc., San Diego, CA, USA) was used at a dilution of 1:500, and the secondary antibody (HRP-labelled anti-rabbit IgG, Sigma) was used at a dilution of 1:4000. All incubations were performed in Tris-buffered saline, pH 7.5, with 0.1% Tween-20 and 5% dry milk, except for the final washing when dry milk was omitted.

Preparation of VLDL-like Emulsion Particles. TG-rich VLDL-like emulsion particles (80 nm) were prepared as described (Rensen et al. 1997). Radiolabeled emulsions were obtained by adding 200 μCi of glycerol tri[^{3}H]oleate (triolein, TO) and 20 μCi of [^{14}C]cholesteryl oleate (CO) to 100 mg of emulsion lipids before sonication.

In Vivo Clearance of VLDL-Like Emulsion Particles. Mice were anesthetized with hypnorm

(fentanyl)/midazolam and injected with the radiolabeled emulsion particles (1.0 mg TG in 200 μl PBS) at 8:00 a.m. into the tail vein. At indicated time points after injection, blood was taken from the tail vein, to determine the serum decay of [^{3}H]TO and [^{14}C]CO by scintillation counting (Packard Instruments, Dowers Grove, IL, USA). At 30 min after injection, mice were sacrified by cervical dislocation and tissues were collected. Tissues were weighed and dissolved overnight in Solvable (Packard Bioscience, Meriden, CT, USA), followed by measurement of ^{3}H- and ^{14}C-activities in Ultima Gold (Packard Bioscience). The total plasma volumes of the mice were calculated from the equation V (ml) = 0.04706 x body weight (g), as determined from ^{125}I-BSA clearance studies as previously described (Jong et al. 1999).

Microarray. RNA was prepared from livers of 24h fasted wild-type and Angptl4-Tg mice using Trizol and subsequently pooled per group (n=7). Pooled RNA was further purified using Qiagen RNeasy columns and the quality verified by lab on a chip analysis (Bioanalyzer 2100, Agilent, Amsterdam, Netherlands). 10 μg of RNA was used for one cycle of cRNA synthesis (Affymetrix, Santa Clara, USA). Hybridization, washing and scanning of Affymetrix Genechip mouse genome 430 2.0 arrays was according to standard Affymetrix protocols. Fluorimetric data were processed by Affymetrix GeneChip Operating software and the gene chips were globally scaled to all the probe sets with an identical target intensity value using MAS5.0 algorithm. Further analysis was performed by Data Mining Tool (Affymetrix). Only probe sets labeled present in both wild-type and Angptl4-Tg mice were considered.

Hyperinsulinemic euglycemic clamp analysis. Wild-type and Angptl4-Tg mice were fasted for 24 hours prior to the clamp studies. The hyperinsulinemic euglycemic clamp and assays for blood glucose and plasma insulin were carried out as previously described (Voshol et al. 2003).

3.3 Results

The Angptl4 transgenic (Angptl4-Tg) mice used in this manuscript have been previously described (Mandard et al. 2006). While in the fed state Angptl4-Tg mice show only mildly increased Angptl4 expression in skeletal muscle, heart, white adipose tissue (WAT) and brown adipose tissue (BAT), in the 24h fasted state dramatic Angptl4 over-expression is observed in all tissues examined, including liver (Figure 3.1).

To examine the effect of Angptl4 over-expression on lipid metabolism, several plasma parameters were determined. While Angptl4 over-expression increased plasma levels of FFA, glycerol, and TG already in the fed state, the increase was much more pronounced in the 24h fasted state (Figure 3.2). During the course of fasting plasma TG levels gradually decreased in WT mice, whereas plasma TG showed a marked increase in the Angptl4-Tg mice, leading to an almost 24-fold difference after 24h of fasting (Figure 3.3a). The severe hypertriglyceridemia in fasted Angptl4-Tg was mainly because of dramatically elevated plasma VLDL levels, as determined by FPLC, although IDL/LDL levels were also increased to some extent (Figure 3.3b).

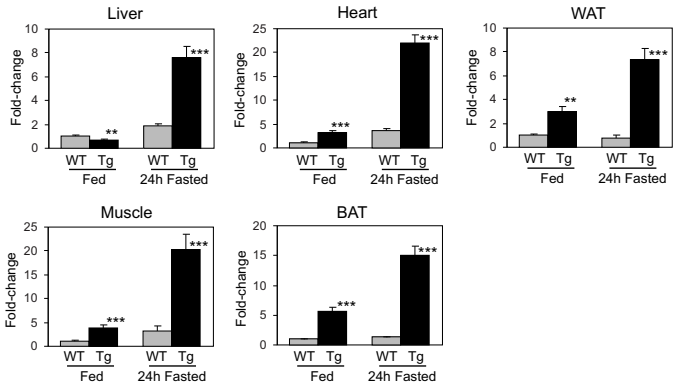


Figure 3.1: *Marked over-expression of Angptl4 mRNA in 24h fasted Angptl4- Tg mice. Angptl4 mRNA expression in liver, heart, white adipose tissue (WAT), skeletal muscle, and brown adipose tissue (BAT) of fed and 24h fasted WT (grey bars) and Angptl4- Tg (black bars) mice. Errors bars represent SEM. n=5-10 mice/group. Statistical significance according to Students t test. **, p<0.01; * * *, p<0.001.*

	FFA (mM)	Glycerol (mM)	Ketones (mM)	TG (mM)
Fed				
Wild-type	0.40±0.02	0.24±0.01	n.d.	1.79±0.15
Angptl4-Tg	0.57±0.05**	0.33±0.04*	n.d.	3.36±0.64**
Fast 24h				
Wild-type	0.58±0.04	0.31±0.02	1.20±0.07	0.74±0.07
Angptl4-Tg	1.32±0.09**	0.99±0.09**	1.38±0.06*	17.58±2.22**

Figure 3.2: *Plasma parameters. FFA, glycerol, ketones bodies and TG concentrations were determined in EDTA plasma of fed and 24h fasted WT and Angptl4-Tg mice. Values are mean±SEM. n=7-10 mice/group. Statistical significance according to Students t test. *, p<0.05; **, p<0.01.*

No difference in HDL-cholesterol levels was observed (Figure 3.3b). Detailed lipoprotein profiling was performed by gel permeation HPLC, which allows for better separation between the lipoprotein classes (Figure 3.4). In addition to VLDL, other lipoprotein fractions were also markedly enriched in TG. Amazingly, chylomicrons were still detected in plasma of 24h fasted Angptl4-Tg mice (Figure 3.4), which was visualized as a distinct peak in front of the VLDL peak (data not shown). Previously, it was shown that Angptl4 inhibits the activity of LPL in vitro (Yoshida et al. 2002). Using an endogenous LPL activity assay, a dramatic 90% reduction in post-heparin plasma LPL activity was observed in 24h fasted Angptl4-Tg compared with WT mice. Similarly, activity of hepatic lipase (HL) was about 50% reduced (Figure 3.5a). In contrast, mRNA levels of LPL in adipose tissue and of HL in liver were significantly increased in Angplt4-Tg mice, likely reflecting a mechanism that attempts to compensate for the decrease in enzyme activity (Figure 3.5b). Recent in vitro data suggest that Angptl4 inactivates LPL by

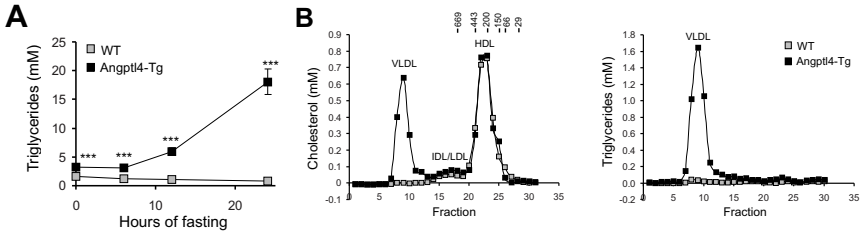


Figure 3.3: *Severe hypertriglyceridemia in Angptl4-Tg mice. (A), Time course of plasma TG during 24-hour fasting in WT mice(gray squares, n=7) and Angptl4-Tg mice (black squares, n=10). Error bars reflect SEM. (B), Pooled plasma of 24-hour fasted WT mice (gray squares, n=7) and Angptl4-Tg mice (black squares, n=10) was analyzed for lipoprotein profiles by FPLC. Elution of molecular weight markers is indicated.*

	Total Cholesterol	CM Cholesterol	VLDL Cholesterol	IDL/LDL Cholesterol	HDL Cholesterol	Total Triglyceride	CM Triglyceride	VLDL Triglyceride	IDL/LDL Triglyceride	HDL Triglyceride	Free Glycerol
Wild-type	3.91	0.00	0.15	0.42	3.33	0.61	0.00	0.36	0.22	0.04	0.28
Angptl4-Tg	5.91	0.23	1.69	0.81	3.19	12.57	1.61	9.09	1.40	0.46	0.82

Figure 3.4: *Lipoprotein cholesterol and TG content. Pooled plasma of 24h fasted wild-type and Angptl4-Tg mice was separated by gel permeation HPLC using lipoprotein analysis service by LipoSEARCH. CM, chylomicron. Concentrations are expressed in mM. n=7-10 mice/group.*

promoting the conversion of catalytically active LPL dimers into inactive monomers (Sukonina et al. 2006). This type of mechanism is expected to cause permanent inactivation of LPL. To verify whether this is the case, recombinant hAngptl4 was either pre-incubated with [^{3}H]TO-labeled VLDL-like emulsion particles, followed by mixing with LPL and measurement of LPL activity, or alternatively pre-incubated with HSPG-bound LPL and removed by washing before addition of the VLDL-like particles and measurement of LPL activity. Whereas the first assay revealed an expected potent inhibition of LPL activity by Angptl4 (Figure 3.5c) (left panel), a similar effect was observed in the second assay (right panel), suggesting that Angptl4 may permanently disable LPL. Because LPL monomers have much lower affinity for HSPG than LPL dimers, an Angptl4-mediated LPL dimer to monomer conversion would be expected to translate into a decrease in LPL dimers in Angptl4-Tg mice in post-heparin plasma, and possibly an increase in LPL monomers in pre-heparin plasma. To visualize LPL monomers and dimers, pre- and post-heparin plasma from 24h fasted WT and Angptl4-Tg mice was separated by heparin-sepharose affinity chromatography and fractions assayed for LPL content (Sukonina et al. 2006). Importantly a marked decrease in LPL dimer content was observed in post-heparin plasma of Angptl4-Tg mice (Figure 3.5d). No LPL was detected in fractions from pre-heparin plasma by ELISA (detection limit 0.2 ng/150 μl). However, using a more sensitive Western blot assay LPL protein was detected in fractions corresponding to LPL monomer in Angptl4-Tg but not WT mice (Figure 3.5e). These data indicate that Angptl4 over-expression promotes the conversion of HSPG-bound LPL dimers

into circulating LPL monomers. To investigate whether Angptl4 might physically associate with circulating LPL monomers, we studied the co-elution of Angptl4 and LPL in pre-heparin plasma fractions from the heparin-sepharose column and gel filtration FPLC column. It was observed that Angptl4, present in the truncated form, was present in exactly the same heparin-sepharose fractions as monomeric LPL (Figure 3.5e). The same observation was made in the FPLC fractions: Angptl4 was present in the fractions corresponding to (large) LDL (Figure 3.5f), which also harbors monomeric LPL, as previously published (Vilella et al. 1993). These data suggest that in blood plasma Angptl4 is physically associated with LDL-bound LPL monomers. To examine whether inhibition of LPL by Angptl4 overexpression resulted in a decrease in LPL-dependent VLDL-TG lipolysis, the decay of protein-free VLDL-like emulsion particles labeled with glycerol tri[^{3}H]oleate ([^{3}H]TO) and [^{14}C]cholesteryl oleate ([^{14}C]CO) was monitored in fed and 24-hour fasted WT and Angptl4-Tg mice. In the fed state, no difference in the clearance of [^{3}H]TO was observed between the two genotypes (Figure 3.6a)(serum half life 27±4.5 min vs. 32.2±5.2 min, respectively). In contrast, in the 24-hour fasted state, clearance of [^{3}H]TO was much lower in Angptl4-Tg mice, as evidenced by an almost 8-fold increase in serum half-life (102±9.3 min vs. 13.8±2.6 min, respectively; $p< 0.01$). In fact, whereas in WT mice fasting accelerated [^{3}H]TO clearance, the opposite was true in Angptl4-Tg mice. This was supported by measurement of tissue uptake of [^{3}H]TO-derived [^{3}H]oleate, which was markedly reduced in heart, liver, skeletal muscle and adipose tissues of 24h fasted Angptl4-Tg mice in comparison with WT mice (Figure 3.6c). These data indicate that Angptl4 overexpression strongly inhibited LPL-dependent VLDL-TG lipolysis. As the VLDL-like emulsion particles also contained [^{14}C]CO, it was possible to monitor clearance of VLDL-cholesteryl esters. Similar to the situation for [^{3}H]TO, in WT mice fasting accelerated [^{14}C]CO clearance, whereas clearance was decelerated in Angptl4-Tg mice (Figure 3.6b)(serum half life 29.5±10.4 min vs. 262±24.5 min, respectively; $p<0.01$). Tissue uptake of [^{14}C]CO, which occurs mainly in liver and is secondary to LPL-and HL-mediated conversion of VLDL to LDL via IDL, was also strongly reduced in Angptl4-Tg mice (Figure 3.6d). To find out if the decreased hepatic uptake of VLDL [^{3}H]TO and [^{14}C]CO in Angptl4-Tg mice was paralleled by changes in liver lipids, we measured hepatic cholesterol and TG content. Whereas in fed state no difference in liver cholesterol was observed between WT and Angptl4-Tg mice, in the fasted state liver cholesterol was modestly but significantly decreased in Angptl4-Tg mice (Figure 3.7a). In contrast, hepatic TG content was not changed significantly in Angptl4-Tg (Figure 3.8). In response to the decrease in hepatic cholesterol uptake and content in the fasted Angptl4-Tg mice, one would expect marked changes in the expression of genes dependent on cholesterol. To investigate the effect of Angptl4 over-expression on hepatic gene expression, we performed micro-array analysis. RNA from liver of several animals was pooled and hybridized to Affymetrix mouse genome 430 2.0 GeneChip arrays. Using a cut-off of 1.4-fold, expression of a total of 103 probe sets was increased in Angptl4-Tg mice compared with WT mice. Of the 103 probe sets, 40 probe sets represented genes directly involved in cholesterol and fatty acid biosynthesis, including

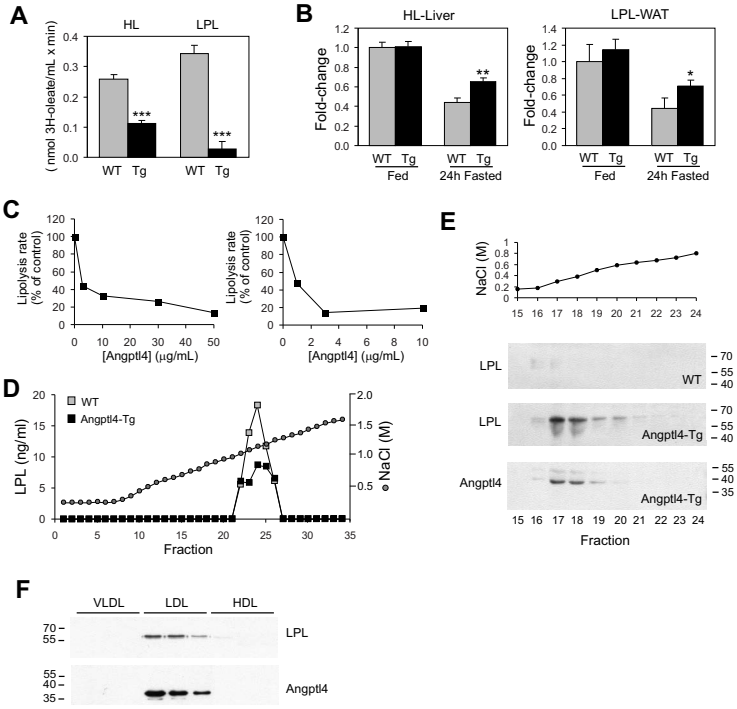


Figure 3.5: *Angptl4 inhibits lipoprotein lipase activity by promoting formation of LPL monomers. (A), Endogenous post-heparin plasma HL and LPL activity in 24-hour fasted mice (n=8 per group). (B), mRNA expression of HL and LPL in liver and WAT, respectively. Errors bars represent SEM. n=7 to 10 mice per group. Statistical significance according to Student t test. *P<0.05; **P<0.01; ***P<0.001. (C), Left panel: Effect of Angptl4 on LPL activity in solubilized LPL assay. Amount of [^{3}H]FFA released at increasing concentrations of hAngptl4 is expressed as a percentage of control treatment. Right panel: Effect of Angptl4 on LPL activity in HSPG-bound LPL assay. (D), Post-heparin plasma from 24-hour fasted WT and Angptl4-Tg mice was applied to a heparin sepharose column and eluted with a NaCl gradient in the presence of BSA. Fractions were analyzed for LPL by ELISA. (E), Pre-heparin plasma from 24-hour fasted WT and Angptl4-Tg mice was applied to a heparin sepharose column and eluted with a NaCl gradient in the absence of BSA. Fractions were analyzed for LPL and Angptl4 by Western blot. Upper panel: NaCl concentration in fractions. (F), Pooled plasma of 24-hour fasted WT mice was fractionated by FPLC. Fractions corresponding to VLDL, LDL, and HDL were analyzed for LPL or Angplt4 protein by Western blot. Molecular weight markers (in kDa) are shown.*

3-hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) synthase 1, HMG-CoA reductase, and Isopentenyl diphosphate isomerase (Figure 3.9). Up-regulation of these genes in Angptl4-Tg mice was verified by qPCR (Figure 3.7b) and (Figure 3.10). The increased expression of cholesterol biosynthetic enzymes at the mRNA level was supported by an increase in plasma lathosterol to

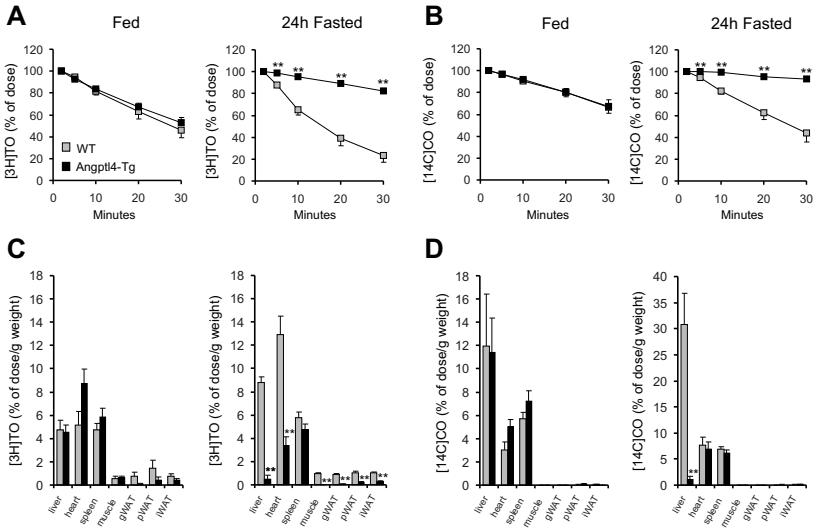


Figure 3.6: *Serum decay and tissue uptake of radiolabeled VLDL-like emulsion particles. VLDL-like particles labeled with glycerol tri[^{3}H]oleate and [^{14}C]cholesteryl oleate were injected into anesthetized mice. Serum samples were collected at indicated times and measured for ^{3}H-activity (A) and ^{14}C-activity (B). After 30 minutes, mice were euthanized and tissues collected for measurement of ^{3}H-activity (C) and for ^{14}C-activity (D) (bottom). Errors bars represent SEM. n=4 to 6 mice per group. Differences between WT and Angptl4-Tg mice were evaluated by MannWhitney test. *P<0.05; **P<0.01.*

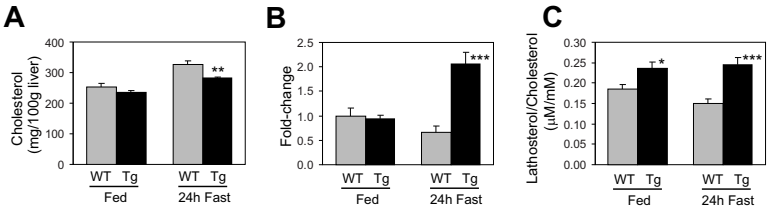


Figure 3.7: *Upregulation of hepatic cholesterol synthesis in Angptl4-Tg mice. (A), Hepatic cholesterol concentration. (B), mRNA expression of 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1. (C), Plasma lathosterol to cholesterol ratio. Errors bars represent SEM. n=7 to 16 mice per group. Statistical significance according to Student t test. *P<0.05; **P<0.01; ***P<0.001.*

cholesterol ratio, which serves as a marker for cholesterol biosynthesis rate (Figure 3.7c). Together, these data indicate that in the 24-hour fasted state Angptl4 overexpression causes a marked reduction in hepatic cholesterol uptake, leading to a decrease in hepatic cholesterol content and

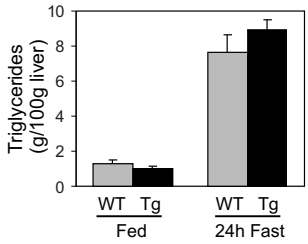
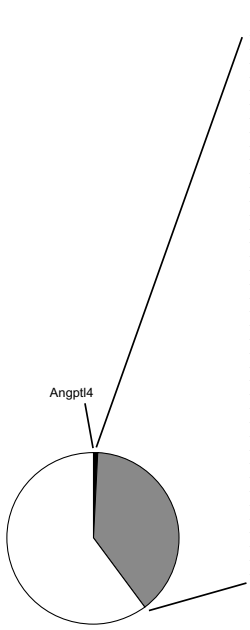


Figure 3.8: *No significant change in hepatic triglycerides in Angptl4-Tg mice. Hepatic triglyceride concentration in fed and 24h fasted WT and Angptl4-Tg mice. Similar results were obtained by Oil red O staining of liver sections.*

subsequent upregulation of cholesterol biosynthesis. Besides major changes in lipid metabolism, Angptl4 over-expression also caused alterations at the level of glucose metabolism. While in the fed state no difference in plasma glucose was observed between WT and Angptl4-Tg mice, in the 24-hour fasted state Angptl4-Tg mice exhibited a modest yet significant increase in plasma glucose (Figure 3.11a). In contrast, no difference in plasma insulin was observed between the two sets of mice in either fed or fasted state (Figure 3.11b). These data suggest that Angptl4-Tg mice may be mildly insulin resistant. To investigate whether this is the case, we performed a hyperinsulinemic-euglycemic clamp in 24-hour fasted mice. Under basal conditions, glucose metabolism was not different between the two sets of mice. However, under hyperinsulinemic conditions glucose utilization was significantly decreased in Angptl4-Tg mice compared to WT mice (Figure 3.11c). Indeed, whereas in WT mice glucose utilization increased by 125% after insulin infusion, in Angptl4-Tg mice glucose utilization only went up 59% (Figure 3.11d). Interestingly, endogenous glucose production was not affected by insulin infusion in WT mice, characteristics of the highly insulin-resistant FVB strain, yet was clearly inhibited by insulin in Angptl4-Tg mice (Figure 3.11e&f). These data suggest that Angptl4-Tg mice are insulin-resistant in the periphery, but more insulin-sensitive at the hepatic level.

3.4 Discussion

In the present paper we took advantage of the sensitivity of our Angptl4 overexpression model to fasting in order to better characterize the metabolic role of Angptl4. The most dramatic metabolic abnormality in Angptl4-Tg mice is the fasting-dependent elevation of plasma TG levels. The severe hypertriglyceridemia, with plasma TG levels reaching 20 mmol/L after 24 hours of fasting, can be attributed to marked inhibition of LPL-dependent VLDL and chylomicron lipolysis. Accordingly, we provide the definitive in vivo evidence that the hypertriglyceridemic effect of Angptl4 observed in several studies is due to impaired clearance of plasma TG via inhibition of



Affy ID	fold-change	gene name	gene title
1433446_at	2.41	Hmgcs1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1
1422973_a_at	2.33	Thrsp	thyroid hormone responsive SPOT14 homolog (Rattus)
1424737_at	2.30	Thrsp	thyroid hormone responsive SPOT14 homolog (Rattus)
1423804_a_at	2.25	Idi1	isopentenyl-diphosphate delta isomerase
1423418_at	2.20	Fdps	farnesyl diphosphate synthetase
1433445_x_at	2.14	Hmgcs1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1
1451122_at	2.13	Idi1	isopentenyl-diphosphate delta isomerase
1416222_at	2.13	Nsdhl	NAD(P) dependent steroid dehydrogenase-like
1433444_at	2.04	Hmgcs1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1
1415993_at	2.04	Sqle	squalene epoxidase
1433443_a_at	2.00	Hmgcs1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1
1421262_at	1.95	Lipg	lipase, endothelial
1423078_a_at	1.89	Sc4mol	sterol-C4-methyl oxidase-like
1437453_s_at	1.85	Pcsk9	proprotein convertase subtilisin/kexin type 9
1459497_at	1.80	Fdft1	Farnesyl diphosphate farnesyl transferase 1
1426913_at	1.77	Lss	lanosterol synthase
1450883_a_at	1.75	Cd36	CD36 antigen
1427229_at	1.69	Hmgcr	3-hydroxy-3-methylglutaryl-Coenzyme A reductase
1448130_at	1.69	Fdft1	farnesyl diphosphate farnesyl transferase 1
1416022_at	1.68	Fabp5	fatty acid binding protein 5, epidermal
1448663_s_at	1.65	Mvd	mevalonate (diphospho) decarboxylase
1438322_x_at	1.56	Fdft1	farnesyl diphosphate farnesyl transferase 1
1418052_at	1.56	Mvk	mevalonate kinase
1434520_at	1.55	Sc5d	sterol-C5-desaturase (fungal ERG3, delta-5-desaturase) homolog
1454671_at	1.53	Insig1	insulin induced gene 1
1439459_x_at	1.52	Acly	ATP citrate lyase
1416021_a_at	1.49	Fabp5	fatty acid binding protein 5, epidermal
1451457_at	1.49	Sc5d	sterol-C5-desaturase (fungal ERG3, delta-5-desaturase) homolog
1435630_s_at	1.48	Acat2	acetyl-Coenzyme A acetyltransferase 2
1449854_at	1.47	Nr0b2	nuclear receptor subfamily 0, group B, member 2 (SHP)
1455011_at	1.45	Stard4	StAR-related lipid transfer (START) domain containing 4
1427893_a_at	1.45	Pmvk	phosphomevalonate kinase
1450188_s_at	1.45	Lipg	lipase, endothelial
1448619_at	1.43	Dhcr7	7-dehydrocholesterol reductase
1423166_at	1.42	Cd36	CD36 antigen
1451263_a_at	1.41	Fabp4	fatty acid binding protein 4, adipocyte
1421821_at	1.40	Ldlr	Low density lipoprotein receptor
1417303_at	1.40	Mvd	mevalonate (diphospho) decarboxylase
1426690_a_at	1.40	Srebf1	sterol regulatory element binding factor 1
1429239_a_at	1.40	Stard4	StAR-related lipid transfer (START) domain containing 4

Figure 3.9: *Upregulation of genes involved in hepatic cholesterol synthesis in Angptl4-Tg mice. Microarray analysis was performed on pooled livers (n=7) of 24h fasted WT and Angptl4-Tg mice. The threshold for fold-induction was set at 1.4. Approximately 40% of the probe sets upregulated in Angptl4-Tg mice represented genes involved in cholesterol synthesis or lipogenesis.*

LPL-dependent lipolysis of TG-rich lipoproteins. The inhibition of LPL activity by Angptl4 was linked to conversion of endothelial-bound catalytically active LPL dimers into circulating catalytically inactive LPL monomers, as previously demonstrated in vitro (Sukonina et al. 2006). One major consequence of inhibition of LPL- and HL-dependent VLDL lipolysis in Angptl4-Tg mice is a reduced formation of LDL, leading to a marked decreased in cholesterol uptake in liver and subsequent decrease in hepatic cholesterol content. The decrease in hepatic cholesterol led to upregulation of almost every single gene that is part of the cholesterol synthesis pathway. In fact, close to 40% of the genes identified by micro-array as being upregulated in livers of Angptl4-Tg mice were involved in either fatty acid or cholesterol synthesis. Thus, by inhibiting LPL and HL, Angptl4 has a major impact on cellular cholesterol-uptake and consequently cholesterol-dependent gene regulation. The changes in gene expression are translated into a functional change in the rate of cholesterol synthesis, as shown by elevated plasma lathosterol to cholesterol ratio. We sus-

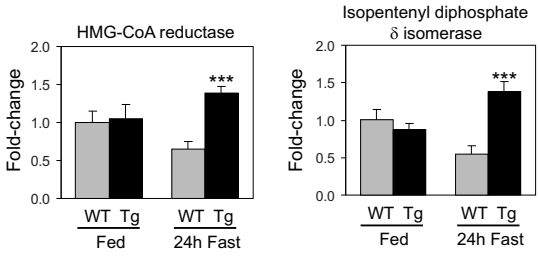


Figure 3.10: *Upregulation of genes involved in hepatic cholesterol synthesis in Angptl4-Tg mice. mRNA expression of two genes involved in cholesterol synthesis was measured by real-time quantitative PCR. Errors bars represent SEM. n=7-10 mice/group. Statistical significance according to Students t test. ***, p<0.001.*

pect that Angptl4 over-expression may similarly suppress fatty acid-dependent gene regulation, especially in tissues that rely heavily on LPL for fatty acid uptake. Fatty acid-dependent gene regulation is mainly mediated by PPARs. Previous studies have indicated that LPL can act on circulating lipoproteins to generate endogenous PPARα ligands, thus providing a potentially important link between lipoprotein metabolism and transcriptional effects of PPARα (Ziouzenkova et al. 2003). Indeed, it was shown that cardiac-specific knock-out of lipoprotein lipase resulted in decreased expression of several PPARα target genes (Augustus et al. 2004). Future studies will have to clarify whether Angptl4 over-expression has any influence on fatty acid and PPARα-dependent gene regulation in tissues that express LPL. Recently, it was shown that the N-terminal domain of Angptl4 (residues 1 to 187) is able to dissociate catalytically active LPL dimers into catalytically inactive LPL monomers in vitro, suggesting a novel mechanism for regulating LPL activity (Sukonina et al. 2006). Our data indicate that overexpression of Angptl4 in mice leads to conversion of endothelial-bound LPL dimers into circulating LPL monomers. Consequently, in Angptl4-Tg mice less LPL dimers are released into the circulation upon heparin injection, providing an explanation for the marked decrease in post-heparin LPL activity. Interestingly, the relative decrease in post-heparin LPL activity exceeded the relative decrease in heparin-releasable LPL dimers, suggesting that Angptl4 may inhibit LPL activity via an additional mechanism. An Angptl4-mediated LPL dimer to monomer conversion was supported by the presence of LPL monomers in pre-heparin plasma of Angptl4-Tg mice but not WT mice. However, the low concentration of LPL monomers suggests that they are rapidly cleared from the circulation. Our data also suggest that in blood plasma Angptl4 is physically associated with LPL monomers, which in turn are bound to LDL. It can be hypothesized that by binding LPL monomers, Angptl4 pulls the equilibrium between LPL dimers and monomers towards monomers, which effectively results in an inhibition of LPL activity. This model conflicts with the mechanism proposed by Sukonina et al. based on experiments carried out in vitro (Sukonina et al. 2006). More research is necessary to elucidate the precise mechanism by which Angptl4 promotes conversion of LPL dimers

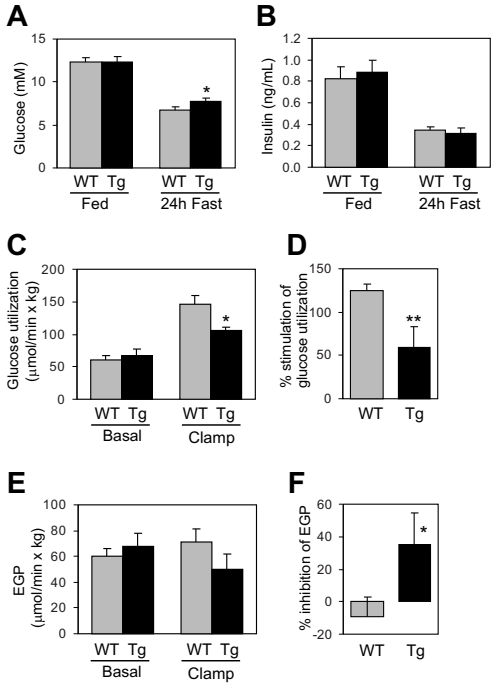


Figure 3.11: *Peripheral insulin resistance in fasted Angptl4-Tg mice. Plasma glucose (A) and insulin (B) levels in fed and 24-hour fasted WT and Angptl4-Tg mice. n=7 to 10 mice per group. A hyperinsulinemic euglycemic clamp was carried out in 24-hour fasted WT and Angptl4-Tg mice. (C), Whole body glucose use under basal and hyperinsulinemic conditions. (D), Percentage induction of glucose use by insulin. (E), Endogenous glucose production (EGP) under basal and hyperinsulinemic conditions. (F), Percentage inhibition of EGP by insulin. Errors bars represent SEM. n=3 to 6 mice per group. Statistical significance according to Student t test. *P<0.05; **P<0.01.*

to monomers in vivo. In addition to inhibition of LPL activity, we also observed that Angptl4 overexpression resulted in a significant decrease in post-heparin plasma HL activity, although the effect was less pronounced compared to LPL. Inhibition of HL may contribute to the elevated levels of IDL/LDL, especially the increase in IDL/LDL-TG content, and likely contributes to the observed decrease in hepatic cholesterol uptake. The latter result could also be linked to increased IDL/LDL-particle size, which has been shown to lead to reduced hepatic uptake via a mechanism independent of the LDL receptor (Rensen et al. 1997). In line with the role of HL in converting HDL_2 to HDL_3, inhibition of HL may contribute to the elevated HDL-TG content in Angptl4-Tg mice. Similar to LPL, HL is functionally active as a dimer (Hill et al. 1996, Berryman et al. 1998). Accordingly, it is possible that inhibition of HL activity by Angplt4 is accounted for by conver-

sion of HL dimers to monomers. In contrast to our observations, Koster et al did not observe any change in post-heparin plasma HL activity in mice with liver-specific Angptl4 overexpression or in Angptl4 KO mice (Koster et al. 2005). The reason for this discrepancy is not clear but may be related to differences in the sensitivity of the HL activity assay employed or to differences in the magnitude of Angptl4 overexpression. In contrast to HDL-TG, HDL-Chol levels were identical between the two sets of mice. Although LPL activity and HDL-Chol levels are positively correlated in humans, no link between LPL activity and HDL-Chol levels has been observed in mice overexpressing LPL or in LPL null mice, which is probably explained by the absence of CETP activity in mice (Clee et al. 1997). Recently, it was reported that Angptl3, which is structurally and functionally highly related to Angptl4, inhibits activity of endothelial lipase, an important determinant of HDL-Chol levels (Shimamura et al. 2007). Mice lacking Angptl3 showed a decrease in plasma HDL-Chol and HDL phospholipid, coupled with increased phospholipase activity in post-heparin plasma. Furthermore, Angptl3 inhibited the phospholipase activity of endothelial lipase in vitro. Whether Angptl4 may inhibit EL as well remains to be investigated. Whereas the impact of Angptl4 on plasma lipoprotein metabolism is well established, much less is known about the role of Angptl4 in glucose homeostasis. Recently it was reported that adenoviral-mediated overexpression of Angptl4 causes an abrupt and dramatic decrease in plasma glucose coupled to a marked improvement in glucose tolerance (Xu et al. 2005). In contrast to the hypertriglyceridemia elicited by Angptl4 overexpression, which was transient, the hypoglycemia was persistent up to two weeks after virus injection, when plasma levels of Angptl4 had almost returned to normal. Infection of primary hepatocytes with Angptl4 adenovirus significantly decreased secretion of glucose into the medium, suggesting that Angptl4 may lower plasma glucose by decreasing hepatic glucose output. In partial support of this notion, we find decreased endogenous glucose production in Angptl4-Tg mice under hyperinsulinemic conditions, whereas no change was found under basal conditions. At the same time, Angptl4 overexpression was associated with a decrease in insulin-mediated glucose disposal, suggesting peripheral insulin resistance. Under conditions of prolonged fasting, the effect of Angptl4 on insulin resistance seems to dominate as plasma glucose was modestly increased in Angptl4-Tg mice compared to wild-type mice, while plasma insulin levels remained unaltered. The decreased peripheral insulin sensitivity in Angptl4-Tg mice may be related to elevated plasma FFAs, which are known to induce insulin resistance (Kahn et al. 2006), although a direct effect of Angptl4 protein on insulin signaling cannot be excluded. In mice, the overall impact of Angptl4 on plasma glucose levels appears to be limited, and may only be evident at higher plasma Angptl4 concentrations since inactivation of the Angptl4 did not result in any change in plasma glucose (Koster et al. 2005). However, this observation does not exclude an effect of Angptl4 inactivation on glucose fluxes. It was reported that in humans, levels of Angptl4 in blood plasma of obese diabetics appear to be significantly decreased in comparison with obese non-diabetics (Xu et al. 2005). Although these data still have to be verified, they suggest that in humans Angptl4 might serve as a marker for insulin sensitivity. Whereas the effects of Angptl4

on lipoprotein metabolism in mice are supported by numerous studies, much less is known about the role of Angptl4 in humans. Recently, it was shown that a variant of the human Angptl4 gene (E40K), which is present in approximately 3% of European Americans, was associated with significantly lower plasma TG levels and higher HDL chol levels (Romeo et al. 2007). These data suggest that, at least with respect to plasma TG, the effect of Angptl4 is similar between mice and humans. The picture is a little bit more complicated for HDL chol. Previously, we found a positive correlation between plasma Angptl4 concentration, assessed semi-quantitatively by immunoblot, and HDL chol levels (Mandard et al. 2004). In the present study, no change in plasma HDL-Chol level was observed in Angptl4-Tg mice, despite a >20-fold increase in plasma TG. Thus, the link between Angptl4 and HDL, and the impact of Angptl4 on effectors of plasma HDL levels, deserve further study. In conclusion, we show that (1) the hypertriglyceridemic effect of Angptl4 is attributable to inhibition of LPL-dependent VLDL lipolysis by converting endothelial-bound LPL dimers to circulating LPL monomers, (2) Angptl4 up-regulates cholesterol synthesis in liver via inhibition of LPL- and HL-dependent hepatic cholesterol uptake, (3) Angptl4 overexpression increases insulin sensitivity in liver but decreases insulin sensitivity in the periphery.

Adapted from: S. Kersten, L. Lichtenstein, E. Steenbergen, K. Mudde, H.F.J. Hendricks, M.K. Hesselink, P. Schrauwen and M. Müller – *"Caloric restriction and exercise increase plasma ANGPTL4 levels in humans via elevated free fatty acids,"* ATVB 2009 Jun;29(6): 969-74

Chapter 4

Caloric restriction and exercise increase plasma ANGPTL4 levels in humans via elevated free fatty acids

Abstract

Plasma lipoprotein levels are determined by the balance between lipoprotein production and clearance. Recently, Angiopoietin-like protein 4 was uncovered as a novel endocrine factor that potently raises plasma triglyceride levels by inhibiting triglyceride clearance. However, very little is known about ANGPTL4 in human.Here we set out to identify physiological determinants of plasma ANGPTL4 levels in humans, focusing on the effect of energy restriction and plasma FFAs. We developed an ELISA for quantitative measurement of ANGPTL4 in human plasma. Using this assay we found major variations in baseline plasma ANGPTL4 levels between individuals. Within an individual, plasma ANGPTL4 levels remain stable throughout the day but increase significantly in response to long term fasting, chronic caloric restriction, and endurance exercise. Intralipid injection as well as treatment with a β-adrenergic agonist, both of which lead to elevated plasma FFA levels, increased plasma ANGPTL4 levels compared to control treatment. Fatty acids markedly induced ANGPTL4 gene expression in rat hepatoma FAO cells, human primary myocytes, and mouse intestinal MSIE cells. In conclusion, our results show that plasma ANGPTL4 levels are increased by fasting, caloric restriction, and exercise, which is likely mediated by elevated plasma FFAs.

4.1 Introduction

Changes in plasma lipoproteins are known to affect atherosclerosis and associated coronary heart disease (CHD). Indeed, it is well established that elevated plasma low-density lipoprotein (LDL) concentrations increase the risk for CHD, whereas elevated high-density lipoprotein (HDL) concentrations are considered atheroprotective. Plasma lipoprotein levels are determined by the balance between lipoprotein production and clearance. Recently, Angiopoietin-like protein 4 was uncovered as a novel endocrine factor that impact plasma lipoprotein levels. ANGPTL4, which is also referred to as fasting induced adipose factor Fiaf, is a secreted protein of about 50 kDa that belongs to the family of Angiopoietin-like proteins (Kim et al. 2000). ANGPTL4 was discovered by screening for target genes of the peroxisome proliferators activated receptors alpha and gamma, which serve as the molecular targets of the hypolipidemic fibrate and the insulin sensitizing thiazolidinedione drugs, respectively (Kersten et al. 2000, Yoon et al. 2000). In mice, ANGPTL4

is produced by a number of tissues including adipose tissue, liver, skeletal muscle, heart, skin and intestine. Evidence abounds that ANGPTL4 plays a major role in the regulation of lipid metabolism (Kersten 2005). ANGPTL4 inhibits the enzyme lipoprotein lipase, thereby suppressing clearance of TG-rich lipoproteins and thus raising plasma TG levels (Yoshida et al. 2002). Inhibition of LPL occurs by inducing dissociation of catalytically active LPL dimers into inactive LPL monomers (Sukonina et al. 2006). Experiments with transgenic mice over-expressing ANGPTL4 suggest that ANGPTL4 inhibits LPL via paracrine and endocrine signaling (Xu et al. 2005, Yu et al. 2005, Koster et al. 2005, Mandard et al. 2006). Furthermore, ANGPTL4 stimulates adipose tissue lipolysis, leading to elevation of plasma free fatty acid levels (Yoshida et al. 2002, Mandard et al. 2006, Lichtenstein et al. 2007). Analogous to numerous other protein hormones, ANGPTL4 is proteolytically processed to produce N-terminal and C-terminal fragments that can be detected in human serum (Mandard et al. 2004, Ge et al. 2004a). Processing seems to occur in a tissue-specific manner. Indeed, the liver actively cleaves ANGPTL4, whereas human adipose tissue shows no proteolytic activity (Mandard et al. 2004). In addition to governing clearance of plasma lipoproteins, ANGPTL4 has been suggested to play a role in angiogenesis. It has been shown that ANGPTL4 is a structural and likely regulatory component of the extra-cellular matrix that affects numerous aspects of endothelial function including angiogenesis, endothelial cell migration, vascular leakage, and cell adhesion (Le Jan et al. 2003, Ito et al. 2003, Cazes et al. 2006, Galaup et al. 2006, Hermann et al. 2005, Padua et al. 2008). Presently, very limited information is available about ANGPTL4 in human. It has been shown that carriers of a rare sequence variant of the ANGPTL4 gene have elevated plasma HDL and decreased TG levels (Romeo et al. 2007). However, due to the absence of a commercial assay to quantitatively assess ANGPTL4, very little is known about plasma ANGPTL4 levels in human. Here, we describe the development, validation and application of a novel ELISA for quantitative determination of ANGPTL4 in human plasma. We used this newly developed ELISA to identify physiological determinants of plasma ANGPTL4, focusing on the role of fasting and plasma FFAs

4.2 Materials and Methods

Human interventions Study 1: Blood was taken after an overnight fast from 25 male mildly hyperlipidemic subjects before and after a 4-week treatment with 250 mg of micronized fenofibrate daily. All subjects had significant coronary artery disease as documented by angiography(Patsouris et al. 2004). Study 2: A fasting blood sample was taken in 36 apparently healthy post-menopausal women varying in body mass index from 20.7 to 35 kg/m^2. (age: 56.5±4.2 years, BMI: 25.4±3.3 kg/m^2, fasting glucose: 5.5±0.4 mM). The women participated in a randomised, open label, placebo-controlled trial that investigated the effects of daily wine or grape juice consumption on markers of insulin sensitivity. Blood samples were obtained from the antecubital vein of the forearm and collected in EDTA-coated tubes (Joosten et al. 2008). Study 3: Two male and seven fe-

male subjects between the age of 24-32 yr came to the laboratory in fasted state (BMI: 19.1±26.6, mean 22.4 kg/m^2; fasting glucose: 4.8±0.6 mM). Blood samples were taken via finger prick at selected time before, after and in between meals. Subjects maintained their normal pattern of activities including meal choice. Study 4: Four healthy male volunteers, between 19 and 22 years of age, were recruited from the Wageningen student population(age:19-22, mean 20.6; BMI: 20.4-22.6, mean 21.3 kg/m^2; fasting glucose: 4.9-5.7, mean 5.2 mM). Volunteers received an identical meal at 1700, before the start of a 48-h fasting period. At baseline and after 24h and 48 h of fasting, blood was drawn from the antecubital vein of the forearm into EDTA-coated tubes (Bouwens et al. 2007). Study 5: Participants were overweight men with a mean (± SEM) age of 34.8±1.3 y and body mass index (in kg/m2) of 28.8±0.5. All 22 subjects reduced their energy intake to 2.1 MJ/d by means of a very-low-energy diet for the duration of 25 days. Fasting blood samples were obtained from the antecubital vein of the forearm and collected in EDTA-coated tubes (Hukshorn et al. 2003). Study 6: Seven healthy, untrained male volunteers participated in the study (age: 22.7±0.6 years, BMI: 23.8±1.0 kg/m^2, height: 1.79±0.03 m, VO_2max: 50.5±2.4 mL/min/kg). After an overnight fast, subjects came to the laboratory at 8:00 a.m.. After local anesthesia, a Teflon cannula was inserted in an antecubital vein for sampling of blood. Subjects rested on a bed, and a baseline blood sample was taken. Immediately thereafter, subjects ingested 1.4 g/kg bodyweight glucose or water. Subject exercised at 50% VO_2 max for 2 h and then rested for 4 h. Blood was samples at regular intervals throughout the study. Also, at regular intervals subjects ingested 0.35 g/kg bodyweight glucose or water. All subjects underwent the experimental protocol two times, once with glucose ingestion and once while fasting (Schrauwen et al. 2002). Study 7: Nine healthy lean male volunteers participated in the present study (age: 20.1±0.5 years, BMI: 21.7±0.6 kg/m^2, bodyfat: 16.0±1.3%). All subjects underwent two euglycaemic-hyperinsulinaemic clamps with 40 mU/m^2/min insulin and variable co-infusion of a 20% glucose solution to reach a blood glucose level of ~5 mmol/L with simultaneous infusion of glycerol (73 mL/h) or intralipid (81 mL/h) in randomised order. Test days were separated by at least 1 week. On both experimental days, subjects arrived at the laboratory at 8.00 h after an overnight fast. Blood was sampled at regular time points throughout the clamp (Hoeks et al. 2003). Study 8: Nine healthy lean male volunteers participated in this study (age: 24.4±1.3 years, BMI: 22.2±0.8 kg/m^2, height: 1.79±0.03 m). Upon arrival at the laboratory after an overnight fast, two Teflon cannulas were inserted into an antecubital vein of each arm. One cannula was used for the infusion of β2-adrenergic agonist and one cannula for sampling of blood. A first blood sample was taken, followed by a continuous infusion of 77 ng/min/kg FFM salbutamol maintained for 3 h. In addition, two doses of 250 mg acipimox or placebo were given orally at -120 min and time 0. Blood samples were taken at regular intervals throughout the study (Aas et al. 2006). All human interventions were approved by the Medical Ethics Committee of the institute involved as outlined in the original research publications.

Elisa: 96-well microtiter plates were coated with the anti-hANGPTL4 polyclonal goat IgG antibody (AF3485, *R&D* Systems) at 0.16μg/well and incubated overnight at 4°*C*. This antibody

was obtained by immunization of goats with NS0-derived recombinant human Angiopoietin-like 4 (aa 26 - 406). After four washes with 300μL PBS-Tween 0.1%, 300 μL of blocking solution (PBS containing 1% BSA) was added per well and left for 1 hour at room temperature under gentle agitation. 100 μL of 20-fold diluted human plasma was applied to each well, followed by 2 hour incubation at room temperature under gentle agitation. A standard curve of increasing amounts of recombinant human ANGPTL4 (3485-AN, *R&D* Systems) was prepared (0.03-0.21 ng/well) and incubated under similar conditions. After four washes, 100 μL of diluted biotinylated anti-hANGPTL4 polyclonal goat IgG antibody (BAF3485, *R&D* Systems) was added at 0.02μg per well, followed by another 2 hour incubation. After four washes, streptavidine-conjugated horseradish peroxidase was added for 1 h. Subsequent reaction with tetramethyla-benzidine substrate reagent was allowed to proceed for 6 min. The reaction was stopped by addition of 50 μl of 10% H_2SO_4 and the absorbance was measured at 450 nm on a MultiSkan Ascent spectrophotometer (Thermo Scientific, Breda, the Netherlands). The ELISA assay used is virtually identical to the DuoSet Elisa hANGPTL4 offered commercially by *R&D* systems with several minor modifications. Importantly, absorbance values remain within the linear range of the standard curve, which does not exceed 2.1 ng/mL.

Cell culture: Partially confluent HepG2 cells were transfected with a GFP control vector (pEGFP-N2) or the expression vector pcDNA3.1/V5-HisA encoding mAngptl4 or hANGPTL4. After transfection, cells were incubated in serum-free DMEM. 24 hours posttransfection, medium and cell lysates were collected and used for ANGPTL4 Elisa assay. Human primary myocytes were prepared from 3 individuals as previously described (Aas et al. 2006). Rat hepatoma FAO cells, human primary myocytes, and mouse intestinal MSIE cells were grown in DMEM containing 10% (vol/vol) fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were incubated with albumin only (control), albumin-bound oleic or linoleic acid (100 μmol/L), or synthetic PPAR agonists for 24 hours, followed by RNA isolation and qRT-PCR. Wy14643 was used at a concentration of 5 μmol/L (FAO, MSIE). GW501516 was used at 1 μmol/L (FAO, MSIE) or 10 μmol/L (primary myocytes), and TTA (Tetradecylthioacetic Acid) was used at 100 μmol/L.

Electrophoresis and Western Blot: 0.25 μL of human plasma was separated by SDS-PAGE and transferred to immobilon-P membrane. Western blotting was carried out using an ECL system (GE Healthcare, Diegem, Belgium) according to the manufacturer's instructions. The primary antibody was used at a dilution of 1:2500, and the secondary antibody (anti-rabbit IgG, Sigma) was used at a dilution of 1:8000. All incubations were performed in 1x Tris-buffered saline, pH 7.5, with 0.1% Tween 20 and 5% dry milk, except for the final washings, when milk was omitted. Primary antibody was the same as used for Elisa or was a human polyclonal antibody directed against the epitope CQGTEGSTDLPLAPE (Mandard et al. 2004).

Quantitative PCR: cDNA was prepared from FirstChoice Human Total RNA Survey Panel (Ambion) using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories BV, Veenendaal, The Netherlands). Real-Time PCR was carried out using platinum Taq polymerase (Invitrogen, Breda,

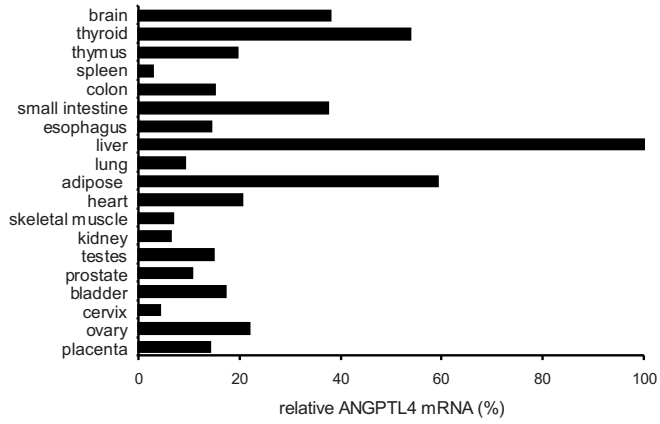


Figure 4.1: *Expression profile of ANGPTL4 mRNA in human tissues. The FirstChoice Human Total RNA Survey Panel (Ambion) was reverse transcribed and used for qPCR using primers specific for human ANGPTL4. Expression levels are expressed relative to liver, which showed the highest expression level (100%).*

the Netherlands) and SYBR green using an iCycler PCR machine (Bio-Rad Laboratories BV). Melt curve analysis was performed to assure a single PCR product was formed. hANGPTL4 cDNA was amplified using primers: 5'-CACAGCCTGCAGACACAACTC-3' and 5'-GGAGGCC AAACTGGCTTTGC-3'; m/rAngptl4 cDNA was amplified using primers: 5'-GTTTGCAGACTC AGCTCAAGG-3' and 5'-CCAAGAGGTCTATCTGGCTCTG-3'. ANGPTL4 expression data were normalized against the housekeeping genes 36B4 (FAO, MSIE) or actin (primary myocytes). Primer sequences are available upon request.

4.3 Results

To better characterize ANGPTL4 in human, we first determined ANGPTL4 gene expression levels in a large number of human tissues. In general, ANGPTL4 expression was relatively ubiquitous. Highest expression levels were found in liver, followed by adipose tissue, thyroid, brain and small intestine (Figure 4.1).

To better understand the role of ANGPTL4 in human, we developed an ELISA assay to quantitatively assess serum or plasma ANGPTL4 levels in human subjects. A standard curve was generated using recombinant human ANGPTL4, showing a clear linear relationship between ANGPTL4 concentration and absorbance at 450 nm (Figure 4.2a). As shown by Western blot, the antibody used in the ELISA detected a single band at 50 kD, corresponding to the molecular weight of

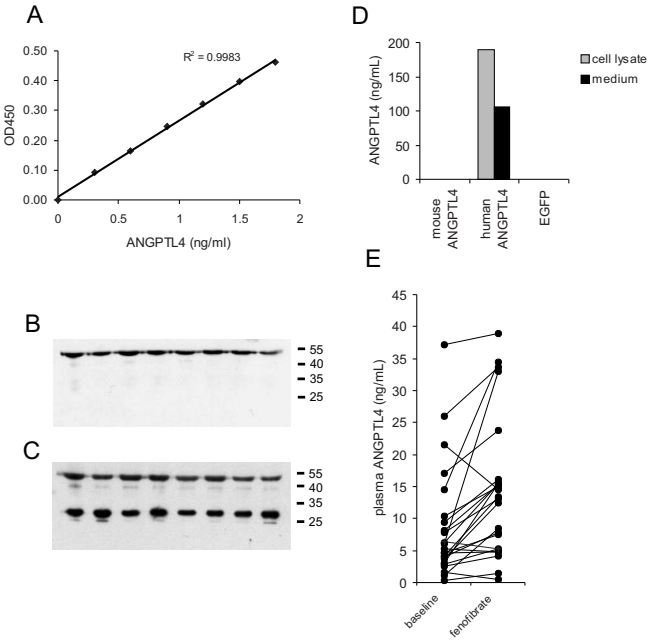


Figure 4.2: *Characteristics of the ANGPTL4 Elisa assay. (A), standard curve showing linear relationship between concentration of ANGPTL4 protein and absorbance. Plasma samples were diluted 20-50 fold to fall within the linear range. (B), western blot of human blood plasma using the same antibody as used in the Elisa assay. (C), western blot of human blood plasma using an antibody directed against a N-terminal epitope of ANGPTL4. Same individuals are shown as in panel B. (D), concentration of ANGPTL4 in medium and cell lysate of HepG2 cells 24h after transfection with GFP control vector or expression vector encoding ANGPTL4. (E), plasma ANGPTL4 levels in 25 individuals before and after 4 weeks of fenofibrate treatment (study1).*

full length ANGPTL4 (Figure 4.2b). The same band and an additional band corresponding to the N-terminal truncated portion of ANGPTL4 were detected using an antibody directed against a N-terminal peptide-epitope (Figure 4.2c). To test the specificity of the assay, HepG2 cells were transiently transfected with expression vectors encoding mouse Angptl4, human ANGPTL4 or EFGP, and cells and medium were harvested 24 hours thereafter. ANGPTL4 was specifically detected in medium and cell lysate of HepG2 cells transfected with hANGPTL4, indicating the absence of any cross-reactivity with mAngptl4 (Figure 4.2d). As a final verification, we confirmed the increase in plasma ANGPTL4 levels in patients after treatment with the PPAR agonist fenofibrate (Figure 4.2e), as previously demonstrated by semi-quantitative Western blot (Mandard et al. 2004). We first determined a number of basic characteristics of the hANGPTL4 ELISA assay.

The intra-assay coefficient of variation (CV) for the assay was determined at 6.8%. The within-subject intra-day CV, which was determined by measuring plasma ANGPTL4 levels at numerous time points throughout the day, was slightly higher at 10%, while the within-subject inter-day CV was similarly low at 14%. In contrast to the low variability within subjects, we found a large inter-individual variation in plasma ANGPTL4 levels, as shown by a between-subject CV of 78%. These data indicate that plasma ANGPTL4 levels are relatively constant within an individual, yet differ markedly between subjects. As plasma ANGPTL4 levels were highly variable between individuals, we were interested to study the potential relationship with plasma TG, which have been shown to be impacted by ANGPTL4 overexpression or deletion in mice. Interestingly, in a samples of 36 middle-aged women (study 2), we did not find any association between plasma ANGPTL4 and plasma TG levels (data not shown). Similarly, no association was found with BMI or other plasma metabolic parameters such as insulin, glucose, and adiponectin. In an effort to ascertain the physiological determinants of plasma ANGPTL4 levels, we first determined the circadian rhythm of plasma ANGPTL4 in nine subjects that took their meals at specific times. While normal post-prandial responses in plasma free fatty acids and glucose were observed (Figure 4.3 a&b), plasma ANGPTL4 levels remained remarkably stable (Figure 4.3c). As mentioned above, however, major variation in plasma ANGPTL4 levels was observed between subjects. To metabolically challenge subjects, 4 young males were fasted for 48 hours (Bouwens et al. 2007). Plasma ANGPTL4 levels went up in all individuals (Figure 4.4a, mean relative increase: 80%, $p<0.01$). Plasma ANGPTL4 also significantly increased in obese patients subjected to 25 days of severe food deprivation (Figure 4.4b), mean relative increase: 79%, $p<0.001$) (Hukshorn et al. 2003) These data indicate that plasma ANGPTL4 levels increase in response to both short term and long term energy restriction, which are associated with elevated plasma FFA levels (Figure 4.5). Expression of ANGPTL4 in numerous tissues was highly sensitive to free fatty acids, as shown by dramatic induction of ANGPTL4 mRNA by the fatty acids oleic acid and linoleic acid in rat FAO hepatoma cells, primary human myocytes, and mouse intestinal MSIE cells (Figure 4.6). Angptl4 induction was mimicked by synthetic agonists of PPARs, which serve as receptors for fatty acids, indicating a role for PPARs. These data suggest that the effect of severe energy restriction on plasma ANGPTL4 may be mediated by elevated plasma FFAs. Another physiological stressor that increases plasma FFA is endurance exercise. Remarkably, we found that fasting plasma ANGPTL4 levels further increased during endurance exercise and especially during subsequent recovery, concurrent with an increase in plasma FFAs (Figure 4.7a) and (Figure 4.8) (Schrauwen et al. 2002). Importantly, the increase in plasma ANGPTL4 was entirely abolished when subjects were given oral glucose, which elicits insulin release and thereby suppresses plasma FFA levels. To further explore the impact of plasma FFA, plasma ANGPTL4 levels were measured in subjects that underwent a hyperinsulinemic clamp while receiving an infusion of either glycerol (control) or lipids together with heparin, which causes a massive increase in plasma FFAs (Figure 4.8) (Hoeks et al. 2006). Whereas plasma ANGPTL4 declined significantly on control treatment, which may

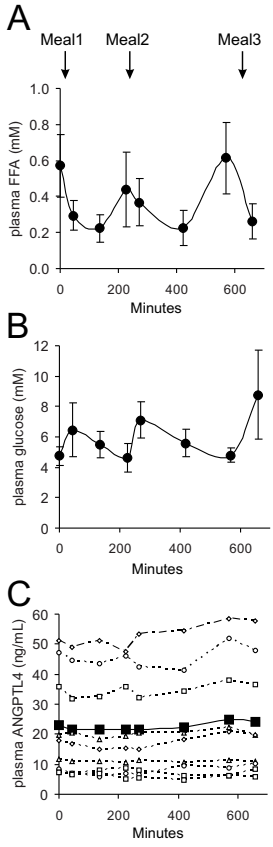


Figure 4.3: *Plasma ANGPTL4 shows little diurnal variation. Blood plasma was sampled from nine subjects before, after and in-between meals for measurement of FFA (A), glucose (B), and ANGPTL4 (C) (study 3). Dotted lines represent plasma ANGPTL4 levels of individual subjects. Straight lines represent means of nine subjects. Error bars represent SD.*

reflect a direct effect of insulin or may be due to an insulin-induced suppression of plasma FFAs, the decrease in plasma ANGPTL4 was significantly diminished after lipid infusion (Figure 4.7b). Similarly, treatment with the -adrenergic agonist salbutamol, which causes a marked increase in plasma FFA (Figure 4.8) (Hoeks et al. 2003), significantly raised plasma ANGPTL4 levels. This response was entirely blunted when subjects were simultaneously given acipimox, a lipolysis inhibitor that reduces plasma free fatty acids (Figure 4.7c). These data suggest that plasma FFAs

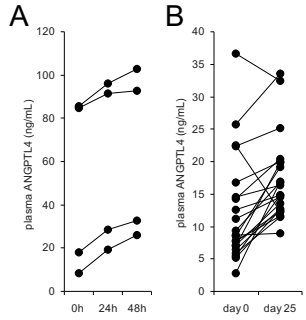


Figure 4.4: *Plasma ANGPTL4 levels are increased by fasting and caloric restriction. (A) Plasma ANGPTL4 was measured in four subjects after a meal, and after 24h or 48h of fasting (study 4). (B) ANGPTL4 levels were measured in overnight fasting plasma from 22 overweight males before and after 25 days of caloric restriction (study 5).*

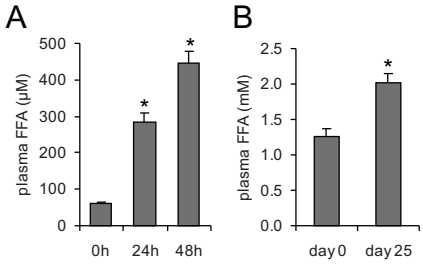


Figure 4.5: *Plasma FFA levels are increased by fasting and caloric restriction. (A) FFA levels were measured in plasma sampled from four young healthy males right after a meal, and after 24h or 48h of fasting (study 4). (B) FFA levels were measured in overnight fasting plasma sampled from 22 healthy overweight males (BMI 25-32) before and after 25 days of subsisting on a proteinenriched formula diet that provided 2.1 MJ/d (study 5). Error bars represent SEM.* $*$ *= significantly different according to Student's T-test* $p < 0.05$.

increase plasma ANGPTL4 levels.

4.4 Discussion

Very little is known about determinants of plasma ANGPTL4 levels in humans. Our data point to a large inter-individual variation in plasma ANGPTL4 levels. In contrast, plasma ANGPTL4 levels are very constant within an individual, which is true during normal circadian rhythm as well as between days. From our data it is evident that major physiological stressors such as fasting

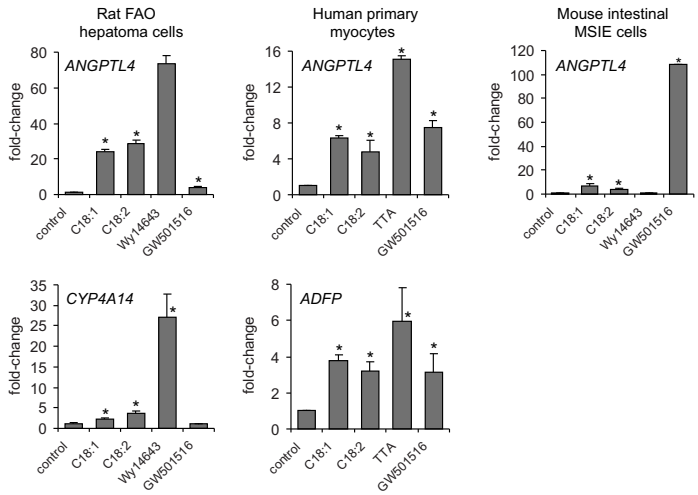


Figure 4.6: *Fatty acids markedly induce ANGPTL4 mRNA. Cultured cells were incubated with oleic or linoleic acid, Wy14643, GW501516 or tetradecylthioacetic acid (TTA) for 24 hours, followed by RNA isolation and qRT-PCR. CYP4A14 or ADFP served as positive PPAR control genes. Error bars represent SEM.* $*$ *= significantly different according to Student's T-test (p<0.05).*

and exercise are required to elicit significant changes in plasma ANGPTL4. Specifically, we observed that 48h of fasting and 25 days of severe caloric restriction both raised plasma ANGPTL4 by an average of 80%. Fasting plasma ANGPTL4 levels were further stimulated by endurance exercise and post-exercise recovery. Additional experiments indicated that the increase in plasma ANGPTL4 levels is likely mediated by elevated FFAs via activation of ANGPTL4 gene transcription via PPARs. Alternatively, it may be argued that instead of or in addition to FFAs raising plasma ANGPTL4, ANGPTL4 may raise plasma FFAs via stimulation of adipose tissue lipolysis. Studies using Angptl4 knock-out or transgenic mice (Mandard et al. 2006), as well as iv injection of recombinant Angptl4 (Yoshida et al. 2002) support a potent pro-lipolytic activity of ANGPTL4 in adipose tissue. It is possible that both mechanisms operate in concert as part of a positive feedback loop. The increase in plasma ANGPTL4 levels during fasting is in line with a wealth of mouse data showing induction of Angptl4 gene expression by fasting in numerous tissues (Kersten et al. 2000, Mandard et al. 2006, Lichtenstein et al. 2007, Ge et al. 2005, Dutton and Trayhurn 2008). By inhibiting lipolysis of TG-rich lipoproteins, which results in decreased tissue uptake of plasma TG-derived fatty acids, while at the same time increasing plasma FFAs by stimulating adipose tissue lipolysis, we hypothesize that ANGPTL4 may cause a shift in overall fuel utilization from plasma TG towards TG stored in the adipose tissue. Depending on whether the

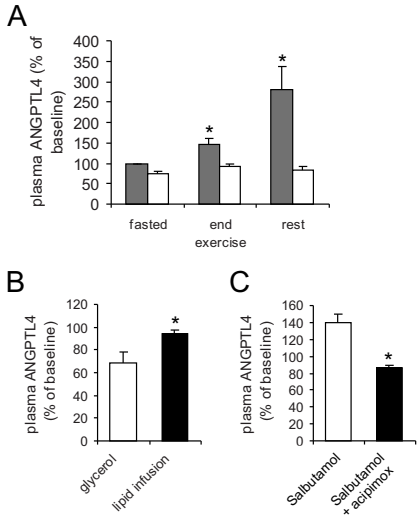


Figure 4.7: *Plasma ANGPTL4 levels increase during exercise in fasted but not fed state. (A) After overnight fasting, seven subjects performed 2 hours of endurance exercise followed by 4 hours of post-exercise recovery while fasted (grey bar) or with glucose ingestion (white bar) (study 6).To normalize for large differences in baseline ANGPTL4 values, levels are shown as % change over baseline (= fasted state, pre-exercise). (B) Plasma ANGPTL4 levels were determined in nine subjects before (baseline) and after a 6h hyperinsulinaemic-euglycaemic clamp with simultaneous infusion of either glycerol or lipid emulsion study 7). The percentage change in plasma ANGPTL4 from 0 to 6 hours is shown. (C) Plasma ANGPTL4 levels in nine males before (baseline) and after receiving a 3-h infusion of salbutamol with or without simultaneous administration of acipimox (study 8). The percentage change in plasma ANGPTL4 from 0 to 3 hours is shown. Error bars represent SEM. $*$ = significantly different according to Student's T-test ($p<0.05$).*

tissue primarily uses plasma TG-derived fatty acids or FFA (Teusink et al. 2003), ANGPTL4 upregulation is expected to either cause a decrease or increase in net fatty acid uptake. Upregulation of ANGPTL4 by fasting may thus be aimed at promoting use of fat in favor of fat storage. This mechanism may be supported by the above mentioned positive feedback loop in which ANGPTL4 stimulates release of FFAs, which in turn stimulates ANGPTL4 production. Depending on the type of tissue involved, the effect of fatty acids on ANGPTL4 mRNA can be mediated by different PPAR isotypes. Our results suggest that fatty acids induce ANGPTL4 mRNA via PPARα in rat hepatoma cells and via PPARβ/δ in mouse intestinal cells MSIE cells. In human myocytes, both PPARα and PPARβ/δ likely play a role in ANGPTL4 gene induction by fatty acids. Using semi-quantitative Western Blot, we previously showed that 4 weeks of treatment with the PPARα agonist fenofibrate significantly raised plasma levels of the N-terminal ANGPTL4 fragment (Mandard et al. 2004). Our present ELISA data similarly reveal a marked increase in plasma levels of

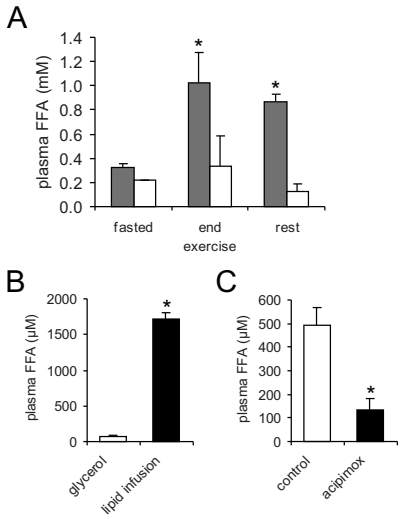


Figure 4.8: *Plasma FFA levels increase during exercise in fasted but not fed state. (A) After an overnight fast, seven subjects underwent a protocol of 2 hours of endurance exercise followed by 4 hours of post-exercise recovery either in the fasted state (grey bar) or with glucose ingestion (white bar) (study 6). Blood was samples at various time points for assessment of plasma FFA. (B) Plasma FFA levels were determined in nine healthy lean male subjects after undergoing a 6-h hyperinsulinaemiceuglycaemic clamp with simultaneous infusion of either glycerol or a lipid emulsion (study 7). (C) Plasma FFA levels were determined in nine lean males after receiving a 3-h infusion of salbutamol with or without simultaneous administration of acipimox (study 8). Error bars represent SEM. ∗ = significantly different according to Students T-test ($p<0.05$).*

full length ANGPTL4 upon fenofibrate treatment. As fibrates primarily target liver, these data suggest that liver is the most important source of plasma ANGPTL4, which is supported by the dominant expression of ANGPTL4 in human liver. Previously, Xu and colleagues found that plasma ANGPTL4 levels were decreased in obese diabetics compared to obese non-diabetics or normal weight individuals (Xu et al. 2005). It should be emphasized that, in contrast to the antibody used by Xu and colleagues, the antibody used in our ELISA specifically detects the full length ANGPTL4 protein and does not recognize any of the truncated variants of ANGPTL4. It is known that ANGPTL4 is cleaved into N- and C-terminal fragments of about equal size, which may each carry an entirely different function (Mandard et al. 2004). N-terminal ANGPTL4 has been shown to potently inhibit lipoprotein lipase, while C-terminal ANGPTL4 influences endothelial cell migration and function, possibly via suppressing the Raf/MEK/ERK signaling cascade (Yang et al. 2008). Future research will have to better address possible changes in full

length ANGPTL4 in diabetic patients. Since Angptl4 over-expression markedly elevates plasma TG levels in mice (Xu et al. 2005, Yu et al. 2005, Koster et al. 2005, Mandard et al. 2006), it is of interest to study whether in humans a positive correlation may exist between plasma ANGPTL4 and plasma TG levels. In a limited sample of 36 women, we could not find such a correlation, nor between plasma ANGPTL4 and other metabolic parameters. Although these data might argue against a major impact of ANGPTL4 on plasma TG levels in humans, recent studies indicate that a rare sequence variant of ANGPTL4 that gives rise to a dysfunctional protein is associated with decreased plasma TG levels (Romeo et al. 2007, Folsom et al. 2008, Shan et al. 2009, Talmud et al. 2008). More extensive investigations using large cohorts will need to be carried out to further explore the relation between plasma ANGPTL4 and various plasma lipid parameters. In conclusion, our data show major variation in plasma ANGPTL4 levels between individuals. Within an individual, plasma ANGPTL4 levels remain stable throughout the day but increase in response to fasting, chronic caloric restriction, and endurance exercise. Long term changes in plasma ANGPTL4 levels are likely mediated by changes in plasma FFA, which potently raise ANGPTL4 gene expression.

In revision: A. Georgiadi, L. Lichtenstein, T. Degenhardt, M. Boekschoten, M. van Bilsen, B. Desvergne, M. Müller and S. Kersten – *"Induction of cardiac Angptl4 by dietary fatty acids is mediated by PPARβ/δ and protects against oxidative stress,"* 2009

Chapter 5

Induction of cardiac Angptl4 by dietary fatty acids is mediated by PPARβ/δ and protects against oxidative stress

Abstract

Little is known about the direct effects of dietary fatty acids on gene expression in the intact heart. In the present paper we show that oral administration of synthetic triglycerides composed of one single fatty acid alters cardiac expression of numerous genes, many of which are involved in the oxidative stress response. The gene most significantly and consistently upregulated by dietary fatty acids encoded angiopoietin-like 4 (Angptl4), a circulating inhibitor of lipoprotein lipase expressed by cardiomyocytes. Induction of Angptl4 was specifically abolished in peroxisome proliferator activated receptor beta/delta (PPARβ/δ) -/- and not peroxisome proliferator activated receptor alpha (PPARα) -/- mice. Consistent with these data, fatty acids stimulated binding of PPARβ/δ but not PPARα to the Angptl4 gene. Upregulation of Angptl4 resulted in decreased cardiac uptake of plasma triglyceride (TG)-derived fatty acids and decreased fatty acid-induced oxidative stress and lipid peroxidation. In contrast, Angptl4 deletion led to enhanced oxidative stress in the heart, both after a acute oral fat load and after prolonged high fat feeding. We conclude that stimulation of cardiac Angptl4 gene expression by dietary fatty acids and via PPARβ/δ is part of a feedback mechanism aimed at protecting the heart against lipid overload and consequently fatty-acid induced oxidative stress.

5.1 Introduction

Cardiac contractility is dependent on the adequate delivery of oxygen and energy substrates to the heart followed by their efficient metabolic degradation to yield adenosine triphosphate (ATP). The energy requirements of the contracting heart are primarily met by fatty acid oxidation, with the remainder of the energy coming from glucose and lactate (Abozguia et al. 2006, Stanley et al. 2005). While fatty acids are thus of major importance to the heart, excessive uptake of fatty acids causes lipid overload or lipotoxicity and may compromise cardiac function, possibly leading to cardiomyopathy (Park et al. 2007). Consequently, cardiac uptake of fatty acids needs to be well adjusted to fatty acid utilization. Since most of the fatty acids taken up by the heart are derived from lipoprotein lipase (LPL)-dependent hydrolysis of circulating triglyceride-rich lipoproteins (Teusink et al. 2003), the activity of LPL needs to be carefully regulated via specific activators and inhibitors, especially after a fatty meal. Besides serving as a major fuel for the heart

and a potential lipotoxic substrate, fatty acids are able to regulate gene expression (Clarke 2004). In vitro experiments in rat cardiomyocytes have shown that fatty acids increase expression of uncoupling protein 2 (Ucp2), carnitine palmitoyltransferase 1 (Cpt1), Cd36 molecule (Cd36), fatty acid binding protein 3 (Fabp3), acyl-CoA synthetase long-chain family member 1 (Acsl1), acyl-coA thioesterase (Acot1), and acyl-Coenzyme A dehydrogenase, long chain (Acadl) (Brandt et al. 1998, Durgan et al. 2006, van der Lee et al. 2000, Lee et al. 2000). As these genes all represent target genes of peroxisome proliferator activated receptor alpha (PPARα) (Mandard, Muller and Kersten 2004, Rakhshandehroo et al. 2007), they suggest an important role of PPARα in fatty acid-dependent gene regulation in the heart (Finck et al. 2002). However, little is known about the direct effects of dietary fatty acids on gene expression in the intact heart. In addition, it is unclear what pathways are activated by fatty acids besides their own catabolism. Here we studied the comprehensive effects of dietary fatty acids on cardiac gene expression in vivo by giving mice a single oral bolus of synthetic triglyceride composed entirely of one single fatty acid, which were either linolenic acid (C18:3), linoleic (C18:2) acid or oleic acid (C18:1). Subsequent microarrays analysis yielded Angptl4 as the gene most highly induced in the heart after oral fat administration. The collective data suggest that induction of Angptl4 by dietary fatty acids is mediated by peroxisome proliferator activated receptor beta/delta (PPARβ/δ) and is part of a feedback mechanism aimed at protecting cardiomyocytes against lipid overload and consequently fatty-acid induced oxidative stress, e.g. lipotoxicity.

5.2 Materials and Methods

Materials: GW501516 was purchased from Alexis (Axxora, Raamsdonkveer, The Netherlands). Wy14643 was obtained from Eagle Picher Technologies laboratories (Lenexa, KS, USA). Trilinolein (9c,12c) and trilinolenin (9c,12c,15c) were from Larodan free chemicals (Malmo, Sweden). SYBR green was from Eurogentec (Seraing, Belgium) and all other chemicals were from Sigma (Zwijndrecht, The Netherlands).

Animals: Pure-bred Sv129 PPARα-/- mice (129S4/SvJae) and corresponding wildtype mice (129S1/SvImJ) were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). The Angptl4-/- and transgenic mice were on C57Bl/6 background and have been previously described (Koster et al. 2005, Mandard et al. 2006). The PPARβ/δ-/- mice were on a mixed background (Sv129/C57Bl/6) and have been previously described (Nadra et al. 2006). Males mice were used at 2.5 to 4 months of age. Mice were anaesthetized with a mixture of isofluorane (1,5%), nitrous oxide (70%), and oxygen (30%). Blood was collected via orbital puncture into EDTA tubes. After sacrifice, the hearts were excised and stored in -80 $^{\circ}C$ until further analysis. The animal studies were approved by the Local Committee for Care and Use of the Laboratory Animals at Wageningen University and the University of Lausanne, Switzerland.

Oral lipid load: Starting at 5 a.m. the animals were fasted for 4 hours followed by an intra-

gastric gavage of 400μL synthetic triglyceride (triolein, trilinolein, and trilinolenin). The control group received only carboxymethylcellulose (CMC). The mice were killed 6 hours thereafter. Four to five mice per group were used.

High fat diet: Angptl4 -/-, +/+ and transgenic mice on C57Bl/6 background received a low fat diet (LFD) or high fat diet (HFD) for 8 weeks, providing 10 or 45 energy percent in the form of triglycerides, respectively (D12450B or D12451, Research Diets, New Brunswick, USA). The major source of fat in the diet was palm oil, with 5 energy percent provided as soybean oil.

RNA isolation and qRT-PCR: Total RNA was isolated with TRIzol Reagent (Invitrogen, Breda, the Netherlands). 1μg of total RNA for the in vivo studies and 350ng of total RNA for the in vitro experiment was reverse transcribed using iScript (Bio-Rad, Veenendaal, the Netherlands). cDNA was amplified on BioRad MyIQ or iCycler machine using Platinum Taq polymerase (Invitrogen, Breda, the Netherlands). PCR primer sequences were taken from the PrimerBank and ordered from Eurogentec (Seraing, Belgium). Sequences of the primers used are available upon request.

Cell culture: Neonatal cardiomyocytes were isolated and cultured as described using differential plating to separate myocytes from non-myocytes (de Vries et al. 1997). The experiments were approved by the Institutional Animal Care and User Committee of Maastricht University. Neonatal cardiomyocytes were incubated with 1μM of GW501516 or 62.5μM, 125μM or 250μM of linolenic for 6h as previously described (de Vogel-van den Bosch et al. 2008). In a second experiment, cardiomyocytes were incubated with 1μM GW501516, 10μM Wy14643, or 250μM linolenic acid for 24h.

Plasma lipid parameters: Plasma was obtained from blood by centrifugation for 10 min at 10,000g. The plasma free fatty acids and TG concentration were determined using kits from Instruchemie (Delfzijl, The Netherlands).

Chromatin immunoprecipitation assay (ChIP): Wildtype C57Bl/6 mice were fasted for 4 hours followed by an oral gavage of Trilinolenin (n=3). Six hours thereafter the mice were killed by cervical dislocation and the hearts excised. The fresh hearts were cut into half and placed into PBS containing 1% formaldehyde. Cross-linking was stopped after 15 min by adding glycine to a final concentration of 0.125M for 5 min at room temperature. The samples were centrifuged for 5 min at 700 g at 4°C to collect the heart pieces, the supernatant was removed and washed once again with ice-cold PBS. Fresh PBS containing protease inhibitors (Roche, Almere, Netherlands) was added and the tissue was disaggregated with a homogenizer Ultra Turrax T25 basic (Ika Werke, Staufen, Germany). The tissue was distributed into 3 tubes (2 mL each), centrifuged for 5 min at 700 g at 4°C. After the supernatant was removed, heart homogenate was resuspended in lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl pH 8.1, protease inhibitors) and the lysates were sonicated with a Bioruptor TM (Diagenode, Lige, Belgium) to achieve a DNA length of 300-800 bp. After removal of cellular debris by centrifugation, supernatants were diluted 1:10 in ChIP dilution buffer (150mM NaCl, 1% Triton X-100, 2mM EDTA, 20 mM Tris-HCl pH 7.5, protease inhibitors). Chromatin was incubated overnight at 4°C with 2μg an-

tibody, 25μl BSA (100 mg/ml) and 2.4μl sonicated salmon sperm (10 mg/ml). The following antibodies were used: anti-PPARα (sc-9000), and anti-PPARβ/δ (sc-7197). All were obtained by Santa Cruz Biotechnologies (Heidelberg, Germany). Immunocomplexes were collected with 25μl MagaCell©Protein A Magnetic beads (Isogen Life Science) for 1hour at room temperature, and subsequently washed with 700μL of the following buffers: ChIP was buffer 1 (150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8, protease inhibitors) two times, ChIP wash buffer 2 (500mM NaCl, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8, protease inhibitors), ChIP wash buffer 3 (250 mM LiCi, 1% NP40, 1% Deoxycholate, 1mM EDTA, 10 mM Tris-HCl pH 8), two times TE buffer (1 mM EDTA, 10 mM Tris-HCl pH 8). Elution of immucomplexes were carried out in 250μL elution buffer (10 mM EDTA, 0.5% SDS, 25mM Tris-HCl pH 7.5) at 64°C for 30 min. After collection of supernatant, elution was repeated with 250μl elution buffer at room temperature for 2 min. After combining the supernatants, cross-linking was reversed at 64°C overnight with 2.5μl Proteinase K (20 mg/ml) for digestion of any remaining proteins. Genomic DNA fragments were recovered by phenol-chloroform extraction with phase lock gel (Eppendorf, Wesseling-Berzdorf, Germany), followed by salt-ethanol precipitation. Samples were diluted in sterile H_2O, and analyzed with qPCR. The ChIP data are normalized against IgG to account for non-specific immunoprecipitation. A fold-enrichment value of 1 represents baseline thus no enrichment and no specific precipitation. Primers were chosen to study binding of PPARs to the transcriptional start site of the Angptl4 and Ucp3 genes, and to the previously identified PPRE within intron 3 of the Angptl4 gene. The ribosomal phosphoprotein P0 (Rplp0) was used as negative control for PPAR binding. The sequences of primers used in ChIP were as follows: Ucp3-TSS: (For:5'-GAGCCCCAGGTCACGGAAG-3', Rev:5'- CTGTGCGTCTAGCCA AGGTTG-3'), Angptl4-TSS: (For:5'-CCAGCAAGTTCATCTCGTCC-3', Rev:5'-TCCCTCCCACTCCCACACC-3'), Angptl4-PPRE: (For:5'-TCTGGGTCTGCCCCCAC TCCTGG-3', Rev:5'-GTGTGTGTGTGGGATACGGCTAT-3'), Rplp0 (For:5'-CGAGGACCGCC TGGTTCTC-3', Rev:5'-GTCACTGGGGAGAGAGAGG-3').

In Vivo Clearance of very low density lipoprotein (VLDL)-Like Emulsion Particles: Tissue uptake of [^{3}H]-labeled TG packaged into VLDL-like emulsion particles was measured as previously described (Lichtenstein et al. 2007). The data shown represent the percentage of injected radioactivity taken up by the heart after 30 min.

Immunohistochemistry: Deep frozen tissues (-80°C). were cryosectioned (5μm) with a cryostat (Leica, CM1900 UV). Immunostaining of protein adducts of the lipid peroxidation byproduct 4-hydroxy-2-nonenal (4-HNE) was performed on freshly cut frozen sections using a rabbit polyclonal antibody (Calbiochem, San Diego, CA, USA). All steps were carried out at room temperature. The tissue was fixed in 70% ethanol for 3 min and then rinsed in PBS 1X for 3 min. To block endogenous peroxidase activity, slides were incubated with 3% H_2O_2 for 10 min. Incubation with the primary antibody (rabbit anti 4-HNE protein-adducts, 1:50 dilution in PBS 1X) was performed for 1h. After rinsing with PBS 1X, tissue was incubated for 45 min with the

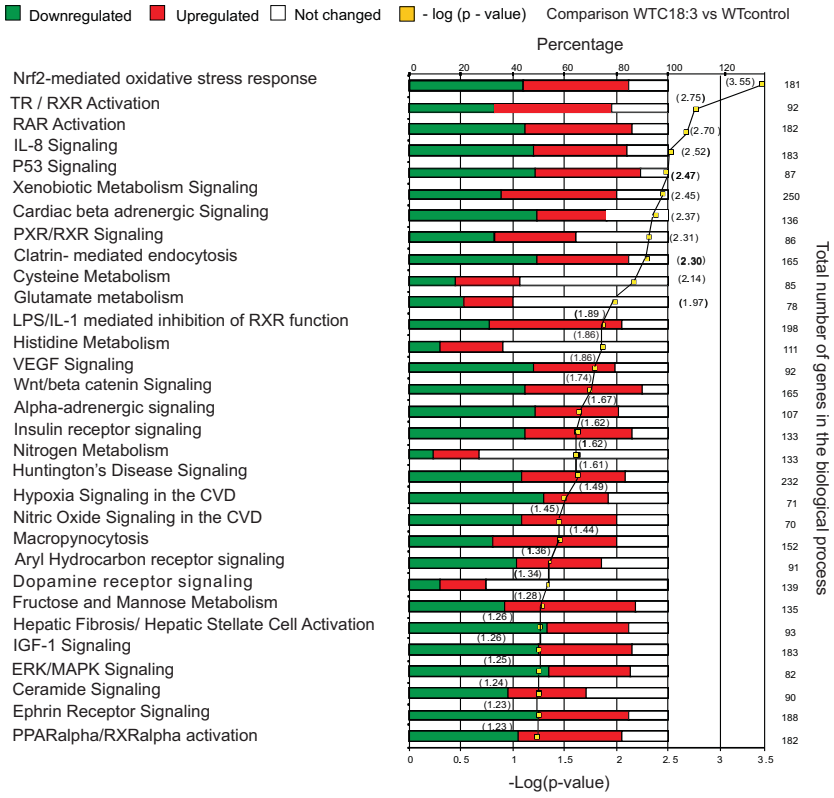


Figure 5.1: *Induction of oxidative stress response 6 hours after the oral ingestion of fatty acids. Bars show the percentage of upregulated genes (red) and downregulated genes (green) out of the total number of eligible genes for each pathway, based on the Ingenuity knowledge database. The white part represents the percentage of genes that do not overlap with the experimental dataset. The pathways are displayed from the direction of the most significantly regulated to least significantly regulated based on Fisher's Exact Test p-value (cut off p<0.05). The -log(p-value) is displayed on the top of each pathway (yellow square).*

secondary antibody (Dako EnVision+ System Labelled Polymer-HRP AntiRabbit). Visualization of the complex was carried out using AEC substrate chromogen (Dako Cytomation) for 15 minutes. Sections were mounted with Kaiser's glycerol gelatin mounting medium (Merck KGaA, Darmstadt, Germany). Normal rabbit serum was used as a negative control (Vector Laboratories). Immunostaining of Angptl4 in human heart was performed using an antibody directed against the C-terminus of Angptl4. Five-micrometer sections of paraffin-embedded human heart were

mounted on Superfrost microscope slides. These sections were dewaxed in xylene and rehydrated in a series of graded alcohols. To block endogenous peroxidase activity, slides were incubated with 3% H_2O_2 for 20 min. Antigen retrieval was performed by placing the slides in citrate buffer (pH 6.0) and heat them in a microwave oven 5 min 700 W (without lid) and 4 times 5 min 500 W (with lid). After cooling down to room temperature, the sections were briefly washed with PBS. Prior to staining, a 20 min preincubation was performed using 20% normal goat serum (Vector Laboratories, Burlingame, CA, USA). Incubation with the primary antibody (1:50) was performed for 1h. After rinsing with PBS 1X, tissue was incubated for 45 min with the secondary antibody (Dako EnVision+ System Labelled Polymer-HRP AntiRabbit). Visualization of the complex was carried out using AEC substrate chromogen (Dako Cytomation) for 15 minutes. After counterstaining with Meyer's hematoxylin, sections were mounted with DePex mounting medium (Gurr, BDH, Poole, Dorset, UK). Negative control staining was performed using only the secondary antibody.

Tissue homigenization and quantification of oxidative stress: The extent of lipid peroxidation in heart homogenates was determined by measuring the levels of hydroxynonenal-histidine (HNE-His) protein adducts and malondialdehyde (MDA) adducts. 25mg of heart tissue were homogenized in 250μL of tissue homogenation buffer (1mM EDTA, PBS 1X pH 7.4 containing protease inhibitors). Heart tissue was homogenized over ice by needle sonication for 15sec at 40V. Heart homogenates were centrifuged at 1600xg for 10min at $4°C$. Protein content was determined in tissue supernatants by BCA Protein assay reagent. Starting from a protein concentration of 10 μg/mL 4-HNE-His protein adducts and MDA adducts content were quantified using the Oxiselect HNE-His Adduct ELISA kit (Cell Biolabs Inc., San Diego, USA) and MDA Adduct ELISA kit (Cell Biolabs Inc.), respectively. The quantity of HNE-His protein adducts was determined by using a standard curve containing known amounts of HNE-BSA (0-10μg/ml). For the quantification of MDA adducts a standard curve of known amounts of MDA-BSA (0-120pmol/mg) was used.

Tissue triglyceride content: Triglycerides content was measured in tissue homogenates with Triglyceride LiquiColor Test (Mono) HUMAN GmbH, kit (Instruchemie, Delfzijl, The Netherlands). 5% tissue homogenates were prepared by needle sonication over ice in tissue homogenization buffer consisting of 10mM Tris, 2mM EDTA, 0.25M sucrose pH 7.5.

Affymetrix microarray and pathway analysis: Expression profiling was carried out on individual mouse hearts. Total RNA (5 μg) was labeled using the Affymetrix One-cycle Target Labeling Assay kit (Affymetrix, Santa Clara, CA). The correspondingly labeled RNA samples were hybridized on Affymetrix Mouse Genome 430 2.0 Arrays, washed, stained and scanned on an Affymetrix GeneChip 3000 7G scanner. Packages from the Bioconductor project, integrated in an in-house developed on-line management and analysis database for multiplatform microarray experiments, were used for analyzing the scanned arrays (Sanderson et al. 2008). Probesets were redefined according to Dai et al. as the genome information utilized by Affymetrix at the time of designing the arrays is not current anymore, resulting in unreliable reconstruction of expression levels (Yoshida et al. 2002). In this study probes were reorganized based on the Entrez Gene database,

build 36, version 2 (remapped CDF v10). Expression estimates were obtained by GC-robust multi-array (GCRMA) analysis, employing the empirical Bayes approach for background adjustment, followed by quantile normalization and summarization. Differentially expressed probesets were identified using linear models, applying moderated t-statistics that implement empirical Bayes regularisation of standard errors (Sukonina et al. 2006). A probeset was found to be significantly changed after treatment if $p<0.05$. For the pathway analysis ingenuity software version 6.5 was used.

5.3 Results

In order to study the acute effects of dietary fatty acids on cardiac gene expression in vivo, SV129 mice were given a single oral gavage of synthetic TGs composed entirely of either C18:1, C18:2, or C18:3 (Sanderson et al. 2008), thus mimicking a post-prandial lipid challenge. Animals receiving carboxymethylcellulose were used as control in order to study the absolute effect of dietary fatty acids. Expression profiling was carried out on individual mouse hearts collected 6 hours after the gavage using Affymetrix Mouse Genome 430 2.0 Arrays. Pathway analysis using Ingenuity revealed that the dominant pathway affected by the oral fat load was nuclear factor-like 2 (Nrf2)-related oxidative stress, indicating that the fatty acids induced oxidative stress (Figure 5.1). This was supported by examination of the top 20 of upregulated genes, most of which were involved in the oxidative stress response, including uncoupling protein 3 (Ucp3), heme oxygenase 1 (Hmox1), FK506 binding protein 5 (Fkbp5), lipocalin 2 (Lcn2), glutathione S-tranferase A3 (Gsta3),and metallothionein 2 (Mt2) (Figure 5.2).

A large correspondence in gene regulation between the fatty acids was observed, especially between C18:2 and C18:3. Indeed, scatter plot analysis indicated that the effects of C18:2 and C18:3 on cardiac gene expression were remarkably similar, whereas effects of C18:1 were somewhat different (Figure 5.3). Therefore the remainder of the paper will focus on effects of C18:3. Apart from genes involved in the oxidative stress response, various genes involved in lipid metabolism were also induced by the fatty acids. Interestingly, the gene most significantly and consistently upregulated by each of the dietary fatty acids was Angptl4 (Figure 5.2), which encodes a secreted protein involved in the regulation of plasma TG levels. Previous studies have shown that Angptl4 potently inhibits LPL and accordingly plasma TG clearance by converting active LPL-dimers into inactive LPL-monomers (Lichtenstein et al. 2007, Yoshida et al. 2002, Sukonina et al. 2006). Although Angptl4 is known to be expressed in heart (Yu et al. 2005, Zandbergen et al. 2006) the specific cardiac cell types that produce Angptl4 remain unclear. Accordingly, we performed immunohistological staining of Angptl4 in human heart samples. The results reveal the presence of Angptl4 protein in cardiomyocytes and vascular smooth muscle cells but not endothelial cells and fibroblasts (Figure 5.4). Significant production of Angptl4 by cardiomyocytes was confirmed by the relatively low Ct values for amplification of Angptl4 cDNA from rat cardiomyocytes (Ct

Figure 5.2: *Cardiac Angptl4 expression is highly sensitive to dietary fatty acids. Top 20 of genes upregulated after oral gavage of synthetic triglyceride composed of either linolenic acid (C18:3), linoleic acid (C18:2), or oleic acid (C18:1). The heatmaps were generated directly from the GCRMA normalized microarray data. Genes are ranked according to mean fold-change with wildtype mice receiving CMC serving as control. Only probesets showing significant upregulation by the different fatty acids were included in the analysis (P<0.05).*

22-23, data not shown).

Long-chain fatty acids are bona fida ligands for the peroxisome proliferator activated receptors

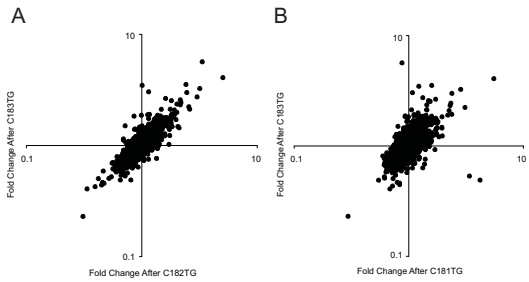


Figure 5.3: *Similarity in cardiac gene regulation between linolenic acid and linoleic acid. Graphs show fold-change in gene expression after treatment with C18:3TG (y-axis) plotted against fold-change in gene expression after treatment with C18:2TG (A) or C18:1TG (B) (x-axis). Analysis shows more significant similarity in gene regulation between C18:3 and C18:2, compared to between C18:3 and C18:1.*

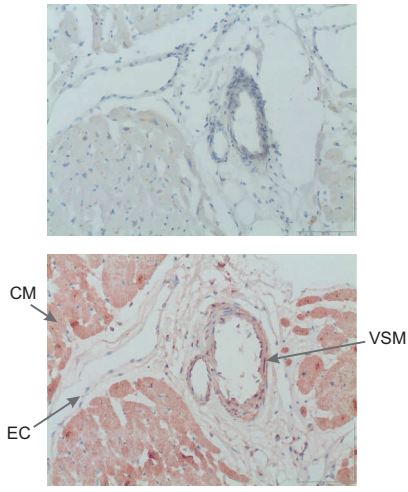


Figure 5.4: *Angptl4 protein localizes specifically to the cardiomyocytes and not to endothelial cells. High magnification image of the human heart tissue stained with an antibody against Angptl4 (bottom) or negative control (top). Arrows point to endothelial cells (EC), vascular smooth muscle cells (VSM) and cardiomyocytes (CM).*

(PPARs). The previous demonstration that Angptl4 is a direct PPAR target gene prompted us to investigate the role of PPARs in Angptl4 gene regulation by dietary fatty acids. Since cardiomyocytes predominantly express PPARα and PPARβ/δ and only low amounts of peroxisome proliferator activated receptor γ (PPARγ) (Gilde et al. 2003), the focus was on PPARα and PPARβ/δ.

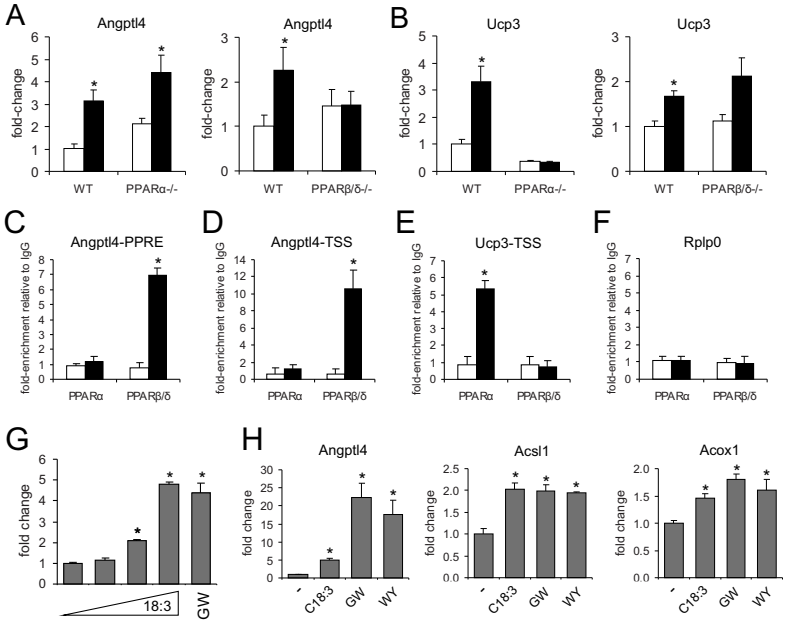


Figure 5.5: *PPARβ/δ but not PPARα mediates the induction of Angptl4 expression by dietary linolenic acid. Wildtype, PPARα-/-, and PPARβ/δ-/- were given a single oral gavage of 0.5% CMC (open bars) or synthetic TG composed entirely of C18:3 (closed bars). mRNA expression levels of Angptl4 (A) and Ucp3 (B) were determined in mouse heart using real-time PCR. Results are expressed as fold-change compared to the WT control mice. (C-F) ChIP was performed on hearts of wildtype mice given an oral gavage of either CMC or C18:3. Chromatin was precipitated using antibodies against PPARα or PPARβ/δ. Rabbit IgG was used as a specificity control. Real-time qPCR was performed on reverse-crosslinked chromatin templates using primers specific to the known PPRE in intron 3 of the Angptl4 gene (C), the TSS of Angptl4 (D), the TSS of Ucp3 (E), and the negative control gene RpLp0 (F). (G) Rat neonatal cardiomyocytes were incubated with increasing concentrations of linolenic acid (0, 62.5, 125 and 250 μM) or GW501516 (1μM) for 6h. Angptl4 expression was determined by qPCR. (H) Rat neonatal cardiomyocytes were incubated with linolenic acid (250μM), GW501516 (1μM), or Wy14643 (10μM), for 24h. Expression of Angptl4 and known PPAR targets Acsl1 and Acox1 was determined by qPCR. Error bars represent SEM. Statistical significance was determined with a Student's T-test (P<0.05).*

PPARα -/- mice , PPARβ/δ -/- mice, and the corresponding wildtype mice were given an oral gavage of C18:3 as synthetic TG. Hearts were collected 6 hours thereafter for analysis of gene expression by quantitave PCR. Remarkable, upregulation of Angptl4 by C18:3 was entirely abolished in the PPARβ/δ -/- mice, while it was retained in the PPARα-/- mice (Figure 5.5a). In contrast, upregulation of Ucp3, another well-characterized PPAR target gene, was retained in PPARβ/δ -/- mice and completely abolished in PPARα-/- mice (Figure 5.5b). No compensatory increase in PPARβ/δ and PPARα expression was observed in PPARα-/- and PPARβ/δ -/- mice, respectively

(Figure 5.6). To examine whether regulation of Angptl4 by PPARβ/δ and not PPARα was supported by binding of PPARβ/δ to the Angptl4 gene, we performed chromatin imunoprecipitation (ChIP). Previously, we located the response element responsible for PPAR-mediated upregulation to intron 3 of the Angptl4 gene (Mandard et al. 2004). Consistent with data on Angptl4 gene regulation, ChIP on hearts of wildtype mice six hours after oral gavage of C18:3 showed enhanced binding of PPARβ/δ but not PPARα to the intronic PPRE (Figure 5.5c). Nuclear receptors and other transcription factors bound to such distal sites likely contact the basal transcription machinery via DNA looping, and accordingly binding of PPAR to distant PPREs can be demonstrated by showing cross-linking of PPAR to the transcriptional start site (TSS) (Palstra et al. 2008, Saramki et al. 2009). Indeed, oral gavage of C18:3 enhanced binding of PPARβ/δ but not PPARα to TSS of the Angptl4 gene (Figure 5.5d), while C18:3 enhanced binding of PPARα but not PPARβ/δ to the TSS of the Ucp3 gene (Figure 5.5e). No binding of either PPARα or PPARβ/δ to the negative control gene Rplp0 was observed (Figure 5.5f). These results demonstrate that the induction of Angptl4 gene expression by dietary C18:3 is mediated by PPARβ/δ.

To further investigate whether the fatty acid- and PPARβ/δ-mediated induction of cardiac Angptl4 expression was occurring in cardiomyocytes, rat neonatal cardiomyocytes were incubated for 6h with increasing concentrations of C18:3 or with the synthetic PPARβ/δ agonist GW501516. The results show that C18:3 caused a dose-dependent increase in Angptl4 mRNA, which at the highest concentration was equivalent to that obtained using GW501516 (Figure 5.5g). Our results do not imply that Angptl4 is an exclusive target gene of PPARβ/δ under any type of circumstances. Indeed, we find that in cardiomyocytes Angptl4 is induced to a similar extent by synthetic PPARα and PPARβ/δ agonists, similar to other cardiac PPAR targets such as Acsl1 and Acox1 (Figure 5.5h). Instead, our data suggest that the stimulatory effect of dietary fatty acids on cardiac Angptl4 expression is mediated specifically by PPARβ/δ.

To study the effect of Angptl4 on the metabolic response to dietary fat, we performed the oral fat load with C18:3 TG in wildtype, Angptl4-/- and Angptl4-Tg mice. In agreement with inhibition of LPL by Angptl4, the post-prandial increase in plasma TG was dramatically increased in Angptl4-Tg mice, whereas it was entirely blunted in Angptl4-/- mice (Figure 5.7a). Consistent with LPL inhibition, Angptl4 overexpression markedly reduced cardiac fatty acid uptake from [^{3}H]triolein-labeled VLDL-like particles (Figure 5.7b). These results suggest that upregulation of Angptl4 by dietary fatty acids will lead to reduced cardiac uptake of fatty acids via inhibition of LPL, thereby suppressing the stimulus that led to induction of Angptl4 expression.

To examine whether the inhibitory effect of Angptl4 on cardiac fatty acid uptake is associated with reduced fatty acid-induced oxidative stress, expression of Fkbp5, Lcn2, and Gsta3 was determined 6h after oral gavage with either control treatment (CMC) or C18:3TG in wildtype, Angptl4-/- and Angptl4-Tg mice. All three genes represent markers of oxidative stress (Roudkenar et al. 2007, Edwards et al. 2003, Black et al. 2008). Consistent with a protective role of Angptl4 against fatty acid-induced oxidative stress, the magnitude of induction of Fkbp5, Lcn2, and Gsta3

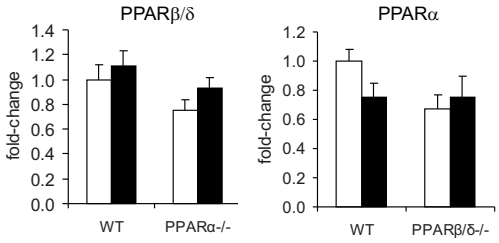


Figure 5.6: *No compensatory increase in PPARβ/δ and PPARα expression in PPARα-/- and PPARβ/δ -/- mice, respectively. Wildtype, PPARα-/-, and PPARβ/δ-/- were given a single oral gavage of 0.5% CMC (open bars) or synthetic TG composed entirely of C18:3 (closed bars). mRNA expression levels of PPARβ/δ (left panel) and PPARα (right panel) were determined in mouse heart using real-time PCR. Results are expressed as fold-change compared to the WT control mice.*

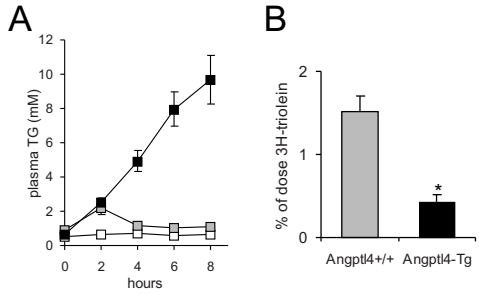


Figure 5.7: *Angptl4 overexpression raises post-prandial plasma TG levels and decreases cardiac fatty acid uptake. (A) Wildtype (grey squares), Angptl4-/- (open squares) and Angptl4-Tg (black squares) mice were given a single oral gavage of synthetic TG composed entirely of C18:3. Plasma TG were determined in blood collected via the tail vein. (B) [^{3}H]-labeled triolein was incorporated into VLDL-like emulsion particles and directly injected into the tail vein of wildtype and Angptl4-Tg mice. The heart was collected 30 min after the injection for determination of radioactivity. ∗ = significantly different between WT and Angptl4-Tg mice according to Student's T-test (P<0.05).*

by C18:3 was inversely correlated with Angptl4 expression (Figure 5.8a & b). Expression of Fkbp5, Lcn2, and Gsta3 after the oral fat load was not related to plasma free fatty acid (FFA) levels, which followed an opposite pattern (Figure 5.8c). Expression of the ER stress marker Herpud1 mimicked the pattern of the oxidative stress markers (Figure 5.8a).

Finally, we examined whether Angptl4 may exert a similar effect in the context of a chronic fat overload. To that end, we measured expression of the oxidative stress markers and performed immunohistochemical and quantitative analysis of 4-HNE protein adducts in wildtype and Angptl4-/-

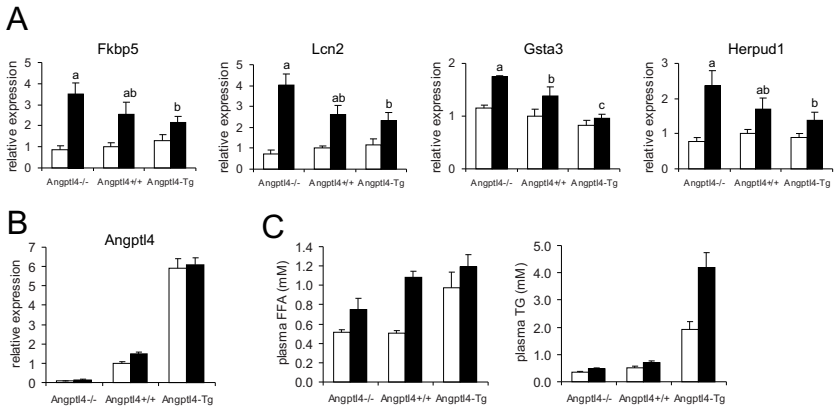


Figure 5.8: *Markers of oxidative stress are inversely correlated with Angptl4 expression after oral fat load. Wildtype, Angptl4-/- and Angptl4-Tg mice were given a single oral gavage of 0.5% CMC (open bars) or synthetic TG composed entirely of C18:3 (closed bars). Mice were sacrificed 6 hours later. (A) mRNA expression levels of oxidative stress genes Fkbp5, Lcn2,and Gsta3, and ER stress marker gene Herpud1 as determined by real-time PCR. Bars with different letters are significantly different (Student's T-test, P<0.05). (B) mRNA expression levels of Angptl4. (C) Plasma levels of FFA and TG.*

mice fed a high fat diet (HFD) for 8 weeks. 4-HNE is one of the major biologically active aldehydes formed during inflammation and oxidative stress. Formation of 4-HNE- protein adducts is a marker for lipid peroxidation. Although high fat feeding did not influence expression levels of Fkbp5, Lcn2 and Gsta3, expression was significantly higher in Angptl4-/- mice fed HFD compared to wildtype mice fed HFD (Figure 5.9a). A similar trend was observed for Herpud1. Furthermore, lipid peroxidation was increased in Angptl4-/- mice fed HFD, as shown by enhanced 4-HNE staining (Figure 5.9b). These results were supported by quantitative analysis of 4HNE protein adducts (Figure 5.9c) and MDA adducts (Figure 5.9d), which were significantly increased in Angptl4-/- mice fed HFD compared to wildtype mice fed HFD. These data indicate that Angptl4 protects against oxidative stress in the context of a chronic fat overload. No differences in cardiac triglyceride levels were observed between wildtype and Angptl4-/- mice (Figure 5.9e).

5.4 Discussion

In the present paper we show that the gene most significantly and consistently upregulated by short term treatment with dietary fatty acids is Angptl4. Induction of Angptl4 by dietary fatty acids is mediated by PPARβ/δ and confers a protective effect against fatty acid-induced oxidative stress by restricting cardiac fatty acid uptake via inhibition of LPL. Overall, our data suggest that upregu-

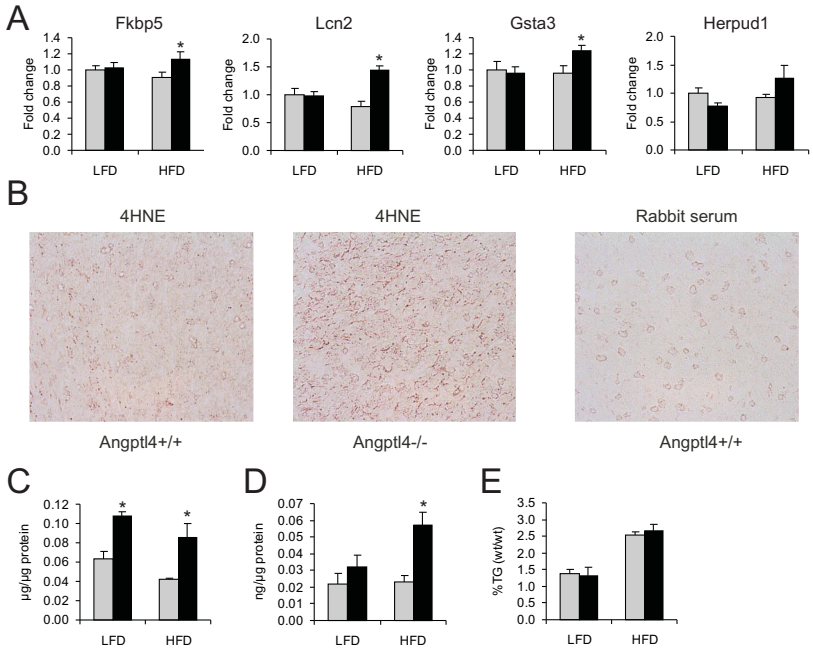


Figure 5.9: *(A) Wildtype and Angptl4-/- mice were fed a LFD or HFD for 8 weeks. Expression levels of Fkbp5, Lcn2, Gsta3 and Herpud1 in the heart were measured by real-time PCR. Results are expressed as fold-change compared to wildtype mice on LFD. (B) Immunohistochemistry of 4-HNE protein adducts in mouse cardiac tissue from mice fed HFD for 8 weeks. Representative sections are shown. Right panel shows negative control obtained using rabbit serum. Quantitative measurement of 4HNE protein adducts (C) and MDA adducts (D). (E) Cardiac triglyceride content. Grey bars = wildtype mice, black bars = Angptl4-/- mice. ∗ = significantly different between WT and Angptl4-/- mice according to Student's T-test (P<0.05).*

lation of Angptl4 by fatty acids is part of a feedback mechanism aimed at preventing myocardial fatty acid accumulation, thereby minimizing lipid-induced oxidative stress and lipotoxicity (Figure 5.10). Lipotoxicity describes the untoward consequences of fat overload in a particular tissue and may be related to fatty acid-induced oxidative stress, accumulation of lipotoxic intermediates such as ceramides and fatty acyl-CoA, and excess storage of triglycerides (Schaffer 2003). Chronic lipotoxicity in the heart has been shown to promote cardiomyopathy in several animal models (Chiu et al. 2001, Chiu et al. 2005, Finck et al. 2002, Yagyu et al. 2003). Most of these models are characterized by a mismatch between myocardial fatty acid uptake and utilization, as in mice with heart-specific overexpression of acyl-CoA synthetase (ACS), fatty acid transport protein 1, or lipoprotein lipase (Chiu et al. 2001, Chiu et al. 2005, Yagyu et al. 2003). Although triglyc-

erides are unlikely to be the actual culprit in cardiac lipoxicity, they may be guilty by association as its levels may be positively correlated with lipotoxic intermediates. However, we did not see increased cardiac triglyceride levels in Angptl4-/- mice compared to wildtype mice after 8 weeks of high fat feeding, suggesting that the increase in fatty acid uptake is limited or that the incoming fatty acids are efficiently oxidized. In the present study, it was not possible to investigate the effect of Angptl4 deletion on cardiac lipid storage, oxidative stress, and parameters of cardiac dysfunction after a more prolonged period of HFD, as a cachectic phenotype progressively emerges after 12 weeks of HFD (Lichtenstein et al., in preparation). In a previous study, heart-specific Angptl4 overexpression reduced cardiac LPL activity and reversed the excessive lipid storage in hearts of lipotoxic ACS-transgenic mice 20. In agreement with these data, we find that Angptl4 overexpression reduced cardiac fatty acid uptake and protects against fatty acid-induced oxidative stress. Angptl4 was discovered by screening for target genes of PPARα and PPARγ in liver and adipose tissue, respectively (Yoon et al. 2000, Kersten et al. 2000). It is produced by a variety of organs and may exert both paracrine/autocrine and endocrine effects. Intravenous injections of recombinant Angptl4 protein as well as numerous studies using Angptl4 transgenic or knockout mice have invariably shown a stimulatory effect of Angptl4 on plasma TG levels, which is achieved by inhibiting LPL activity (Yoshida et al. 2002, Yu et al. 2005, Backhed et al. 2004, Ge, Yang, Yu, Pourbahrami and Li 2004, Xu et al. 2005, Koster et al. 2005, Mandard et al. 2006, Desai et al. 2007). These data have established Angptl4 as an important circulating regulator of plasma TG levels. The present data suggest that Angptl4 not only acts as an endocrine factor, but also acts locally in an autocrine/paracrine manner in response to dietary fatty acids to inhibit local LPL activity and consequently fatty acid uptake. The PPAR-dependent upregulation of Angptl4 and Ucp3 by dietary linolenic acid underscores the importance of TG-rich lipoproteins as a potent source of PPAR ligands in the heart, which are liberated via the action of LPL (Ziouzenkova et al. 2003, Augustus et al. 2004). Remarkably, upregulation of Angptl4 by C18:3 was mediated by PPARβ/δ and not PPARα, while the induction of Ucp3 was regulated exclusively via PPARα. These data were supported by ChIP data showing C18:3-induced binding of PPARβ/δ and not PPARα to the Angptl4 gene. This is somewhat surprising as Angptl4 was originally identified as PPARα target gene in liver and has been shown to be potently activated by PPARα agonists in numerous organs and cell lines, including liver, small intestine, kidney, and C2C12 myotubes (Yu et al. 2005, Mandard et al. 2004a, Kersten et al. 2000, Ge et al. 2005, Bunger et al. 2007).

At the same time, Angptl4 has been shown to be highly inducible by PPARβ/δ agonists, as observed in liver, skeletal muscle, adipose tissue, keratinocytes, fibroblasts, and C2C12 myotubes (Akiyama et al. 2004, Schmuth et al. 2004, Schug et al. 2007, Rieck et al. 2008); our unpublished observations). It is unclear what mechanism may underlie the differential regulation of Angptl4 and Ucp3 by PPARβ/δ vs. PPARα, respectively. Gel shift and transactivation studies have failed to provide convincing evidence for the existence of response elements or promoters that are specifically or selectively bound or regulated by a particular PPAR isotype (Juge-Aubry

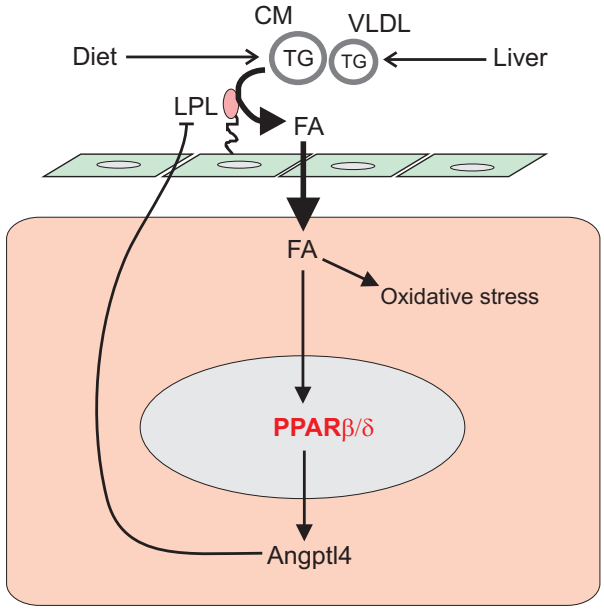


Figure 5.10: *Model of the role of Angptl4 in the heart. Triglycerides arrive at the heart as TG packaged into chylomicrons or VLDL particles. Plasma TG are hydrolyzed by lipoprotein lipase to release fatty acids, which are taken up by the cardiomyocyte. Excess uptake of fatty acids gives rise to oxidative stress and leads to induction of Angptl4 expression via PPARβ/δ. Angptl4 will feed back on fatty acid uptake by inhibiting lipoprotein lipase.*

et al. 1997, Heinaniemi et al. 2007). Indeed, in vitro transactivation experiments revealed that all three PPARs are inherently able to transactivate the human and mouse Angptl4 gene (Mandard et al. 2004). Clearly, our knowledge of the molecular determinants of PPAR binding and promoter regulation in vivo is insufficient. In the present study, induction of cardiac Angptl4 by dietary fatty acids in vivo was exclusively mediated by PPARβ/δ. In cultured cardiomyocytes we found that Angptl4 expression was equally induced by C18:3 and the synthetic PPARβ/δ agonist GW501516. In these cells, Angptl4 is also upregulated by synthetic agonists for PPARα (our unpublished data), which may account for the inhibitory effect of PPARα activation on LPL activity in these cells (Carroll and Severson 2001). Currently, the effect of PPARβ/δ activation on LPL activity in cardiomyocytes remains unclear. Recently, targeted PPARβ/δ overexpression was shown to have a clear differential effect on cardiac metabolism compared to PPARα overexpression (Burkart et al. 2007). In contrast to PPARα, PPARβ/δ overexpression did not impact fatty acid transport and failed to induce myocardial lipid accumulation. Based on the data presented here it can be hypothesized that PPARβ/δ is neutral towards cardiac lipid storage by inducing Angptl4

expression, which in turn feeds back on fatty acid uptake. Multiple studies support an effect of Angptl4 on angiogenesis and endothelial function, although presently it is unclear whether its role is primarily pro- or anti-angiogenic (Le Jan et al. 2003, Ito et al. 2003, Cazes et al. 2006, Galaup et al. 2006, Hermann et al. 2005, Padua et al. 2008). Our immunohistochemical results indicate that Angptl4 is absent from vascular endothelial cells in the heart, whereas it is abundantly present in cardiomyocytes. These data are in line with previous studies showing that Angptl4 is absent from a number of different endothelial cells, yet is dramatically induced under hypoxic conditions 53, 55. Hypoxia also upregulates Angptl4 in cardiomyocytes (Belanger et al. 2002). Induction of Angptl4 by hypoxia and the associated inhibition of fatty acid uptake may be an adaptive mechanism to shift fuel use towards glucose, which requires less oxygen for oxidation. In conclusion, our data show that an acute oral load of triglycerides stimulates an oxidative stress response in the heart. The concomitant upregulation of Angptl4 by dietary fatty acids is mediated by PPARβ/δ and is part of a feedback mechanism aimed at protecting the heart against lipid overload and consequently fatty-acid induced oxidative stress, one of the hallmarks of lipotoxic cardiomyopathy.

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Chapter 6

Angptl4 protects against severe pro-inflammatory effects of dietary saturated fat

Abstract

Consumption of saturated fat is linked to numerous chronic diseases, including cardiovascular disease and inflammatory bowel disease. Here we identify Angiopoietin-like protein 4 as a fat-sensitive intestinal hormone produced by specific enteroendocrine cells whose expression is critical for the proper response to dietary saturated fats. Strikingly, in mice lacking Angptl4 dietary saturated fat induces a severe and ultimately lethal phenotype consisting of fribrinopurulent peritonitis, intestinal inflammation and fibrosis, and cachexia. These clinical abnormalities are preceded by a massive acute phase response, which is specifically induced by a high saturated but not unsaturated fat diet. Further analysis revealed that Angptl4-/- mice exhibit decreased mucosal immune function. Angptl4 was shown to bind integrin αVβ5 which was localized to intestinal neutrophils, suggesting Angptl4 modulates mucosal immunity via integrin αVβ5. Taken together, we demonstrate that Angptl4 governs mucosal immunity and protects against the pro-inflammatory and ultimately lethal effects of dietary saturated fat.

6.1 Introduction

Elevated saturated fat consumption is associated with increased risk for numerous chronic diseases, including cardiovascular disease, inflammatory bowel disease, and type 2 diabetes (Mann 2002). However, the underlying mechanism and why specifically saturated fat is harmful largely remains unknown. Consequently, there is a need to better understand the physiological and molecular mechanisms that underlie the response to high fat consumption. While much is known about the acute effects of dietary fats, including effects on satiety and satiation, there is poor understanding of the regulatory mechanisms invoked upon chronic fat overconsumption. The gastro-intestinal (GI) tract serves as the entry point for nutrients and energy in the body and hence represents a key organ for therapeutic targeting of diseases related to nutrient overconsumption. Besides carrying out nutrient digestion and absorption via the production of numerous digestive enzymes, the GI-tract also produces a variety of hormones that play pivotal roles in nutrient handling and energy homeostasis. These gut hormones are synthesized by specialized enteroendocrine cells located in the epithelial layer along the GI-tract. Functions of gut hormones include priming

the body for subsequent absorption of dietary nutrients, controlling the delivery of bile and pancreatic juice into the gut lumen, orchestrating the renewal of the gut epithelium, and regulating food intake (Drucker 2007, Sanger and Lee 2008). Release of several gut hormones is known to be influenced by fat entering GI tract, including GLP-1 and cholecystokinin (Beglinger and Degen 2004, Herrmann et al. 1995). In addition, dietary fat may induce gut hormones at the level of gene expression, representing a more chronic type of regulation. We hypothesized that by studying the changes in expression of secreted proteins in response to chronically elevated saturated fat consumption, we would be able to identify key hormones involved in the handling of dietary saturated fats.

6.2 Materials and Methods

Animals: All animal studies were done using pure-bred WT and Angptl4 -/- mice on a C57Bl/6 background (Koster et al. 2005). In study 1 male 11-week old mice were fed a LFD or HFD for 8 or 19 weeks, providing 10 or 45% energy percent as triglycerides (D12450B or D12451, Research Diets Services, Wijk bij Duurstede, The Netherlands). Mice were anaesthetized with a mixture of isoflurane (1.5%), nitrous oxide (70%) and oxygen (30%). Blood was collected by orbital puncture into EDTA tubes. The mice were killed by cervical dislocation, after which tissues were excised and directly frozen in liquid nitrogen or prepared for histology. In study 2 male 10-18 week old mice were fed HFD for 3 weeks. The fat source of the HFD was either palm oil (HFD used in the first study), lard, MCT oil, or safflower oil. Blood was collected from the tail vein at weekly intervals. The animal studies were approved by the Local Committee for Care and Use of Laboratory Animals at Wageningen University. Two terminally ill animals were transferred to the Small Animal Pathology laboratory of the Faculty of Veterinary Medicine at Utrecht University for a formal autopsy by a licensed animal pathologist. A detailed macroscopic, microscopic and cytologic report was prepared.

Lipid excretion and absorption: During week 3-5 of high fat feeding, the amount of lipid excreted was measured in the faecal samples using established methods. Measurement of intestinal lipid absorption using ^{3}H-labeled triolein and ^{14}C-labeled palmitic acid was carried out exactly as previously described (Goudriaan et al. 2002).

Ascites: Diagnostic assays on the ascites fluid (WBC, RBC, albumin, protein, triglycerides) was carried out at the clinical chemistry laboratory of the Gelderse Vallei Hospital (Ede, the Netherlands) using standard methodology. Lipoprotein profiling of the ascites fluid was carried out by FPLC as previously described (Lichtenstein et al. 2007).

Plasma analyses: Plasma levels of serum amyloid A (SAA) and endotoxins (Limulus Amoebocyte Lysate) were determined by ELISA (Biosource International, Breda, The Netherlands and SanBio, Uden, The Netherlands) following instructions from the manufacturer. Plasma concentrations of multiple inflammation markers were measured by multiplex immunoassay (Ro-

dentMAP) (Rules Based Medicine, Austin, TX). Liver triglycerides were determined as previously described (Mandard et al. 2006).

Gene expression analysis: Isolation of RNA and subsequent analysis of gene expression by qPCR were carried out as previously described (Sanderson et al. 2008). Cyclophilin, 18S, or 36B4 were used as housekeeping genes. PCR primer sequences were taken from the PrimerBank and ordered from Eurogentec (Seraing, Belgium). Sequences of the primers used are available upon request. The small intestine was divided into three equal parts along the longitudinal axis (referred to as part 1, 2 and 3). Affymetrix Genechip analysis was carried out on part 1 and 2 combined and on part 3 as previously described (de Wit et al. 2008). Data were analyzed using MAS5.0 software. Analysis of Gene ontology classes over-represented in the Angptl4-/- mice was carried out using ErmineJ.

Histology/Immunohistochemistry: For IgG immunostaining in small intestine, five-micrometer sections of paraffin-embedded distal part of the small intestine were mounted on Superfrost microscope slides. These sections were dewaxed in xylene and rehydrated in a series of graded alcohols. To block endogenous peroxidase activity, slides were incubated with 3% H_2O_2 for 20 minutes. Antigen retrieval was performed by placing the slides in citrate buffer (pH 6.0) and heat them in a microwave oven 5 min 700 W (without lid) and 4 times 5 min 500 W (with lid). After cooling down to room temperature, the sections were briefly washed with PBS. Prior to staining, a 20 minutes preincubation was performed using 20% normal rabbit serum (Vector Laboratories, Burlingame, CA, USA). The sections were incubated for 30 minutes at room temperature using HRP-conjugated rabbit anti-mouse IgG antibodies (Serotec), diluted 1:250 in PBS. Diaminobenzidine tetrahydrochloride (DAB, Vector Laboratories) was used as substrate to visualize the bound antibodies. After counterstaining with Meyer's hematoxylin, sections were mounted with DePex mounting medium (Gurr, BDH, Poole, Dorset, UK). For detection of liver macrophages, immunohistochemistry was performed using an antibody against Cd68 respectively (Serotec, Oxford, UK). Sections were pre-incubation with 20% normal goat serum followed by overnight incubation at $4°C$ with the primary antibody diluted 1:50 in PBS/ 1% Bovine Serum Albumin (BSA). After incubation with the primary antibody, a goat anti rat IgG conjugated to horseradish peroxidase (Serotec) was used as secondary antibody. Visualization of the complex was done using AEC Substrate Chromogen for 6 minutes. Negative controls were used by omitting the primary antibody. Haematoxylin and Eosin staining of sections was done using standard protocols. For Angptl4 and β5-integrin immunostaining, paraffin embedded samples of the terminal human ileum were obtained from Dr. Andries Mulder at the Rijnstaete Hospital (Arnhem, the Netherlands). Immunohistochemistry was performed using an antibody directed against the C-terminus of Angptl4 (AA186-406) or against β5 integrin (Abcam, Cambridge, UK). Sections were incubated one hour with the primary antibody diluted 1:50 in PBS 1X. A goat anti-rabbit IgG conjugated to horseradish peroxidase (Serotec) was used as secondary antibody. Visualization of the complex was done using AEC Substrate Chromogen for 10 minutes. For Sirius Red staining

paraffin-embedded samples of the small intestine were mounted on Superfrost microscope slides. These sections were dewaxed in xylene and rehydrated in a series of graded alcohols. Slides were stained in picrosirius red 0.1% picric acid for 90 minutes and rinced in acidified H_2O 0.5% acetic acid.

Binding assay Angptl4 and integrin $\alpha V\beta 5$: In the first assay, 96-well microtiter plates were coated with 100 μl/well of integrin $\alpha V\beta 5$ (USBiological, 37K, Swampscott, MA, USA; 5 μg/ml in PBS 1X, pH 7.35) and incubated overnight at 4°*C*. The plate was washed four times with wash buffer (PBS, pH 7.4, 0.1% Tween-20), and 300 μl/well of blocking buffer (PBS, pH 7.35, 1% BSA) was added for 1 hour at room temperature, 100 rpm. Various amounts (0, 0.31 μg, 0.62 μg, 1.25 μg, 2.5 μg, or 5 μg) of hAngptl4 (3485-AN, *R&D* Systems) were prepared in diluent buffer (0.1% BSA, PBS 1X, pH 7.4, 0.5% Tween 20) and 100 μl/well was added to the plate for 2 hours at room temperature, 100 rpm. Plates were washed with buffer and 100 μl of diluted biotinylated anti-hAngptl4 polyclonal goat IgG antibody (BAF3485, *R&D* Systems) was added at 0.02 mg per well, followed by 2 hours incubation. After four washes, 100 μl/ well streptavidine-conjugated horseradish peroxidase (1X PBS, 1%BSA, 1:200 HRP *R&D* DY998) was added for 20 min at room temperature, 100 rpm. After washing, 100 μl/well of tetramethylbenzidine (TMB) (Sigma T4444) was added and incubated in the plates for 6 min at room temperature. 50 μl/well Stop solution (10% H_2SO_4) was added to stop the reaction. Absorbance was read at 450 nm. In the second assay, 96-well microtiter plates were coated with 100 μl/well of integrin $\alpha V\beta 5$ (5 μg/ml in PBS 1X, pH 7.35) and incubated overnight at 4°*C*. The plate was washed four times with wash buffer (PBS, pH 7.4, 0.1% Tween-20), and 300 μl/well of blocking buffer (PBS, pH 7.35, 1% BSA) was added for 1 hour at room temperature, 100 rpm. hANGPTL4 (5 μg/ml) alone, or with anti-$\beta 5$ antibody (5 μg/ml; Ab15459 Abcam) or with IgG-hAngptl4-Cterminal (5 μg/ml; AA186-406), or with anti-albumin antibody (Sigma A0433), were prepared in diluent buffer (0.1% BSA, PBS 1X, pH 7.4, 0.5% Tween 20), and incubated for 30 minutes. Samples were added to plates (100 μl/well in the amounts incubated above) and incubated for 2 hours at room temperature, 100 rpm. Plates were washed with buffer and 100 μL of diluted biotinylated anti-hAngptl4 polyclonal goat IgG antibody (BAF3485, *R&D* Systems) was added at 0.02 mg per well, followed by 2 hours incubation. After four washes, 100 μl/ well streptavidine-conjugated horseradish peroxidase (1X PBS, 1%BSA, 1:200 HRP *R&D* DY998) was added for 20 min at room temperature, 100 rpm. After washing, 100 μl/well of TMB was added and incubated in the plates for 6 min at room temperature. 50 μl/well Stop solution (10% H_2SO_4) was added to stop the reaction. Absorbance was read at 450 nm. In the third assay, human recombinant Vitronectin (2349-VN, *R&D* Systems), human recombinant Angptl4, or BSA (5 μg/ml in PBS1X, pH 7.35) were coated on a 96-well microtiter plates and incubated overnight at 4°*C*. The plate was washed four times with wash buffer (PBS, pH 7.4, 0.1% Tween-20), and 300 μl/well of blocking buffer (PBS, pH 7.35, 1% BSA) containing either IgG-hAngptl4-Cterminal (5 μg/ml; AA186-406) or anti-albumin antibody was added for 1 hour at room temperature, 100 rpm. Plate was washed with buffer and 100 μL

of $\alpha V\beta 5$ (5μg/ml in diluent buffer; 0.1% BSA, PBS 1X, pH 7.4, 0.5% Tween 20) was added and incubated for 2 hours at room temperature, 100 rpm. Plate was washed and anti-$\beta 5$ antibody (5 μg/ml) was prepared in diluent buffer, and incubated for 2 hours at room temperature with gentle agitation. After washing anti-rabbit IgG-HRP conjugated (1:3000, Sigma A0545) was added for 1 hour with gentle agitation. After washing, 100 μl/well of TMB was added and incubated in the plates for 6 min at room temperature. 50 μl/well Stop solution (10% H_2SO_4) was added to stop the reaction. Absorbance was read at 450 nm.

6.3 Results

To screen for novel gut hormones that are under control of dietary saturated fat, we gave mice a chronic fat overload and analyzed changes in expression of genes encoding secreted proteins using transcriptomics (de Wit et al. 2008). One of the genes that was consistently upregulated by dietary saturated fat in small intestine encoded Angptl4 (Figure 6.1a), a secreted protein belonging to the family of angiopoietins also known as fasting-induced adipose factor and PPARγ angiopoietin-related (Kersten et al. 2000, Yoon et al. 2000). Angptl4 has been shown to govern lipid metabolism by inhibiting plasma triglyceride clearance and stimulating adipose tissue lipolysis (Kersten 2005). In addition, several studies support an effect of Angptl4 on endothelial function (Cazes et al. 2006, Ito et al. 2003, Padua et al. 2008). Expression of Angptl4 was highest in the distal part of the small intestine (Figure 6.1b). Immunohistological staining of Angptl4 protein on sections of human ileum showed that Angptl4 is present in isolated cells within the mucosa, which were identified as enteroendocrine cells by counter staining using the specific enteroendocrine marker chromogranin A (Figure 6.1c). These data establish Angptl4 as a gut hormone that is induced by dietary saturated fat. To examine the role of Angptl4 in the response to saturated fat, WT and Angptl4-/- mice were fed a high fat diet (HFD) rich in palm oil. As expected, weight gain was increased in mice receiving HFD. Remarkably, bodyweights of Angptl4-/- mice fed HFD reached a plateau around 12 weeks and declined thereafter (Figure 6.2a). The decrease in bodyweight was related to anorexia, which was noticeable after about 10 weeks of HFD (Figure 6.2b). If left undisturbed all Angptl4-/- mice fed HFD would ultimately die anywhere between 15 and 25 weeks. The cause of death was identified by an animal pathologist as severe fibrinopurulent peritonitis connected with ascites In the Angptl4-/- mice fed HFD, large amounts of fibrin exudate covered the abdominal organs (Figure 6.2c). Other macroscopic abnormalities included intestinal fibrosis (Figure 6.2d), a compressed liver, hyperplasic spleen, and subcutaneous hyperemia (data not shown). No such abnormalities were observed in Angptl4-/- mice fed a LFD, even at advanced age (>1.5 years). We performed routine clinical tests on the ascites fluid, which varied in color from purulent white to purulent red. Ascites white blood cell count was extremely high in all animals (25.5-34.1*10^9/L), as was the endotoxin concentration (50-120 EU/mL). Ascites fluid of some animals tested positive for E. Coli. The combination of high protein concentration (3.43-

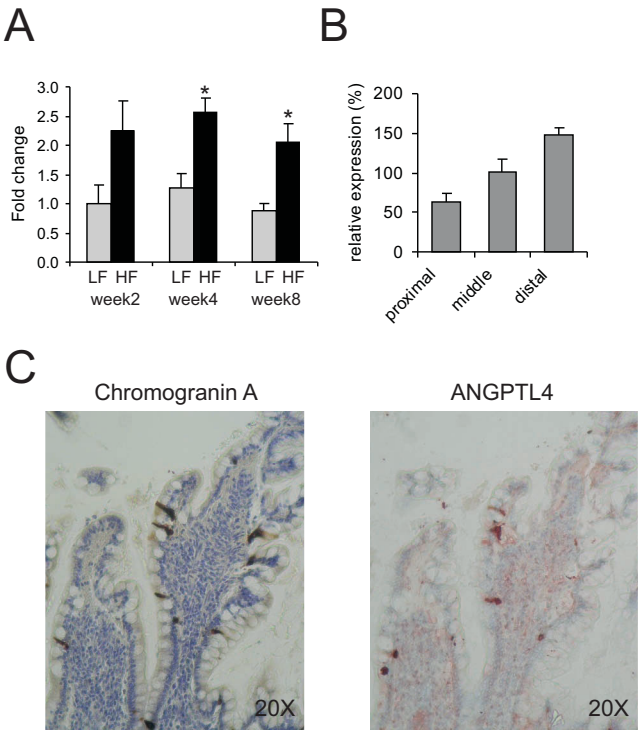

Figure 6.1: *Angptl4 is produced by enteroendocrine cells and is stimulated by HFD. (A) Angptl4 mRNA expression in total small intestine of C57Bl/6 mice after 2, 4 or 8 weeks of LFD or HFD. Grey bars = WT, Black bars = Angptl4-/-. Error bars represent SEM. ∗ = significantly different between WT and Angptl4-/- mice according to Student's T-test ($p<0.05$). (B) Angptl4 mRNA expression was determined in different parts of the mouse small intestine by qPCR. (C) High magnification image of serial sections of human intestinal ileum stained with an antibody against ANGPTL4 (right panel), or chromogranin A (left panel), which is a specific marker for neuroendocrine cells. ANGPTL4 positive cells are present in the mucosa adjacent to goblet cells and enterocytes.*

4.28 g/dL) and low serum-ascites albumin gradient (SAAG, 0.11-0.34 g/dL) indicated exudative ascites. The ascites triglyceride concentration was highly variable but clearly elevated (4.8-75.5 mM). Analysis of ascites fluid by lipoprotein profiling indicated an abundance of chylomicrons (Figure 6.3a). Plasma endotoxin levels were significantly elevated in Angptl4-/- mice fed HFD (Figure 6.3b). Microscopic examination indicated that the fibrin exudate contained an abundance of foam cells, polynuclear giant cells, and various other leukocytes (data not shown). The same cells as well as focal lymphocyte infiltrates were observed in the mesentery and small intestine

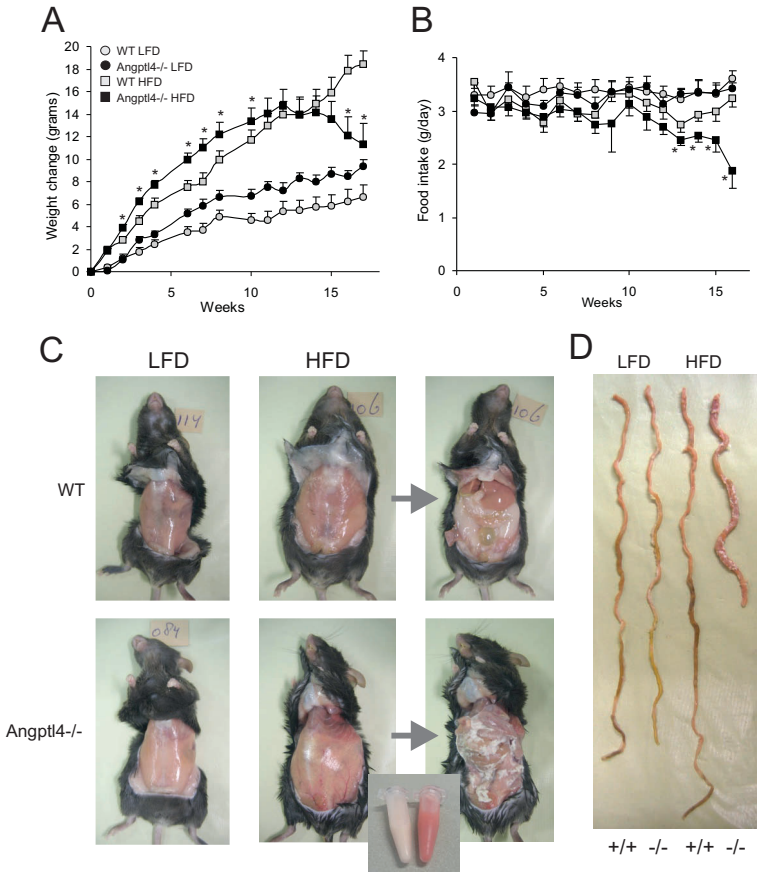

Figure 6.2: *: Angptl4-/- mice chronically fed HFD develop fibrinopurulent peritonitis and ascites. (A) Bodyweight changes in WT and Angptl4-/- mice fed LFD or HFD for 19 weeks. (B) Mean daily food intake in WT and Angptl4-/- mice fed LFD or HFD for 19 weeks. (C) Whole animal photographs taken immediately following sacrifice of representative WT and Angptl4-/- mice fed either LFD or HFD for 19 weeks. Pictures on the far right were taken after removal of the peritoneum. Ascites fluid collected from two Angptl4-/- mice fed HFD is shown at the bottom. (D) Photograph of small intestine of WT and Angptl4-/- mouse fed LFD or HFD for 19 week.*

(Figure 6.4a & b), in the latter encapsulated by collagen as shown by Sirius Red staining (Figure 6.4c) Adipose tissue was very reddish and exhibited coagulation necrosis and steatitis as shown by presence of granulocytes and other infiltrates, mainly at the periphery (Figure 6.5a & b). In the

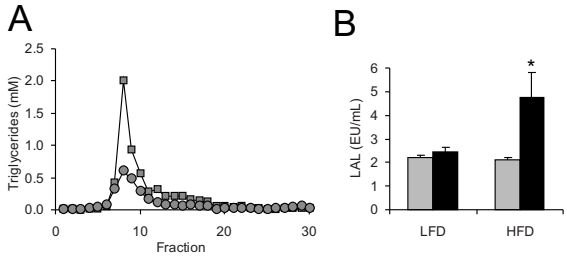

Figure 6.3: *: Chylous ascites and endotoxemia in Angptl4-/- mice fed HFD. (A) Lipoprotein profiling of ascites fluid by FPLC reveals markedly elevated levels of chylomicrons. Results from two mice varying in ascites fluid TG concentration are shown. (B) Plasma endotoxin levels in WT and Angptl4-/- mice fed LFD or HFD for 19 weeks, as determined by LAL Chromogenic endotoxin assay. Grey bars = WT, Black bars = Angptl4-/-. Error bars represent SEM.* $*$ *= significantly different between WT and Angptl4-/- according to Student's T-test ($p<0.05$).*

liver, which generally looked ischemic, portal triads were poorly visible and cords and sinusoids were missing, indicating collapse of liver (Figure 6.5c & d). Additionally, clumping of nuclei and aggregates of eosinophils were observed,and liver fat was almost absent (Figure 6.5e). Weights of liver and adipose tissue were significantly lower in Angptl4-/- mice after 19 weeks of HFD (Figure 6.5f & g). From these data it is evident that Angptl4-/- mice fed a HFD develop a severe phenotype characterized by anorexia,cachexia, intestinal inflammation and fibrosis, ascites, and fibrinopurulent peritonitis, ultimately leading to the death of the animal Previously it was shown that Angptl4-/- mice on a mixed Sv129-C57Bl/6 background die shortly after birth due to defective separation of the intestinal lymphatic and blood microvasculature (Bäckhed, Crawford, O'Donnell and Gordon 2007). Although we did not observe these abnormalities in Angptl4-/- mice on a pure C57Bl/6 background and adult mice in the proper Mendelian ratios were obtained, it is conceivable that there is an underlying weakness in intestinal lymph vessels that only becomes manifest under condition of increased chyle flow associated with HFD. Subsequent leakage of chyle into intestinal lumen and peritoneal cavity may lead to impaired fat absorption and chylous ascites, respectively. However, according to that scenario ascites should develop shortly after starting the HFD, which was not the case. Additionally, one would expect loss of protein, fat and water from lymph into the GI lumen, leading to osmotic diarrhea and steatorrhea. No diarrhea was observed and surprisingly, fecal fat excretion was markedly decreased in Angptl4-/- mice, indicating more efficient fat absorption (Figure 6.6a). An acute intestinal lipid absorption test using ^{3}H-triolein and ^{14}C-palmitic acid failed to show any differences in rate of appearance of either label in blood between WT and Angptl4-/- mice (Figure 6.6b & c), suggesting that chylomicron formation and release into the bloodstream is similar between WT and Angptl4-/- mice. In contrast, in all parts of the intestine accumulation of both labels five hours after the lipid load was markedly higher in the

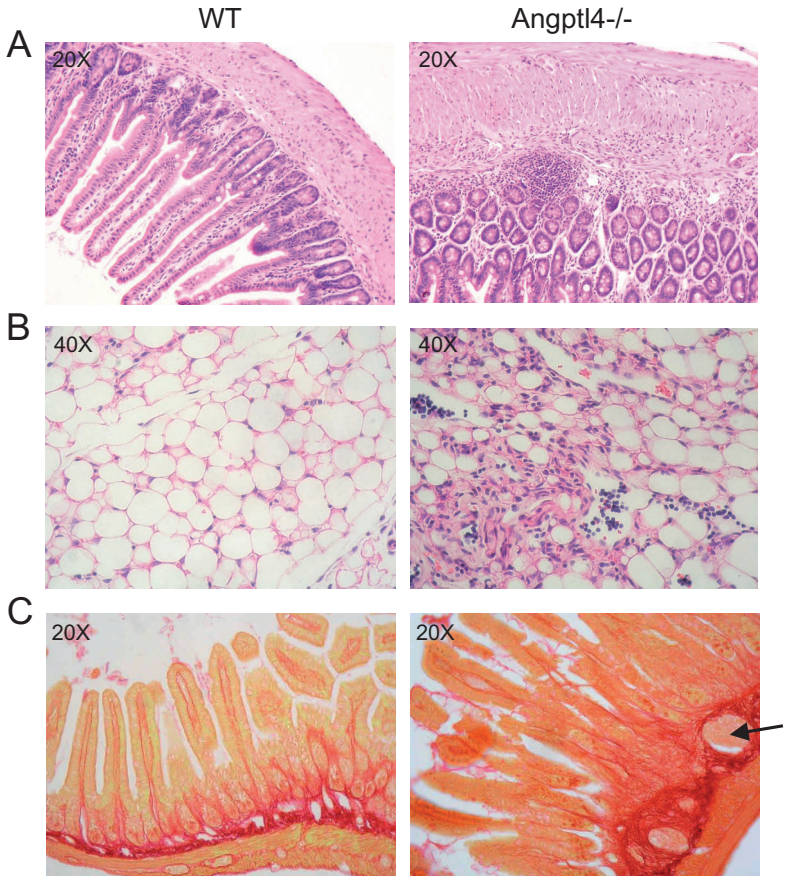

Figure 6.4: *Severe intestinal inflammation in Angptl4 -/- mice chronically fed HFD. H&E staining of small intestine (A) or mesentery (B) of WT and Angptl4-/- mice fed HFD for 19 weeks. (C) Sirius red staining of small intestine of WT and Angptl4-/- mice fed HFD for 19 weeks. Arrow indicates inflammatory infiltrate.*

Angptl4-/- mice (Figure 6.6d&e). Similar data for ^{3}H-triolein and ^{14}C-palmitic acid argue against an effect of Angptl4 inactivation on triglyceride digestion but instead suggest enhanced fatty acid uptake into enterocytes. This is supported by elevated expression of target genes of the fatty acid-activated transcription factor PPAR α in small intestine of Angptl4-/- mice, indicating enhanced gene regulation by fatty acids (Figure 6.6f & g). Overall, these data do not support the notion that ascites and intestinal inflammation are caused by a primary lymph vessel defect, suggesting a

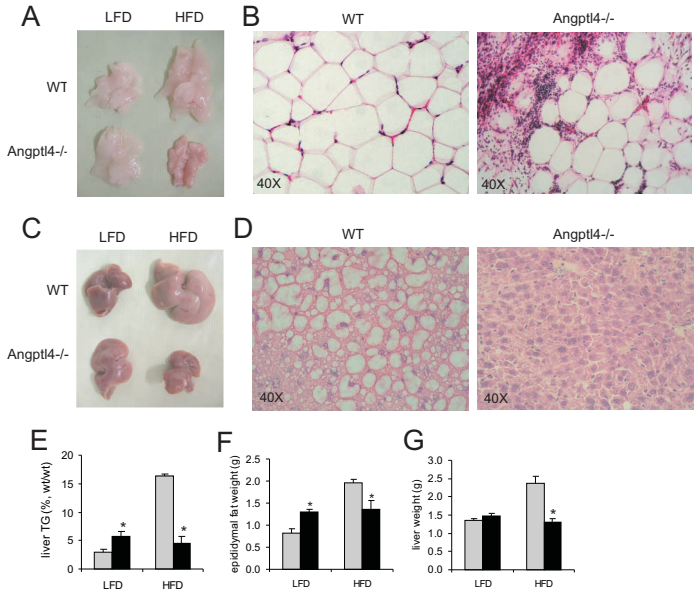

Figure 6.5: *Micro- and macroscopic alteration in liver and adipose tissue. (A) Photograph of epididymal fat of WT and Angptl4-/- mouse fed LFD or HFD for 19 week. Adipose tissue of Angptl4-/- mice fed HFD was reddish and small in size compared to WT mice fed HFD. (B) H&E staining of epididymal fat of WT and Angptl4-/- fed HFD for 19 week. Angptl4-/- mice reveal steatitis as shown by massive infiltration of granulocytes and other leukocytes around the periphery. (C) Photograph of livers of WT and Angptl4-/- mouse fed LFD or HFD for 19 week. Livers of WT mice fed HFD were enlarged and pale, indicating steatosis. Livers of Angptl4-/- mice fed HFD were rigid and compressed. (D)H &E staining of livers of WT and Angptl4-/- mouse fed HFD for 19 weeks. Staining reveals missing cords and sinusoids, poorly visible portal triads and lack of hepatic fat in Angptl4-/- mice fed HFD. (E) Liver triglycerides after 19 weeks of LFD or HFD. Weights of epididymal fat (F) and liver (F) after 19 weeks of LFD or HFD. Grey bars = WT, Black bars = Angptl4 -/-. Error bars represent SEM ∗ = significantly different according to Student's T-test* $p < 0.05$

different etiology. To investigate the cause of the diverse clinical abnormalities in the Angptl4-/- mice, we studied WT and Angptl4-/- mice after 8 weeks of HFD before the onset of any clinical signs. Importantly, at that point no ascites, intestinal fibrosis, fibrin exudate, or other macroscopic abnormalities were observed. Also, anorexia and cachexia were absent (Figure 6.2a & b). As mentioned above, the ascites in the Angptl4-/- mice was characterized as exudative ascites. Exudative ascites excludes portal hypertension as the primary origin and indicates increased capillary and/or lymphatic permeability, which is often related to inflammation and/or infection. To investigate whether the ascites is preceded by excessive inflammation and/or infection, inflammatory mark-

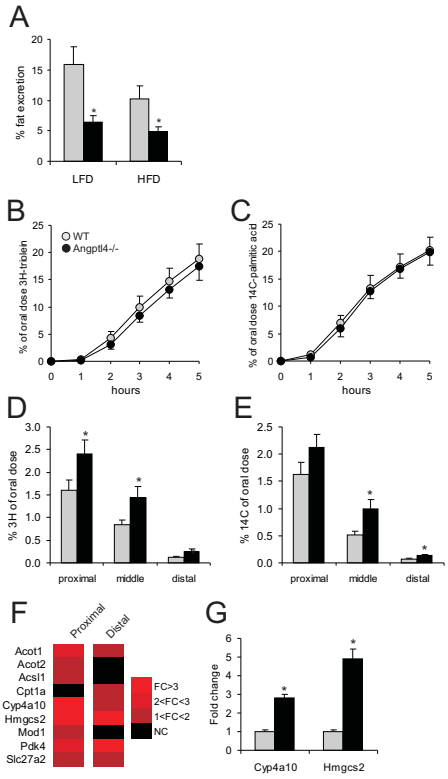

Figure 6.6: *Absence of fat malabsorption in Angptl4-/- mice. (A) Fat excretion expressed as a percentage of dietary fat intake during week 2-5 of LFD or HFD. (B)(C) Mice fasted for 5h were given 400 L of olive oil containing 7 Ci glyceryl-tri[^{3}H] oleate and 2 Ci [^{14}C]palmitate. Blood was collected every hour. ^{3}H- and ^{14}C-radioactivity are expressed as a percentage of total oral dose. (D)(E) Five hours thereafter, small intestine was collected and cut into 3 parts. ^{3}H- and ^{14}C-radioactivity are expressed as a percentage of total oral dose. (F) Heat map showing fold changes in expression of PPAR -target genes in small intestine of Angptl4-/- vs. WT mice. (G) mRNA expression of selected PPAR targets in small intestine of WT and Angptl4-/- mice. Grey bars = WT, Black bars = Angptl4-/-. Error bars represent SEM. ∗ = significantly different between WT and Angptl4-/- mice according to Student T-test ($p<0.05$).*

ers were measured in WT and Angptl4-/- mice after 8 weeks of HFD. Strikingly, plasma levels of the acute phase protein Serum Amyloid A were dramatically increased in Angptl4-/- mice fed HFD (Figure 6.8a), which was also true for numerous cytokines, chemokines, and other inflammatory markers (Figure 6.7). These changes were paralleled by similar massive induction of hepatic mRNA for serum amyloid 2 (Figure 6.8b), haptoglobin, and lipocalin 2 (Figure 6.8c)

Antigen	units	WT	Angptl4-/-
CD40 Ligand	pg/mL	470±93	1553±182
MCP-1	pg/mL	32±7	55±5
MCP-3	pg/mL	73±9	136±10
MCP-5	pg/mL	5.0±1.4	18.5±4.6
MDC	pg/mL	249±5	347±27
IL-1beta	ng/mL	6.5±0.1	7.3±0.3
IL-5	ng/mL	0.9±0.1	1.4±0.0
IL-6	pg/mL	nd	63±26
MIP-1gamma	ng/mL	13±1	24±3
MIP-3beta	ng/mL	0.8±0.0	1.1±0.1
MMP-9	ng/mL	78±5	180±28
MPO	ng/mL	47±2	188±23
TIMP-1	ng/mL	0.6±0.0	2.5±0.6
vWF	ng/mL	97±5	225±83
Factor VII	ng/mL	5.7±0.3	7.1±0.4
CRP	µg/mL	6.7±0.1	10.5±0.7
Haptoglobin	µg/mL	34±12	201±32
IgA	µg/mL	18±1	34±3
Fibrinogen	mg/mL	10±1	36±7
Apo A1	µg/mL	35±1.2	28±0.5

Figure 6.7: *Plasma cytokine profiling in WT and Angptl4-/- mice fed HFD for 8 weeks*

Hepatic expression of the inflammatory mediator Stat3 and macrophage (Kupffer cell) marker Cd68 was also markedly induced (Figure 6.8c), and Cd68 immunostaining was increased (Figure 6.8d). Furthermore, Angptl4-/- mice fed HFD had dramatically enlarged mesenteric lymph nodes (see below), and the mesentery showed an abundance of leukocytes, indicating intestinal infection (Figure 6.9). These data indicate that Angptl4-/- mice fed HFD exhibit chronic inflammation and a massive acute phase response several weeks prior to development of ascites and other external clinical symptoms, which is likely related to intestinal infection. The HFD used contains predominantly palm oil, which is rich in saturated fat. To assess the time course of induction of the inflammation and to study whether induction was exclusive to saturated fat, WT and Angptl4-/- mice were fed diets of different fat composition and plasma SAA levels monitored. Remarkably, HFDs based on palm oil or lard caused a dramatic increase in plasma SAA levels in the Angptl4-/- mice, which was already noticeable after one week of feeding (Figure 6.8e). In contrast, HFDs based on safflower oil, which contains almost exclusively unsaturated fatty acids, or based on medium chain triglycerides (MCT) did not raise plasma SAA levels. Consistent with these data, size of mesenteric lymph nodes was dramatically enlarged in Angptl4-/- mice fed palm oil or lard compared to safflower or MCT oil (Figure 6.8f). Since safflower oil promotes chyle flow compared to medium chain triglycerides, these data argue against increased chyle flow and consequent leakage as a critical early event in the development of the acute phase response and

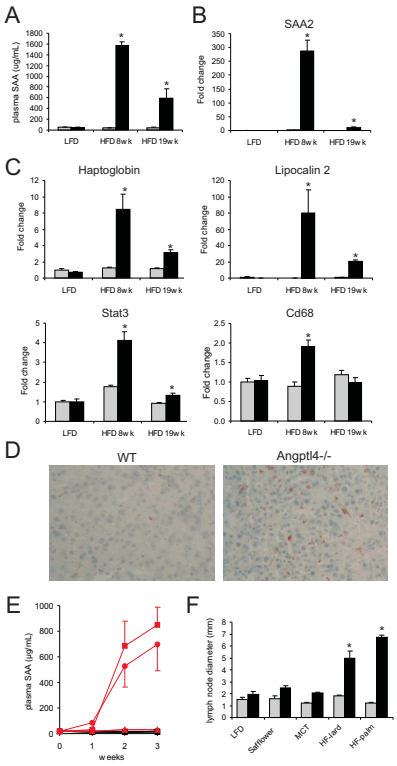

Figure 6.8: *High fat feeding provokes a massive acute phase response in Angptl4-/- mice. (A) Plasma serum amyloid (SAA) levels in WT and Angptl4-/- mice fed LFD or HFD. (B) Hepatic SAA2 mRNA levels, as determined by qPCR. (C) Hepatic mRNA levels of acute phase proteins haptoglobin and lipocalin 2, the transcription factor Stat3 and the macrophage marker Cd68, as determined by qPCR. Expression data were normalized against 36B4. Grey bars = WT, Black bars = Angptl4-/-. Error bars represent SEM. ∗ = significantly different according to Student T-test ($p<0.05$). (D) Cd68 immunostaining of liver sections of WT and Angptl4-/- mice fed HFD for 8 weeks. (E) Plasma SAA levels in WT mice (black symbols) and Angptl4-/- mice (red symbols) fed LFD or HFD for 3 weeks. The fat source of the HFD was either lard (squares), palm oil (circles), MCT oil (triangle), or safflower oil (reverse triangle). (F) Size of mesenteric lymph nodes in WT and Angptl4-/- mice fed the various diets for 8 weeks. ∗ = significantly different from LFD according to Student T-test ($p<0.05$).*

clinical abnormalities. Taken together, these data indicate that saturated but not unsaturated fat exerts a marked pro-inflammatory effect, which is suppressed by Angptl4 expression. Recent studies have indicated that high saturated fat feeding causes changes in the intestinal microflora, which in turn may lead to inflammatory stress, e.g. metabolic endotoxemia (Cani et al. 2008, Turnbaugh

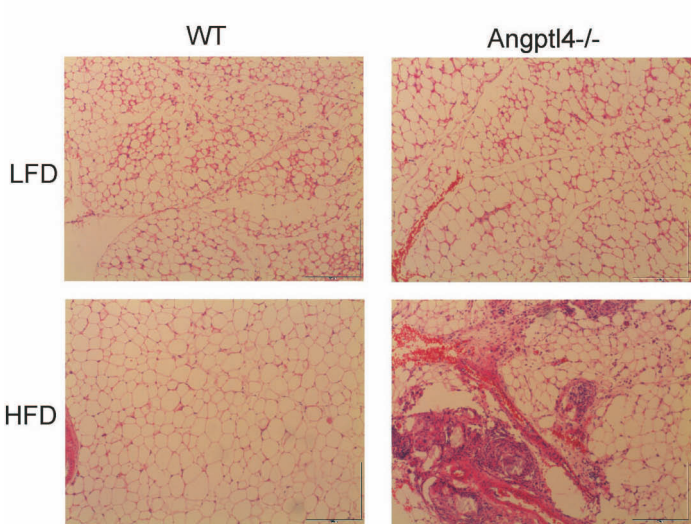

Figure 6.9: *Inflamed mesentery in Angptl4-/- mice fed HFD. H&E staining of mesentery of WT and Angptl4-/- fed HFD for 6 week. Angptl4-/- mice fed HFD exhibit massive infiltration of granulocytes and other leukocytes.*

et al. 2008). To study if the impaired tolerance to saturated fats in Angptl4-/- mice may be related to a poor defense to the changing microflora, we assessed indicators of intestinal immune function. Remarkably, already under LFD the number of IgG-positive cells was significantly decreased in Angptl4-/-mice (Figure 6.10a). In support of these data, expression of genes involved in immune function was suppressed in Angptl4-/- mice, including the B-cell marker CD79 and Igj, a marker for differentiation of B-cells to plasma cells (Figure 6.10b). In addition, expression of the bactericidal lectin Reg3 was severely reduced. Further analysis by gene expression profiling confirmed a dramatic and comprehensive down-regulation of numerous genes involved in the immune response in the intestine (Figure 6.10c), Consistent with these data, analysis of gene ontology revealed highly significant alterations in humoral immune response, antigen presentation, acute phase response, immune cell activation and defense response to bacteria (Figure 6.11). These data strongly suggests that deletion of Angptl4 is associated with decreased mucosal immunity, which is already evident on LFD Impaired immune function in the intestine of Angptl4-/- mice is hard to reconcile with the function of Angptl4 as LPL inhibitor. Analogous to Angptl3 and consistent with the presence of an RGD sequence representing a cell attachment signal that mediates interactions with certain integrins, Angptl4 may bind to integrins as well (Camenisch et al. 2002). Indeed, we found that Angptl4 directly interacts with integrin $\beta 5$ and regulates migration, proliferation and differentiation of keratinocytes (Goh et al, submitted). Using Elisa, dose-dependent binding

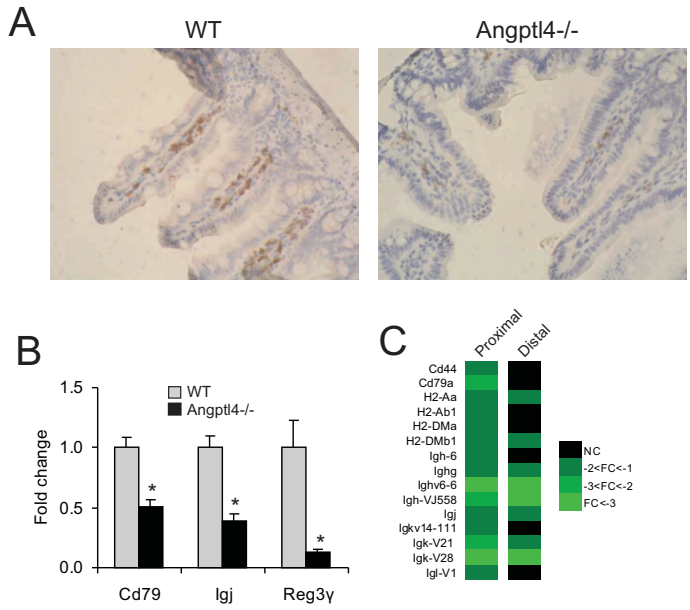

Figure 6.10: *Decreased innate immunity in small intestine of Angptl4-/- mice. (A) IgG immunostaining of sections of small intestine from WT and Angptl4-/- mice fed LFD. (B) mRNA expression of B cell markers Cd79 and Igj and the anti-bacterial lectin Reg3 in small intestine of WT and Angptl4-/- mice fed LFD. Grey bars = WT, Black bars = Angptl4-/-. Error bars represent SEM. ∗ = significantly different according to Student T-test ($p<0.05$). (C) Heat map showing fold changes in expression of genes involved in immune response of Angptl4-/- vs. WT mice fed LFD.*

of coated integrin αVβ5 to Angptl4 was observed (Figure 6.12a). Importantly, this interaction was reduced by co-incubation with an antibody against Angptl4 and integrin β5, but not albumin (Figure 6.12b). Conversely, coated Angptl4 bound to integrin αVβ5, which was abolished by the anti-Angptl4 antibody but not control antibody (Figure 6.12c). Vitronectin, which binds integrin αVβ5, and BSA were used as positive and negative controls, respectively. To study whether the effects of Angptl4 on intestinal immune function may be exerted via integrin αVβ5, we assessed the location of integrin β5 in the small intestine by immunohistochemistry. In agreement with a role of integrin β5 in intestinal immune function, specific staining of isolated immune cells in the lamina propria was observed (Figure 6.12d & e). Based on the distribution of integrin β5-positive cells, which differed from CD3 (T-cells), CD20 (B-cells) and CD35 (dendritic cell) positive cells, their absence from Peyers patches (data not shown), and their lobulated nucleus (Figure 6.12e), these cells very likely represent neutrophil granulocytes. Together with the data by Goh et al.,

ID	P value	Name
GO:0006959	6.62E-09	humoral immune response
GO:0042591	1.67E-06	antigen presentation, exogenous antigen via MHC class II
GO:0006953	1.91E-06	acute-phase response
GO:0019882	2.13E-06	antigen presentation
GO:0045321	2.59E-05	immune cell activation
GO:0019884	3.10E-05	antigen presentation, exogenous antigen
GO:0016064	3.16E-05	humoral defense mechanism (sensu Vertebrata)
GO:0042742	3.27E-05	defense response to bacteria
GO:0019886	9.26E-05	antigen processing, exogenous antigen via MHC class II
GO:0009617	1.47E-04	response to bacteria
GO:0006954	1.90E-04	inflammatory response
GO:0048002	2.01E-04	antigen presentation, peptide antigen
GO:0045576	2.01E-04	mast cell activation
GO:0050728	2.31E-04	negative regulation of inflammatory response
GO:0050777	2.31E-04	negative regulation of immune response
GO:0045582	2.58E-04	positive regulation of T-cell differentiation
GO:0030333	2.68E-04	antigen processing
GO:0042116	5.73E-04	macrophage activation

Figure 6.11: *Overrepresented gene ontology classes in distal small intestine of Angptl4-/- mice fed LFD*

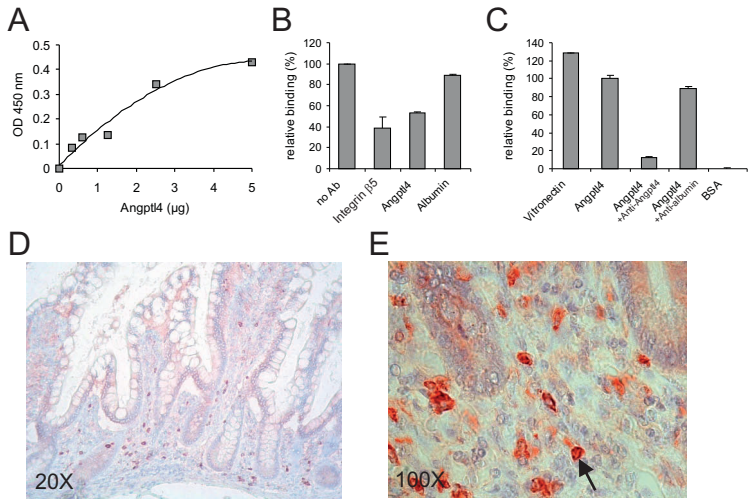

Figure 6.12: *Effect of Angptl4 on immunity may be mediated via binding to integrin β5 on intestinal neutrophils. (A) Binding assay showing dose-responsive binding of recombinant Angptl4 to coated integrin αvβ5. (B) Coated integrin αVβ5 interacts with Angptl4, which is reduced by antibodies directed against integrin β5 and Angptl4 but not albumin. (C) Coated vitronectin and Angptl4 but not BSA interact with integrin αVβ5. Binding between Angptl4 and integrin αVβ5 is abolished by antibody against Angptl4 but not control antibody. Immunostaining of the human ileum using an antibody against integrin β5 at low (D) or high (E) magnification.*

these results suggest that Angptl4 may influence mucosal immune function via integrin-mediated signaling in specific immune cells in the small intestine .

6.4 Discussion

Our studies support a major pro-inflammatory effect of dietary saturated fat, which is suppressed by Angptl4. Recent studies have indicated that ultra high fat feeding in mice (72en% fat) induces a state of metabolic endotoxemia, characterized by mildly but chronically elevated plasma LPS levels (Cani et al. 2007, Cani et al. 2008). These effects are likely related to changes in intestinal microflora and may be associated with increased gut permeability. Our observations are consistent with a scenario in which Angptl4 deletion reduces protection against the intestinal microflora, which becomes highly pro-inflammatory upon high saturated fat feeding but not high unsaturated fat or MCT feeding. Subsequent translocation of bacteria elicit mesenteric lymphadenitis and a full blown acute phase response, followed by cachexia, intestinal inflammation, and capillary and lymphatic leakage, which in turn causes chylous ascites and accumulation of fibrin exudate. Alternatively, it is conceivable that saturated fat has a direct pro-inflammatory effect in the intestine, which is suppressed by Angptl4. Recent studies indicate that saturated fatty acids can activate Toll-like receptor 4, which plays a critical role in the innate immune system by activating pro-inflammatory signaling pathways in response to microbial pathogens (Hwang 2001, Lee et al. 2001, Lee et al. 2003, Shi et al. 2006, Suganami et al. 2007). Whether Angptl4 impacts Toll-like receptor signaling is unknown and requires further investigation. Our data indicate that in addition to its role in lipid metabolism and angiogenesis, Angptl4 plays a major role in intestinal immunity. Gene expression analysis of small intestine specifically points to a role for Angptl4 in the humoral immune response, including antigen presentation and the defense response to bacteria. Indeed, already under baseline conditions, numerous immunoglobulins and class II histocompatibility proteins, as well as specific lymphocyte markers such as Cd44 and Cd79 and the anti-bacterial Reg3 were markedly downregulated in small intestine of Angptl4-/- mice. By serving as a ligand for integrin $\alpha V\beta 5$, it was shown that Angptl4 activates integrin-mediated intracellular signaling (Goh et al. submitted). Interestingly, αV integrins have been shown to be involved in mucosal immunity (Lacy-Hulbert et al. 2007). The dominant production of Angptl4 by specific enteroendocrine cells raises the possibility of paracrine activity by Angptl4 on adjacent integrin a $\alpha V\beta 5$-positive neutrophils to modulate mucosal immunity. Clearly, additional research is required to elucidate the effect of Angptl4 on intestinal immune cell signaling and its specific role in regulation of mucosal immune function. Expression of Angptl4 in the intestine was previously shown to diminish after introducing a normal intestinal microbiota into the intestine of germ free mice, which was proposed to account for the stimulatory effect of the intestinal microflora on bodyfat (Backhed et al. 2004, Backhed, Manchester, Semenkovich and Gordon 2007). Whether the induction of Angptl4 by HFD is related to changes in intestinal microbial population elicited by HFD or reflects a direct effect of fatty acids on Angptl4 mRNA levels remains unclear (Cani et al. 2007). It can be speculated that induction of Angptl4 by dietary saturated fat may be an adaptive response to the pro-inflammatory and metabolic consequences of saturated fat con-

sumption. (Chylous) ascites represents a rare phenotype among transgenic mouse models. It has been observed in mice heterozygous for the transcription factor Prox1 as well as in mice lacking angiopoietin-2. Both proteins are essential for development of the lymphatic vasculature (Gale et al. 2002, Harvey et al. 2005). Accordingly, it is tempting to hypothesize a similar role for Angptl4. However, it should be emphasized that the Prox1+/- and Ang2-/- mice already develop chylous ascites shortly after birth, reflecting a severe developmental defect. In contrast, Angptl4-/- mice do not show any changes in lymphatic endothelial integrity and do not exhibit ascites unless challenged with HFD for at least 12 weeks. Furthermore, clinical abnormalities are exclusive to mice fed a diet rich in saturated fat but not unsaturated fat, suggesting a mechanism independent of chyle flow. Taken together, we demonstrate that Angptl4 protects against the pro-inflammatory and ultimately lethal effects of chronic overconsumption of saturated fat. Our data characterize Angptl4 as a novel modulator of intestinal immune function and suggest that dysfunctional regulation of Angptl4 may contribute to the pathology of chronic inflammatory intestinal diseases. Angptl4 can be added to a growing list of secreted factors involved in regulation of fat metabolism that also impact inflammation and immunity (Tilg and Moschen 2006).

Chapter 7

General discussion

The objective of this thesis was to functionally characterize the ANGPTL4 protein in mouse and human. Since its discovery in 2000, research on ANGPTL4 has evolved from in vitro studies to in vivo mouse studies, and most recently to human genetic studies. It is now very evident that ANGPTL4 is a key regulator of lipoprotein metabolism by interacting with the key enzyme lipoprotein lipase (LPL). LPL is a rate-limiting enzyme in the clearance of the triglyceride rich lipoproteins chylomicrons (CM) and very-low density lipoprotein (VLDL). Many genetically modified mouse models have been developed in the last 2 decades that illustrate the importance of this enzyme in plasma lipid metabolism. Lipid metabolism differs substantially post-prandially and after a period of fasting. During fasting a fall in plasma glucose is followed by a decrease in insulin secretion, and an increase in secretion of glucagon. In adipose tissue those changes inhibit lipogenesis, lead to inactivation of LPL and activate hormone sensitive lipase. As a consequence, plasma free fatty acids (FFA) rise. On the contrary, insulin levels increase post-prandially, which leads to activation of LPL in adipose tissue, thereby allowing the ingested fat to be stored. When there is a chronic dietary fat overload, for examples during chronic high fat feeding, elevated fatty acid uptake into the adipose tissue may lead to obesity. Although obesity and fasting appear to represent metabolic opposites, a number of parallels can be drawn. For example, both conditions are associated with elevated plasma free fatty acid levels. Depending on the particular tissue, both fasting and high fat induced obesity increase ANGPTL4 mRNA and protein expression. We have taken advantage of those two nutritional extremes to study the role of ANGPTL4 in lipid metabolism.

LPL exerts it action at the surface of endothelial cells bound to heparin sulphate proteoglycans (HSPG). Once secreted LPL adopts a head to tail subunit orientation to form the active homodimer (Wong et al. 1997). Dissociation of LPL monomers leads to loss of activity, and consequently to aggregation of the inactive monomers (Hata et al. 1992, Osborne et al. 1985). Within the blood circulation, LPL binds to lipoproteins via apolipoproteins. Markku et al showed that native LDL binds to monomeric LPL. However. Vilella et al. showed that both LPL mass and activity of postheparin (PHP) plasma eluted with LDL. Molecular research described that this binding is allowed by LPL recognition and binding to ApoB (Choi et al. 1997). In vitro studies have shown that ANGPTL4 promotes the conversion of LPL dimers into monomers (Sukonina et al. 2006), which we are able to confirm in vivo using our ANGPTL4-Tg mice (chapter 3). We showed

that in blood plasma ANGPTL4 is physically associated with LDL-bound LPL monomers. One could hypothesize that ANGPTL4 binds to LPL monomers, thereby pulling the equilibrium towards LPL monomers and away from LPL dimers. The complex between LPL monomers and ANGPTL4 may then bind to LDL and is subsequently taken away for further processing. In contrast to Angptl3, ANGPTL4 seems to cause permanent inactivation of LPL activity which is in agreement with the scenario explained above. A surprising observation that we made was that PHP LPL activity was reduced to almost zero in ANGPTL4-Tg mice, while the reduction in LPL-dimers in post-heparin plasma was much less. Recently, it was suggested that much of the LPL released upon heparin injection may represent extravascular LPL, which may be bound to HSPGs in subendothelial compartments or on the surface of adipocytes or myocytes and is not involved in lipolysis of TG-rich lipoproteins (Weinstein et al. 2008). Accordingly, it is possible that ANGPTL4 only inactivated the functional LPL present on the endothelium yet had no effect on ”subendothelial LPL”. In this context it is interesting to mention that the dramatic decrease in PHP LPL activity in ANGPTL4-Tg mice was observed using a modified LPL activity assay, yet no decrease in PHP LPL activity was found when using a traditional LPL assay (data not shown). One could speculate that the modified LPL assay only measures functional LPL on the endothelial surface while the traditional LPL assay measures all LPL (total extracellular LPL mass), the major portion of which is not targeted by ANGPTL4. Beside LPL inhibition, we observed inhibition of hepatic lipase HL in ANGPTL4-Tg mice. HL is a triglyceride lipase as well as phospholipid (PL) lipase for IDL and HDL, and thus completes the work of LPL. Genetic mutations of HL has been shown to lead to an increase in both HDL cholesterol and PL (Connelly et al. 1990, Breckenridge et al. 1982), explaining the rise in IDL and HDL cholesterol/triglyceride we observed in Angptl4-Tg mice. Interestingly, we are the only group to report HL inhibition by ANGPTL4. Since our ANGPTL4-Tg mice overexpress ANGPTL4 in numerous tissue including liver, it is possible that HL inhibition is mediated exclusively by liver-derived ANGPTL4. In turn, ANGPTL4 produced extra-hepatically may specifically serve to target LPL. However, adenoviral or transgene-induced liver-specific ANGPTL4 overexpression was associated with decreased PHP LPL activity. Whether this reflects spillover of ANGPTL4 into the blood plasma as a result of massive ANGPTL4 overexpression is unclear. Whereas Angptl3 has been described to inhibit endothelial lipase (EL), no comparable investigations have been done supporting the same role for ANGPTL4. EL is a recently discovered enzyme that hydrolyzes TG and especially PL hydrolysis within HDL (Jaye et al. 1999). Both genetic ablation and loss of function mutations for EL lead to elevated HDL cholesterol levels (Brown et al. 2009). We observed that ANGPTL4 overexpression was associated with elevated HDL-phospholipid and -cholesterol levels, whereas the opposite effects were observed in ANGPTL4-/- mice. These results suggest that ANGPTL4 may inhibit EL. However, further evidence including from in vitro studies is necessary.

In human, different tissues are capable of synthesizing ANGPTL4 protein, with the highest expression levels found in liver, followed by adipose tissue, thyroid, brain and small intes-

tine (chapter 4). Once synthesized, ANGPTL4 protein is partially cleaved into N-terminal and C-terminal fragments, which can be detected in human plasma (Mandard et al. 2004). A limited number of studies has measured ANGPTL4 protein levels in human plasma. Steyskal et al developed an ELISA assay using a polyclonal antibody directed against AA26-229 of human ANGPTL4. The median plasma ANGPTL4 concentration was determined at ± 8 ng/ml. No difference in plasma ANGPTL4 levels was observed between patients with metabolic syndrome and healthy patients. There was no association of plasma ANGPTL4 with BMI, waist circumference or QUICKI (quantitative insulin sensitivity check index). However, ANGPTL4 correlated significantly with plasma HDL-cholesterol, FGF21, glucose and TG levels. Staiger et al. measured a mean plasma ANGPTL4 concentration of 1.73 ± 0.11 ng/ml (means ± SE; range 0.37-8.00 ng/ml). ANGPTL4 muscle mRNA correlated significantly with plasma ANGPTL4 levels. Importantly, plasma ANGPTL4 levels were positively associated with plasma FFAs. In contrast, no significant correlation were found between plasma ANGPTL4 levels and plasma glucose, insulin, or triglyceride concentrations. Although Staiger and Stejskal used the same supplier for the antibody, it is unclear whether they used the exact same antibody. Importantly, it is also not clear whether their ELISA assays measure total plasma ANGPTL4 or only a particular subfraction (e.g. N-terminal ANGPTL4 or full length ANGPTL4). Xu et al used monoclonal antibody ED12B9 to measure plasma ANGPTL4 protein in patients with type diabetes mellitus. This antibody recognizes both full length and the N-terminal fragment of ANGPTL4. Mean plasma ANGPTL4 concentration was determined at 345.04 ng/ml. Serum levels of ANGPTL4 were significantly lower in obese diabetic patients compared to obese non-diabetic patients. We measured plasma ANGPTL4 using an ELISA protocol that was later adopted and commercialized by R&D systems. Plasma levels of ANGPTL4 in our analysis were in the same order as Stejskal et al. Importantly, in Northern blot the antibody used in our ELISA analysis only detected full length ANGPTL4, which led us to conclude that we are only measuring full length ANGPTL4. Previous work has shown that the N-terminal fragment of ANGPTL4 is sufficient to inhibit LPL activity. However, it is not clear whether nANGPTL4 truly represents the metabolically active form. While it would be interesting to know the plasma concentration of the N-terminal fragment of ANGPTL4, it is equally important to assess the plasma concentration of the full-length protein. The most striking finding was that plasma ANGPTL4 levels varied dramatically between subjects yet were relatively stable within subjects. So far we have not been able to get a handle on the origin of the large inter-individual variation. A specific link with polymorphisms within the ANGPTL4 gene can likely be excluded. One interesting possibility is that individuals differ in the formation of ANGPTL4 oligomers, which may be differentially recognized by the ELISA assay. Despite the large inter-individual variation, we found that conditions associated with elevated plasma FFA such as fasting, caloric restriction and exercise increased plasma ANGPTL4 level. In several cell lines, fatty acid markedly induced ANGPTL4 expression, suggesting a direct stimulatory effect of plasma FFA on ANGPTL4 production. Besides dyslipidemia ANGPTL4 has also been connected with several

other clinical conditions including cancer. Accordingly, it may be of interest to explore plasma ANGPTL4 as a cancer biomarker.

Overload of lipid into the heart can dramatically impair heart function, thereby predisposing to heart failure. Accordingly, strict regulation of cardiac lipid uptake is vital. Under normal conditions, the heart utilizes fatty acids as its principal energy substrate. However, lipid accumulation within cardiomyocyte can lead to lipotoxicity and contractile dysfunction. We observed that cardiac Angptl4 gene expression is highly induced by dietary fatty acids, which feeds back to lower cardiac fatty acid uptake. It has been previously shown that heart specific overexpression of ANGPTL4 leads to impaired heart function due to LPL inhibition and subsequent inhibition of fatty acid uptake (Yu et al. 2005). ANGPTL4-Tg mice showed a strong overexpression of ANGPTL4 in the heart, especially after 24 hours fasting. We showed that fasting-mediated upregulation of ANGPTL4 impaired cardiac uptake of TG-derived FA. Augustus et al. showed that by specifically knocking out cardiac-LPL, the expression of FA metabolism key genes were down regulated. Poly-unsaturated fatty acids (PUFA) have been described to have deleterious effects depending on the position of the double bond. While n-3 are considered to be beneficial, n-6 PUFA has been described to be pro inflammatory and may increase cardiovascular risk. This is explained by the fact that n-6 PUFA may lead to the formation of arachidonic acid, which serves a precursor for numerous inflammatory mediators. Limiting the excess uptake of FA in the heart would be expected to be relevant to limit the deleterious effects on cardiomyocyte function. This principle has been illustrated in chapter 5. Lipotoxicity has a major effect on the mitochondrial electron transport chain, leading to formation of reactive oxygen formation (ROS) as a consequence of incomplete reduction of oxygen. The formation of superoxide radical has been shown to be at the origin of lipid peroxidation. As this process occur in parallel to ATP synthesis, one of the ways to lower ROS formation is to uncouple ATP synthesis. Uncoupling proteins family (UCPs) are composed of several members which display tissue specific expression. UCP1 is expressed in brown adipose tissue, UCP2 is expressed ubiquitously, and UCP3 is expressed preferentially in fast glycolytic fibers of skeletal muscle and in mouse heart. Expression of UCP3 has been shown to be increased after prolonged increases in plasma free fatty acid concentration as a consequence of acute exercice (Schrauwen et al. 2002). In the same study we observed that levels of ANGPTL4 protein in plasma increased in parallel with expression of UCP3. UCP3 has been proposed to protect against ROS production in muscle (Vidal-Puig et al. 2000). The induction of cardiac ANGPTL4 by free fatty acid and by an acute oral fat load may represent a protective mechanism against fatty acid-induced lipotoxicity, which is correlated with induction of UCP3. Recently, a link was made between the small intestine and obesity via the microbiota. It was shown that introduction of microbiota into conventionalized mice promoted weight gain. Additionally, evidence was provided that dietary induced obesity is associated with changes in the intestinal microbiota, which may contribute to chronic inflammation. Recent work on diet induced obesity showed that ANGPTL4 is induced along the longitudinal axis of small intestine already after two weeks of high fat diet

(HFD) (de Wit et al. 2008). Based on our data we propose that either full length ANGPTL4 or its C-terminal fragment constitutes a new gut hormone that is secreted by enteroendocrine cells, especially after a fat-rich meal (chapter 6). Bäckhed et al reported the importance of the protective effect of ANGPTL4 to obesity development using germ free mice. It was suggested that the effect of microbiota on bodyweight may be mediated by ANGPTL4, expression of which is suppressed by introduction of microbiota into germ free mice. Reduced ANGPTL4 expression was suggested to contribute to the enhanced fat storage of conventionalized mice via increased activity LPL activity (Backhed et al. 2004). ANGPTL4 has also been proposed to be involved in regulating lymphangiogenesis-via Prox1 (Backhed, Manchester, Semenkovich and Gordon 2007), a determinant factor for normal lymph vessel development. It can be speculated that ANGPTL4 deficiency impacts weight gain control via regulation of lymphangiogenesis, similar to what has been proposed for Prox1 (Harvey et al). It is important to emphasize that using ANGPTL4 deficient mice on C57Bl/6 background we fail to see any of the lymph abnormalities reported by Backhed et al. The reason for the discrepancy is not clear but may be related to differences in the genetic background of the mice. Instead, we find that ANGPTL4 deletion only leads to a severe phenotype when mice are fed a high fat diet. Serum Amyloid-A (SAA) is an acute phase response protein that is synthesized by the liver upon inflammatory stimuli (Benditt et al. 1971, Levin et al. 1972), including high fat feeding (Scheja et al. 2008). The acute phase response is the immediate response of an organism to an inflammation and is aimed at restoring normal physiological functions. Inflammatory cascades are initiated by the activation and recruitment of plasma monocytes and macrophages to the site of injury. Upon activation, macrophages synthesize and release pro-inflammatory cytokines such as IL-1b, IL-5, IL-6, and monocyte chemoattractant protein. Induction of SAA by lipopolysaccharide was abolished in IL-6 deficient mice, suggesting its involvement in SAA gene regulation (Bopst et al. 1998, Betts et al. 1993). Hepatic SAA2 mRNA expression as well as plasma SAA concentration were strongly increased in ANGPTL4-/- mice already shortly after starting saturated fat feeding. After short term high saturated fat feeding in ANGPTL4 -/- mice, the mesenteric lymph nodes were massively increased, suggesting their involvement in the acute phase cascade in ANGPTL4-/- mice. Also, expression of IL-6 and TNFalpha was increased in mesenteric lymph nodes of ANGPTL4 -/- mice, which was not observed in small and large intestine. These results suggests that the initial inflammatory event in ANGPTL4-/- mice occurs in the lymph nodes. Currently, we are exploring two different scenarios. These scenarios need to take account the specific effect of high saturated fat as opposed to high unsaturated fat on inflammation. Second, they need to incorporate an important modulating role for ANGPTL4 since the inflammation only occurs in ANGPTL4-/- mice. The first scenario is based on the role of ANGPTL4 as LPL inhibitor. LPL is expressed in a number of cell types including muscle and adipose tissue. However, LPL is also well expressed in macrophages which are abundant in mesenteric lymph nodes. Mesenteric lymph nodes are exposed to large amounts of chylomicron-rich chyle which passes through the lymph nodes before being taken up into the circulation. Since ANGPTL4 in-

hibits LPL, in the absence of ANGPTL4, LPL activity and thus uptake of fatty acids from lymph chylomicrons into macrophages may be elevated, which is further enhanced by high fat feeding. According to this scenario, medium chain fats do not elicit an inflammatory response since they are not processed through the lymph. Similarly, unsaturated fats do not have any effect because macrophages are only activated by saturated fatty acids. One potential mediator for the effect of saturated fatty acids on macrophage inflammation is the Toll-like receptor 4, which has been shown to be activated by saturated fatty acids. Activation of TLR4 would initiate an inflammatory cascade leading to synthesis of IL-6 and numerous other cytokines. This model is based on the well established ability of ANGPTL4 to inhibit LPL. Alternatively, we consider a scenario in which the pro-inflammatory effect of high fat diet is not exerted by the high fat content perse but is related to the changes in intestinal microbiota elicited by high saturated fat feeding. Recently, it was shown that high fat feeding alters the intestinal microbiota and leads to elevated production of endotoxins. Accordingly, the ability of high saturated fat but not medium chain fat or unsaturated fat to induce inflammation may be due to specific changes in intestinal microflora uniquely elicited by saturated fat. The reason why inflammation specifically develops in ANGPTL4-/- mice may be related to the effect of ANGPTL4 on immune function. As explained in chapter 6, ANGPTL4 promotes intestinal immune function. We hypothesize that the effect of ANGPTL4 on immune function is related to its C-terminal domain, and is independent of its ability to inhibit LPL. Our immunohistochemical staining results in human intestine suggest that C-ter ANGPTL4 is secreted by enteroendocrine cells within the gut lumen, thus playing an endocrine function. While most of the research so far has focused on the N-terminal fragment of ANGPTL4, studies suggest that the C-terminal fragment is important for governing angiogenic function (Yang et al. 2008). Indeed, the other family members such as Angiopoietin 1 and 2 have been described to have angiogenic properties mediated by binding to the Tie2 receptor. However, ANGPTL4 does not bind to Tie1 or Tie2 receptors. In analogy with Angptl3, which bind to $\alpha V\beta 3$ integrin, ANGPTL4 may be hypothesized to bind to integrin homologues. Recently, Goh et al. demonstrated that the C-terminal domain of ANGPTL4 binds to integrin $\alpha V\beta 5$ on keratinocytes promoting extra-cellular matrix remodeling in a wound-healing context. We were able to confirm ANGPTL4 binding to $\alpha V\beta 5$ integrins using an ELISA assay. Since integrins contribute to the regulation of immune function within the intestine, one could expect that the effect of ANGPTL4 on immune function to be mediated by $\alpha V\beta 5$ integrin, leading to efficient protection against deleterious effects of saturated fat consumption. To further substantiate this second scenario, the role of ANGPTL4 in intestinal immune function needs to be better characterized. Furthermore, specific effect of saturated fat vs unsaturated fat on the intestinal microbiota need to be demonstrated, for instance by using the MITChip. To differentiate against these two scenarios we have put ANGPTL4-/- mice on a high saturated fat diet with or without oral antibiotics. We will then follow the acute phase protein concentration in plasma. If induction of plasma SAA is maintained in the presence of antibiotics, we will conclude that the effect of saturated fat on inflammation is independent of the intestinal

microbiota. In that case, we will focus specifically on the effect of saturated fatty acids on lymph node macrophages. Beside angiopoietins, another protein that shows homology with ANGPTL4 is ficolin-A. Ficolin-A binds to surface bacterial sugar residues and plays a role in innate immunity by activating the lectin complement pathway (Endo et al. 2006). Ficolin A is characterized by a fibrinogen-like domain (FBG) responsible of recognition of microorganisms, and plays an important role in internal defense (Endo et al. 2007, Honor et al. 2007). In invertebrate, several FBG-like domain containing proteins have been described to be involved in nonself recognition, also known as fibrinogen-related proteins (FREP). Invertebrates only rely on innate immune response, while vertebrates have both innate and adaptive immune response. These data lend credence to the notion that ANGPTL4 may carry multiple functions, part of which are exerted via its C-terminal FBG domain which lacks the ability to inhibit LPL.

This thesis has contributed important new information to our understanding of ANGPTL4 function. We showed that ANGPTL4 is a strong regulator of lipid metabolism via a number of approaches. We first confirmed in vivo that ANGPTL4 inhibits LPL by converting active dimers into inactive monomers. Our work showed that ANGPTL4 is also able to critically inhibit HL. As a consequence, uptake of cholesterol in the liver is decreased in ANGPTL4-Tg mice, leading to upregulation of hepatic cholesterol synthesis. Furthermore, we showed that ANGPTL4 plays a protective role against lipotoxicity in both the heart and small intestine. We have developed an accurate and reproducible ELISA assay to quantitatively measure ANGPTL4 protein in plasma and showed that plasma ANGPTL4 are regulated by plasma FFA levels. Although a number of important aspects related to ANGPTL4 function have been resolved, the research described here has also raised a number of important new questions. Importantly, future research must be directed towards the C-terminal fragment of ANGPTL4 and study how it influences cellular signaling. In addition, future research should be aimed at understanding the role of ANGPTL4 in human and to further explore the applicability of plasma ANGPTL4 as a possible biomarker.

Chapter 8

Summary

Obesity is caused by an imbalance between energy intake and energy expenditure. Excessive fat accumulation in the adipose tissue gives birth to a succession of metabolic disturbances, such as insulin resistance, hypertension and a pro-thrombotic state, collectively termed metabolic syndrome. Often metabolic syndrome is also associated with dysregulation of lipoprotein metabolism, leading to changes in the plasma level LDL-cholesterol, HDL-cholesterol and/or triglycerides (TG). TG circulate in the bloodstream in the form of chylomicrons (CM) originating from the small intestine, and very low density lipoproteins produced by the liver. The enzyme lipoprotein lipase (LPL) hydrolyses the TG within CM and VLDL, making it a key regulator of plasma TG clearance. Numerous studies have attempted to find a link between changes in the adipose tissue consequent to obesity and the development of metabolic syndrome. Part of the research focuses on factors secreted from adipose tissue and that have profound effects elsewhere in the body: the so-called adipocytokines. A new adipocytokine was discovered in 2000 by several groups which is referred to as FIAF/ANGPTL4 (Fasting Induced Adipose Factor/angiopoietin-like protein 4). Angptl4 is a 50 KDa secreted protein that belongs to the family of fibrinogen/angiopoietin-like proteins. In this thesis we further characterized the function of Angptl4 in lipid metabolism, focusing on its impact on plasma lipoproteins, on fatty acid-induced inflammation and lipotoxicity, and on the regulation of the level of Angptl4 in human plasma.

In chapter 3 we explored the metabolic function of Angptl4 using transgenic (Tg) mice overexpressing Angptl4. Taking advantage of the induction of the Angptl4 transgene by fasting, we investigated plasma lipoprotein and hepatic cholesterol metabolism in wildtype and Angptl4 transgenic mice after 24 hr of fasting. Angptl4 overexpression increased plasma levels of free fatty acids, glycerol, total cholesterol, and triglycerides. Increased plasma triglycerides could be attributed to inhibition of LPL. It was found that Angptl4 overexpression decreased post-heparin LPL activity by stimulating conversion of endothelial-bound LPL dimers to circulating LPL monomers. Fasting augmented the inhibitory effect of Angptl4 on plasma TG clearance: while no effect of Angptl4 over-expression on VLDL clearance could be detected in the fed state, a dramatic decrease in VLDL clearance and associated tissue cholesterol and fatty acid uptake was found in the 24 hr fasted state. Consequently, hepatic cholesterol content was significantly decreased, leading to universal up-regulation of cholesterol and fatty acid synthesis pathways.

In chapter 4 we report the development, validation and utilization of an ELISA assay to quan-

titatively assess Angptl4 levels in human plasma. While the impact of Angptl4 on plasma lipids is relatively clear, little is known about the physiological determinants of plasma Angptl4 in humans. We found that plasma Angptl4 levels are relatively stable within an individual but they are highly variable between individuals. Treatment of patients with the drug fenofibrate significantly increased plasma hAngptl4 levels. Fasting also significantly increased plasma Angptl4 level, which was further augmented by subsequent endurance exercise. The exercise-induced increase in plasma Angptl4 was completely abolished when subjects were given glucose. Intralipid injection as well as treatment with a β-adrenergic agonist, both of which lead to elevated plasma FFA levels, increased plasma ANGPTL4 levels compared to control treatment. Finally, no relationship was observed between plasma hAngptl4 levels and adipose tissue mRNA expression. Overall, these data reveal a large inter-individual variation in plasma Angptl4 levels, the origin of which is unclear. Within an individual, Angptl4 levels rise in response to elevation of plasma free fatty acids.

In chapter 5 we investigated the role of Angptl4 in the heart. Intramyocardial lipid overload can have dramatic consequences for heart function, possibly leading to non-ischemic heart failure. Accordingly, strict regulation of cardiac lipid uptake is vital. In healthy conditions, the heart mainly utilizes fatty acids as its principal energy substrate. However, when lipids are taken up in excess subsequent lipid accumulation within cardiomyocyte may lead to lipotoxicity and contractile dysfunction. Dietary unsaturated fatty acids have a major impact on human health, which is likely achieved via changes in gene expression. Fatty acids are believed to regulate gene expression mainly via specific nuclear receptors, including the PPARs. Angptl4 was shown to be significantly upregulated in hearts of mice fed with synthetic triglycerides composed of either linoleic acid or linolenic acids. Angptl4 regulation by dietary fat was entirely dependent on PPARβ/δ, and independent of PPARα, in direct contrast to UCP3. Upregulation of Angptl4 resulted in decreased cardiac uptake of plasma triglyceride (TG)-derived fatty acids and decreased fatty acid-induced oxidative stress and lipid peroxidation. In contrast, Angptl4 deletion led to enhanced oxidative stress in the heart, both after an acute oral fat load and after prolonged high fat feeding. The data suggest that stimulation of cardiac Angptl4 gene expression by dietary fatty acids and PPARβ/δ is part of a feedback mechanism aimed at protecting the heart against lipid overload and consequently fatty-acid induced oxidative stress.

In chapter 6 we investigated the possible role of Angptl4 in the small intestine. It is well established that elevated saturated fat consumption is associated with increased risk for numerous chronic diseases, including cardiovascular disease, inflammatory bowel disease, and obesity. However, the underlying mechanisms and why specifically saturated fat is harmful largely remain unknown. Besides carrying out nutrient digestion and absorption via the production of numerous digestive enzymes, the GI-tract also produces a variety of hormones that play pivotal roles in nutrient handling and energy homeostasis. These gut hormones are synthesized by specialized enteroendocrine cells located in the epithelial layer along the GI-tract. Several gut hormones

are known to be influenced by dietary fat, including GLP-1 and cholecystokinin. In chapter 6 we report that Angptl4 is fat sensitive hormone produced by enteroendocrines cells. Noticeably, chronic saturated fat feeding led to complex and dramatic phenotype in Angptl4-/- mice. Angptl4-/- fed saturated fat developed a severe pathology consisting of fibrinopurulent perotinitis, intestinal fibrosis connected with ascites and cachexia. This lethal phenotype is preceded by excessive inflammation as shown by a dramatic hepatic acute phase response. The data show that Angptl4 protects against the pro-inflammatory and ultimately lethal effects of chronic overconsumption of saturated fat.

Taken together, the studies described in this thesis have increased our understanding of Angptl4 function. In addition, they have yielded some exciting new insights that will provide the basis for future investigations. It is expected that the important role of Angptl4 in regulation of lipid metabolism will be increasingly acknowledged.

Chapter 9

Samenvatting

Obesitas wordt veroorzaakt door een dysbalans tussen energie inname en energiegebruik. De abnormale vetophoping in het vetweefsel zorgt voor verstoringen in de stofwisseling zoals insuline resistentie, hypertensie, en een verhoogde stollingsneiging, wat gezamenlijk metabool syndroom wordt genoemd. Vaak gaat metabool syndroom tevens gepaard met verstoringen in het lipoproteine metabolisme, wat leidt tot veranderingen in het plasma gehalte aan LDL-cholesterol, HDL-cholesterol en/of triglyceriden (TG). TG circuleren in het bloed in de vorm van chylomicronen afkomstig uit de dunne darm, en very-low density lipoproteinen geproduceerd door de lever. Het enzym lipoproteine lipase (LPL) hydrolyseert de TG in CM en VLDL en fungeert aldus als een belangrijke regulator van de klaring van plasma TG. Talloze studies hebben gezocht naar een link tussen de veranderingen in vetweefsel die ontstaan als gevolg van obesitas en de ontwikkeling van metabool syndroom. Een deel van het onderzoek concentreert zich op factoren die door vetweefsel worden uitgescheiden en elders in het lichaam hun effect uitoefenen: de zogenaamde adipocytokinen. Een nieuw adipocytokine dat in 2000 door een aantal groepen werd ontdekt heet FIAF/ANGPTL4 (Fasting Induced Adipose Factor/angiopoietin-like protein 4). Angptl4 is een eiwit van 50 kDa behorende tot de familie van fibrinogen/angiopoietin-like proteins. In dit proefschrift hebben we de functie van Angptl4 in het vetmetabolisme verder gekarakteriseerd waarbij de nadruk lag op de effecten op plasma lipoproteinen, op vetzuur-geinduceerde ontsteking , en op de regulatie van het plasma Angptl4 gehalte bij de mens.

In hoofdstuk 3 hebben we de metabole functie van Angptl4 onderzocht met behulp van transgene muizen die Angptl4 tot overexpressie brengen. Gebruik makend van de inductie van het Angptl4 transgen door vasten hebben we plasma lipoproteinen en het cholesterol metabolisme in lever onderzocht in wildtype en Angptl4 transgene muizen na 24 uur vasten. Angptl4 overexpressie leidde tot een verhoging in plasma gehalte aan vrije vetzuren, glycerol, totaal cholesterol en TG. De verhoging in plasma TG kon worden toegeschreven aan remming van LPL. Angptl4 overexpressie leidde tot een verlaging van de postheparine LPL activiteit door de omzetting van endotheel-gebonden LPL dimeren tot circulerende LPL monomeren te stimuleren. Het remmende effecten van Angptl4 op klaring van plasma TG werd verstrekt door vasten: er werd geen effect gevonden van Angptl4 over-expressie op VLDL klaring in de gevoede toestand. Daarentegen werd na 24 uur vasten een sterke verlaging waargenomen in VLDL klaring en de daarmee gepaard gaande opname van cholesterol en vetzuren in de weefsels. Als gevolg daarvan was de opname

van cholesterol in de lever significant verlaagd, wat aanleiding gaf tot een algemene inductie van cholesterol en vetzuursynthese.

In hoofdstuk 4 beschrijven we de ontwikkeling, validatie en toepassing van een ELISA assay om de Angptl4 concentratie in humaan plasma kwantitatief te kunnen bepalen. Ondanks het sterke effect van Angptl4 op plasma lipiden is er nog maar weinig bekend over de fysiologische determinanten van het plasma Angptl4 gehalte in mensen. Het plasma Angptl4 gehalte bleek relatief stabiel te zijn binnen een persoon maar grote variatie te vertonen tussen personen. Behandeling van patinten met het medicijn fenofibraat zorgde voor een significante verhoging in plasma Angptl4 gehalte. Plasma Angptl4 ging ook omhoog na landurig vasten, wat verder versterkt werd door duurinspanning. De verhoging in plasma Angptl4 door inspanning werd volledig teniet gedaan door tevens glucose toe te dienen. Plasma Angptl4 werd verder verhoogd door intralipid injectie alsmede behandeling met een β-adrenerge agonist. Tenslotte werd er geen relatie gevonden tussen plasma Angptl4 gehalte en Angptl4 mRNA expressie in vetweefsel. Deze data laten zien dat Angptl4 in plasma erg variabel is tussen personen. Binnen een persoon gaat plasma Angptl4 omhoog als de plasma vrije vetzuur spiegels stijgen.

In hoofdstuk 5 hebben we de rol van Angptl4 in het hart onderzocht. Een ophoping van vet in het myocardium kan ernstige consequenties hebben voor het functioneren van het hart en mogelijk leiden tot hartfalen. Om die reden is het belangrijk de vetopname in het hart strict te reguleren. In gezonde toestand gebruikt het hart voornamelijk vetzuren als energiebron. Wanneer de vetzuuropname verhoogd is kan echter vet gaan stapelen in de hartcel wat aanleiding kan geven tot lipotoxiciteit en verstoorde contracties. Onverzadigde vetzuren in de voeding benvloeden onze gezondheid op diverse manieren, onder andere door de expressie van genen te benvloeden. Vetzuren reguleren genexpressie voornamelijk via de zogenaamde nucleaire hormoon receptoren, waaronder de PPARs. Wij vonden dat de expressie van Angptl4 significant omhoog ging in muizen die gevoed werden met synthetische triglyceriden bestaande uit linolzuur of linoleenzuur. De regulatie van Angptl4 door vet was volledig afhankelijk van PPARβ/δ en onafhankelijk van PPARα, in tegenstelling tot UCP3. De inductie van Angptl4 leidde tot een verminderde opname van vetzuren door het hart en tot verminderde oxidatieve stress en vetzuurperoxidatie in het hart. Daarentegen ging de afwezigheid van Angptl4 gepaard met verhoogde oxidatieve stress, zowel na een acute vet bolus als na langdurig gebruik van een vetrijk dieet. De data suggereren dat de inductie van Angptl4 door vetzuren in de voeding en door PPARβ/δ deel uitmaakt van een feedback mechanisme dat erop gericht is het hart te beschermen tegen een overdaad aan vet en de daarmee gepaard gaande vetzuur genduceerde oxidatieve stress.

Hoofdstuk 6 richt zich op de mogelijke rol van Angptl4 in de dunne darm. Het is bekend dat een hoge consumptie van verzadigde vetten het risico op diverse ziekten verhoogt, waaronder hart en vaatziekten, IBD (inflammatory bowel disease) en obesitas. Het echter is grotendeels onbekend waarom nu precies verzadigd vet schadelijk is. Naast haar betrokkenheid bij de vertering en opname van voedsel door middel van productie van talloze enzymen produceert de darm ook diverse

hormonen die betrokken zijn bij de verwerking van voedingsstoffen en energie homestase. Deze darmhormonen worden geproduceerd door gespecialiseerde enteroendocriene cellen die aanwezig zijn in het darmepitheel. De productie van een aantal hormonen zoals CCK (cholecystokinin) en GLP-1 (glucagon-like peptide 1) wordt beinvloed door vetconsumptie. In hoofdstuk 6 laten we zien dat Angptl4 een darmhormoon is wiens productie wordt gereguleerd door de hoeveelheid vet in de voeding. De afwezigheid van Angptl4 in muizen in combinatie met het consumeren van een vetrijk voer leid tot een ernstig ziektebeeld waaronder fibrinopurulente peritonitis, intestinale fibrose gecombineerd met ascites en cachexie. Dit lethale ziektebeeld wordt voorafgegaan door ernstige ontsteking wat blijkt uit een extreme acute fase respons in de lever. De resulaten laten zien dat Angptl4 beschermt tegen de pro-inflammatoire en uiteindelijk dodelijke effecten van chronische overconsumptie van verzadigd vet.

De in dit proefschrift beschreven studies hebben belangrijke nieuwe informatie opgeleverd over de functie van Angptl4. Bovendien zijn er een aantal interessante ontdekkingen gedaan die de basis zullen vormen voor toekomstig onderzoek. Het ligt in de lijn der verwachting dat het belang van Angptl4 in de regulatie van het vetmetabolisme steeds meer erkend zal worden.

Chapter 10

Sommaire

L'obésité est la cause d'une mauvaise balance entre la prise et la dépense d'énergie. L'accumulation excessive de lipides dans le tissus adipeux donne naissance à une succession de perturbation métaboliques, comme la résistance à l'insuline, l'hypertension ainsi qu'un état prothrombotique, collectivement appelé syndrôme métabolique. Le syndrôme métabolique est le plus souvent associé à une dérégulation du métabolisme lipoprotéique, menant à des changements des taux plasmatiques de cholestérol LDL, HDL et/ou de triglycérides (TG). Les TG circulent dans le compartiment sanguin sous la forme de chylomicrons (CM) originaires de l'intestin grêle, et de VLDL, d'origine essentiellement hépatique (very low density lipoprotein). Dans ce contexte la LPL (lipase lipoprotéique), capable d'hydrolyser les TG des CM et des VLDL, joue un rôle clef de la clairance plasmatique des TG. De nombreuses études ont tenté d'identifier le lien entre les changements ayant lieu dans le tissus adipeux lors de l'obésité et le développement du syndrôme métabolique. Une partie de ces recherches s'est concentrée sur les facteurs sécrétés par le tissus adipeux ayant un effet important dans le corps: les adipokynes. Découverte en 2000 par différentes équipes, Angptl4 (FIAF/ANGPTL4 : Fasting Induced Adipose Factor/angiopoietin-like protein 4) est une protéine sécrétée de poids molculaire égal à 50KDa, appartenant à la famille des fibrinogène/angiopoietin-like proteins. Dans cette thèse nous avons caractérisé en détail la fonction d'Angptl4 dans le métabolisme lipidique, son impact au sein des lipoprotéines, son implication dans l'inflammation et la lipotoxicité induite par les acides gras, ainsi que sa régulation au niveau plasmatique.

Dans le chapitre 3 nous avons exploré la fonction métabolique d'Angptl4 en utilisant des souris transgéniques (Tg) qui surexpriment Angptl4. Prenant avantage de l'induction du transgène d'Angptl4 par le jeûne, nous avons étudié le métabolisme des lipoprotéines plasmatiques et du cholestérol hépatique dans les souris normale et transgéniques, après 24 heures de jeûne. La surexpression d'Angptl4 augmente les concentrations plasmatiques d'acide gras libres, de glycérol, de cholestérol total et de triglycérides. L'augmentation de la concentration plasmatique de triglycérides a été attribuée à l'inhibition de l'activité de la LPL. Nous avons démontré que la surexpression d'Angptl4 diminue l'activité post-héparine de l'enzyme LPL, stimulant la conversion des dimères attachés à l'endothélium en monomères circulants. Nous avons observé par le jeûne une augmentation de l'effet inhibiteur d'Angptl4 sur la clairance des TG plasmatiques alors qu'aucun effet n'a été observé sur la production de VLDL à l'état nourris dans les souris transgéniques,

une diminution dramatique de la clairance des VLDL, de même que le stockage de cholestérol et d'acide gras ont été drastiquement perturbé dans les souris transgéniques après 24h de jeûne. Par conséquent, le contenue en cholestérol hépatique s'est trouvé significativement diminué, menant à une régulation à la hausse des voix de synthèse de cholestérol et d'acide gras.

Dans le chapitre 4, nous rapportons la mise au point d'un essais ELISA évaluant les quantités d'Angptl4 dans le plasma humain. Alors que l'impact d'Angptl4 sur les lipides plasmatiques est relativement clair, les déterminants physiologiques d'Angptl4 plasmatique chez les humains restent peu connus. Nous avons constaté que les niveaux plasmatiques d'Angptl4 sont relativement stables au sein d'un individu mais hautement variables entre les individus. Le traitement de patients avec fénofibrate augmente sensiblement les niveaux plasmatiques d'hAngptl4. Chez des sujets ayant subit un exercice d'endurance, le jeûne augmente également les niveaux plasmatiques d'Angptl4. Cette augmentation induite par l'exercice a été complètement aboli lorsque les sujets ont pris du glucose. Une injection d'intralipides, ainsi que le traitement avec un agoniste β-Adrénergiques, ont conduit à des niveaux plasmatiques élevés d'acide gras libres, augmentant la concentration plasmatique d'Angptl4, par comparaison avec le groupe control. Enfin, aucune relation n'a été observée entre les concentrations plasmatiques d'hAngptl4 et l'expression d'Angptl4 dans le tissus adipeux. Dans l'ensemble, ces données révèlent une grande variation interindividuelle des niveaux plasmatiques d'Angptl4, dont l'origine est incertaine. Au sein d'un individu, les niveaux d'Angptl4 augmentent en réponse à une élévation des acides gras libres plasmatiques.

Dans le Chapitre 5, nous avons examiné le rôle d'Angptl4 dans le cœur. Une surcharge de lipides dans le myocarde peut avoir des conséquences dramatiques pour la fonction cardiaque, menant éventuellement à un arrêt cardiaque non-ischémique. En conséquence, une réglementation stricte de l'assimilation cardiaque de lipides est vitale. Dans des conditions normales, le cœur utilise les acides gras comme source principale de substrat énergétique. Toutefois, lorsque les lipides sont repris en excès, une accumulation de lipides au sein de cardiomyocyte a lieu pouvant conduire à une lipotoxicité accompagnée de dysfonctionnement contractile. Les acides gras insaturés alimentaires ont un impact majeur sur la santé humaine, pouvant être la conséquence de modifications dans l'expression génétique. Les acides gras régulent l'expression génétique principalement via l'intermédiaire de récepteurs nucléaires spécifiques, y compris les PPARs. Angptl4 a été montré être considérablement uprégulé dans le cœur des souris nourris avec des triglycérides synthétiques composés d'acide linoléique ou d'acide linolénique. La régulation d'Angptl4 par les acides gras alimentaires est entièrement dépendante de PPARβ/δ mais indépendante de PPARα, en directe opposition avec UCP3. L'uprégulation d'Angptl4 a entraîné une absorption cardiaque diminuée des acides gras dérivés des TG plasmatiques diminuant ainsi le stress oxydant ainsi que la péroxydation des lipides. En revanche, la suppression d'Angptl4 a augmenté le stress oxydatifs dans le cœur, aussi bien après une consommation aiguë d'acide gras tant après une consommation chronique comme durant une intervention high fat diet (HFD). Les données suggèrent que stimulation de l'expression d'Angptl4 au niveau du cœur par les acides gras alimentaires et de PPARβ/δ

fait partie d'une mécanisme rétroactif visant à protéger le cœur contre la surcharge lipidique et par conséquent le stress oxydant induit par les acides gras.

Dans le chapitre 6 nous avons examiné le rôle possible de Angptl4 dans l'intestin grêle. Il est bien établi qu'une consommation élevée d'acides gras saturés est associée à un risque accru pour de nombreuses maladies chroniques, y compris les maladies cardiovasculaires, maladies inflammatoires de l'intestin, et l'obésité. Toutefois, les mécanismes sous-jacents permettant d'expliquer pourquoi les matières grasses saturée sont spécifiquement nocives restent en grande partie inconnu. En plus d'être responsable de la digestion et de l'absorption des éléments nutritifs, aidé par la production de nombreuses enzymes digestives, les voies digestives produisent également une variété d'hormones jouant un rôle pivot dans la supplémentation d'éléments nutritifs ainsi que dans l'homéostasie énergétique. Ces hormones intestinales sont synthétisés par cellules spécialisées, les cellules enteroendocrines, situées dans la couche épithéliale tout au long des voies digestives. Plusieurs hormones intestinales sont connus pour être influencés par les acides gras alimentaires, y compris GLP-1 et cholécystokinine. Dans le chapitre 6 nous avons démontré qu'Angptl4 est une hormone sensible aux acides gras, produite par les cellules enteroendocrines. Visiblement, une alimentation chronique riche en acides gras saturés conduit à un phénotype complexe et dramatique chez les souris déficientes pour Angptl4. Angptl4-/- nourries avec des lipides saturés développent une pathologie grave consistant en une péritonite fibrinopurulente, fibrose intestinale liée une ascite et cachexie. Ce phénotype létal est précédé d'une inflammation excessive illustrée par une phase aiguë hépatique spectaculaire. Les données montrent qu'Angptl4 protège contre les effets pro-inflammatoires et finalement létales de surconsommation chronique de lipides saturés.

En conclusion, les études décrites dans cette thèse ont permit d'augmenter notre compréhension de la fonction d'Angptl4. Ces études ouvrent de nouvelles perspectives qui serviront de base pour les recherches futures qui devraient mettrent en évidence un rôle essentiel de Angptl4 dans la réglementation du métabolisme lipidique.

Bibliography

Aas, V., Rokling-Andersen, M. H., Kase, E. T., Thoresen, G. H. and Rustan, A. C.: 2006, Eicosapentaenoic acid (20:5 n-3) increases fatty acid and glucose uptake in cultured human skeletal muscle cells., *J Lipid Res* **47**(2), 366–374.

Abozguia, K., Clarke, K., Lee, L. and Frenneaux, M.: 2006, Modification of myocardial substrate use as a therapy for heart failure., *Nat Clin Pract Cardiovasc Med* **3**(9), 490–498.

Adachi, H., Fujiwara, Y., Kondo, T., Nishikawa, T., Ogawa, R., Matsumura, T., Ishii, N., Nagai, R., Miyata, K., Tabata, M., Motoshima, H., Furukawa, N., Tsuruzoe, K., Kawashima, J., Takeya, M., Yamashita, S., Koh, G. Y., Nagy, A., Suda, T., Oike, Y. and Araki, E.: 2009, Angptl 4 deficiency improves lipid metabolism, suppresses foam cell formation and protects against atherosclerosis., *Biochem Biophys Res Commun* **379**(4), 806–811.

Akiyama, T. E., Lambert, G., Nicol, C. J., Matsusue, K., Peters, J. M., Brewer, H. B. and Gonzalez, F. J.: 2004, Peroxisome proliferator-activated receptor beta/delta regulates very low density lipoprotein production and catabolism in mice on a western diet., *J Biol Chem* **279**(20), 20874–20881.

Ando, Y., Shimizugawa, T., Takeshita, S., Ono, M., Shimamura, M., Koishi, R. and Furukawa, H.: 2003, A decreased expression of angiopoietin-like 3 is protective against atherosclerosis in apoe-deficient mice., *J Lipid Res* **44**(6), 1216–1223.

Augustus, A., Yagyu, H., Haemmerle, G., Bensadoun, A., Vikramadithyan, R. K., Park, S.-Y., Kim, J. K., Zechner, R. and Goldberg, I. J.: 2004, Cardiac-specific knock-out of lipoprotein lipase alters plasma lipoprotein triglyceride metabolism and cardiac gene expression., *J Biol Chem* **279**(24), 25050–25057.

Bäckhed, F., Crawford, P. A., O'Donnell, D. and Gordon, J. I.: 2007, Postnatal lymphatic partitioning from the blood vasculature in the small intestine requires fasting-induced adipose factor., *Proc Natl Acad Sci U S A* **104**(2), 606–611.

Backhed, F., Crawford, P. A., O'Donnell, D., Gordon, J. I., Backhed, F., Ding, H., Wang, T., Hooper, L. V., Koh, G. Y., Nagy, A., Semenkovich, C. F. and Gordon, J. I.: 2004, The gut microbiota as an environmental factor that regulates fat storage, *Proc Natl Acad Sci U S A* **101**(44), 15718–23. eng.

Bäckhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A. and Gordon, J. I.: 2005, Host-bacterial mutualism in the human intestine., *Science* **307**(5717), 1915–1920.

Backhed, F., Manchester, J. K., Semenkovich, C. F. and Gordon, J. I.: 2007, Mechanisms underlying the resistance to diet-induced obesity in germ-free mice, *Proc Natl Acad Sci U S A* **104**(3), 979–84. eng.

Beglinger, C. and Degen, L.: 2004, Fat in the intestine as a regulator of appetite–role of cck, *Physiol Behav* **83**(4), 617–21. eng.

Beigneux, A. P., Davies, B. S., Gin, P., Weinstein, M. M., Farber, E., Qiao, X., Peale, F., Bunting, S., Walzem, R. L., Wong, J. S., Blaner, W. S., Ding, Z. M., Melford, K., Wongsiriroj, N., Shu, X., de Sauvage, F., Ryan, R. O., Fong, L. G., Bensadoun, A. and Young, S. G.: 2007, Glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 plays a critical role in the lipolytic processing of chylomicrons, *Cell Metab* **5**(4), 279–91. eng.

Beigneux, A. P., Franssen, R., Bensadoun, A., Gin, P., Melford, K., Peter, J., Walzem, R. L., Weinstein, M. M., Davies, B. S., Kuivenhoven, J. A., Kastelein, J. J., Fong, L. G., Dallinga-Thie, G. M. and Young, S. G.: 2009, Chylomicronemia with a mutant gpihbp1 (q115p) that cannot bind lipoprotein lipase, *Arterioscler Thromb Vasc Biol* **29**(6), 956–62. eng.

Beigneux, A. P., Gin, P., Davies, B. S., Weinstein, M. M., Bensadoun, A., Ryan, R. O., Fong, L. G. and Young, S. G.: 2008, Glycosylation of asn-76 in mouse gpihbp1 is critical for its appearance on the cell surface and the binding of chylomicrons and lipoprotein lipase, *J Lipid Res* **49**(6), 1312–21. eng.

Belanger, A. J., Lu, H., Date, T., Liu, L. X., Vincent, K. A., Akita, G. Y., Cheng, S. H., Gregory, R. J. and Jiang, C.: 2002, Hypoxia up-regulates expression of peroxisome proliferator-activated receptor gamma angiopoietin-related gene (pgar) in cardiomyocytes: role of hypoxia inducible factor 1alpha, *J Mol Cell Cardiol* **34**(7), 765–74. eng.

Benditt, E., Eriksen, N., Hermodson, M. and Ericsson, L.: 1971, The major proteins of human and monkey amyloid substance: Common properties including unusual n-terminal amino acid sequences., *FEBS Lett* **19**(2), 169–173.

Berbée, J. F. P., van der Hoogt, C. C., Sundararaman, D., Havekes, L. M. and Rensen, P. C. N.: 2005, Severe hypertriglyceridemia in human apoc1 transgenic mice is caused by apoc-i-induced inhibition of lpl., *J Lipid Res* **46**(2), 297–306.

Berryman, D. E., Mulero, J. J., Hughes, L. B., Brasaemle, D. L. and Bensadoun, A.: 1998, Oligomeric structure of hepatic lipase: evidence from a novel epitope tag technique, *Biochim Biophys Acta* **1382**(2), 217–29. Division of Biological Sciences, Cornell University, Ithaca, NY 14853, USA.

Betts, J. C., Cheshire, J. K., Akira, S., Kishimoto, T. and Woo, P.: 1993, The role of nf-kappa b and nf-il6 transactivating factors in the synergistic activation of human serum amyloid a gene expression by interleukin-1 and interleukin-6., *J Biol Chem* **268**(34), 25624–25631.

Black, A. T., Gray, J. P., Shakarjian, M. P., Laskin, D. L., Heck, D. E. and Laskin, J. D.: 2008, Increased oxidative stress and antioxidant expression in mouse keratinocytes following exposure to paraquat., *Toxicol Appl Pharmacol* **231**(3), 384–392.

Bopst, M., Haas, C., Car, B. and Eugster, H. P.: 1998, The combined inactivation of tumor necrosis factor and interleukin-6 prevents induction of the major acute phase proteins by endotoxin., *Eur J Immunol* **28**(12), 4130–4137.

Bouwens, M., Afman, L. A. and Mller, M.: 2007, Fasting induces changes in peripheral blood mononuclear cell gene expression profiles related to increases in fatty acid beta-oxidation: functional role of peroxisome proliferator activated receptor alpha in human peripheral blood mononuclear cells., *Am J Clin Nutr* **86**(5), 1515–1523.

Brandt, J. M., Djouadi, F. and Kelly, D. P.: 1998, Fatty acids activate transcription of the muscle carnitine palmitoyltransferase i gene in cardiac myocytes via the peroxisome proliferator-activated receptor alpha., *J Biol Chem* **273**(37), 23786–23792.

Breckenridge, W. C., Little, J. A., Alaupovic, P., Wang, C. S., Kuksis, A., Kakis, G., Lindgren, F. and Gardiner, G.: 1982, Lipoprotein abnormalities associated with a familial deficiency of hepatic lipase., *Atherosclerosis* **45**(2), 161–179.

Brown, R. J., Edmondson, A. C., Griffon, N., Hill, T. B., Fuki, I. V., Badellino, K. O., Li, M., Wolfe, M. L., Reilly, M. P. and Rader, D. J.: 2009, A naturally occurring variant of endothelial lipase associated with elevated hdl exhibits impaired synthesis., *J Lipid Res* .

Burkart, E. M., Sambandam, N., Han, X., Gross, R. W., Courtois, M., Gierasch, C. M., Shoghi, K., Welch, M. J. and Kelly, D. P.: 2007, Nuclear receptors pparbeta/delta and pparalpha direct distinct metabolic regulatory programs in the mouse heart., *J Clin Invest* **117**(12), 3930–3939.

Camenisch, G., Pisabarro, M. T., Sherman, D., Kowalski, J., Nagel, M., Hass, P., Xie, M. H., Gurney, A., Bodary, S., Liang, X. H., Clark, K., Beresini, M., Ferrara, N. and Gerber, H. P.: 2002, Angptl3 stimulates endothelial cell adhesion and migration via integrin alpha vbeta 3 and induces blood vessel formation in vivo, *J Biol Chem* **277**(19), 17281–90. eng.

Cani, P. D., Amar, J., Iglesias, M. A., Poggi, M., Knauf, C., Bastelica, D., Neyrinck, A. M., Fava, F., Tuohy, K. M., Chabo, C., Waget, A., Delmee, E., Cousin, B., Sulpice, T., Chamontin, B., Ferrieres, J., Tanti, J. F., Gibson, G. R., Casteilla, L., Delzenne, N. M., Alessi, M. C. and Burcelin, R.: 2007, Metabolic endotoxemia initiates obesity and insulin resistance, *Diabetes* **56**(7), 1761–72. eng.

Cani, P. D., Bibiloni, R., Knauf, C., Waget, A., Neyrinck, A. M., Delzenne, N. M. and Burcelin, R.: 2008, Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice, *Diabetes* **57**(6), 1470–81. eng.

Carroll, R. and Severson, D. L.: 2001, Peroxisome proliferator-activated receptor-alpha ligands inhibit cardiac lipoprotein lipase activity., *Am J Physiol Heart Circ Physiol* **281**(2), H888–H894.

Cazes, A., Galaup, A., Chomel, C., Bignon, M., Brechot, N., Le Jan, S., Weber, H., Corvol, P., Muller, L., Germain, S. and Monnot, C.: 2006, Extracellular matrix-bound angiopoietin-like 4 inhibits endothelial cell adhesion, migration, and sprouting and alters actin cytoskeleton, *Circ Res* **99**(11), 1207–15. eng.

Chiu, H.-C., Kovacs, A., Blanton, R. M., Han, X., Courtois, M., Weinheimer, C. J., Yamada, K. A., Brunet, S., Xu, H., Nerbonne, J. M., Welch, M. J., Fettig, N. M., Sharp, T. L., Sambandam, N., Olson, K. M., Ory, D. S. and Schaffer, J. E.: 2005, Transgenic expression of fatty acid transport protein 1 in the heart causes lipotoxic cardiomyopathy., *Circ Res* **96**(2), 225–233.

Chiu, H. C., Kovacs, A., Ford, D. A., Hsu, F. F., Garcia, R., Herrero, P., Saffitz, J. E. and Schaffer, J. E.: 2001, A novel mouse model of lipotoxic cardiomyopathy., *J Clin Invest* **107**(7), 813–822.

Choi, S. Y., Pang, L., Kern, P. A., Kayden, H. J., Curtiss, L. K., Vanni-Reyes, T. M. and Goldberg, I. J.: 1997, Dissociation of lpl and ldl: effects of lipoproteins and anti-apob antibodies., *J Lipid Res* **38**(1), 77–85.

Chomel, C., Cazes, A., Faye, C., Bignon, M., Gomez, E., Ardidie-Robouant, C., Barret, A., Ricard-Blum, S., Muller, L., Germain, S. and Monnot, C.: 2009, Interaction of the coiled-coil domain with glycosaminoglycans protects angiopoietin-like 4 from proteolysis and regulates its antiangiogenic activity, *FASEB J* **23**(3), 940–9. eng.

Clarke, S. D.: 2004, The multi-dimensional regulation of gene expression by fatty acids: polyunsaturated fats as nutrient sensors., *Curr Opin Lipidol* **15**(1), 13–18.

Clee, S. M., Zhang, H., Bissada, N., Miao, L., Ehrenborg, E., Benlian, P., Shen, G. X., Angel, A., LeBoeuf, R. C. and Hayden, M. R.: 1997, Relationship between lipoprotein lipase and high density lipoprotein cholesterol in mice: modulation by cholesteryl ester transfer protein and dietary status, *J Lipid Res* **38**(10), 2079–89. Department of Medical Genetics, University of British Columbia, Vancouver, Canada.

Conklin, D., Gilbertson, D., Taft, D. W., Maurer, M. F., Whitmore, T. E., Smith, D. L., Walker, K. M., Chen, L. H., Wattler, S., Nehls, M. and Lewis, K. B.: 1999, Identification of a mammalian angiopoietin-related protein expressed specifically in liver, *Genomics* **62**(3), 477–82. eng.

Connelly, P. W., Maguire, G. F., Lee, M. and Little, J. A.: 1990, Plasma lipoproteins in familial hepatic lipase deficiency., *Arteriosclerosis* **10**(1), 40–48.

Davies, B. S., Waki, H., Beigneux, A. P., Farber, E., Weinstein, M. M., Wilpitz, D. C., Tai, L. J., Evans, R. M., Fong, L. G., Tontonoz, P. and Young, S. G.: 2008, The expression of gpihbp1, an endothelial cell binding site for lipoprotein lipase and chylomicrons, is induced by peroxisome proliferator-activated receptor-gamma, *Mol Endocrinol* **22**(11), 2496–504. eng.

de Vogel-van den Bosch, H. M., de Wit, N. J. W., Hooiveld, G. J. E. J., Vermeulen, H., van der Veen, J. N., Houten, S. M., Kuipers, F., Mller, M. and van der Meer, R.: 2008, A cholesterol-free, high-fat diet suppresses gene expression of cholesterol transporters in murine small intestine., *Am J Physiol Gastrointest Liver Physiol* **294**(5), G1171–G1180.

de Vries, J. E., Vork, M. M., Roemen, T. H., de Jong, Y. F., Cleutjens, J. P., van der Vusse, G. J. and van Bilsen, M.: 1997, Saturated but not mono-unsaturated fatty acids induce apoptotic cell death in neonatal rat ventricular myocytes., *J Lipid Res* **38**(7), 1384–1394.

de Wit, N. J., Bosch-Vermeulen, H., de Groot, P. J., Hooiveld, G. J., Bromhaar, M. M., Jansen, J., Muller, M. and van der Meer, R.: 2008, The role of the small intestine in the development of dietary fat-induced obesity and insulin resistance in c57bl/6j mice, *BMC Med Genomics* **1**, 14. eng.

Desai, U., Lee, E. C., Chung, K., Gao, C., Gay, J., Key, B., Hansen, G., Machajewski, D., Platt, K. A., Sands, A. T., Schneider, M., Van Sligtenhorst, I., Suwanichkul, A., Vogel, P., Wilganowski, N., Wingert, J., Zambrowicz, B. P., Landes, G. and Powell, D. R.: 2007, Lipid-lowering effects of anti-angiopoietin-like 4 antibody recapitulate the lipid phenotype found in angiopoietin-like 4 knockout mice, *Proc Natl Acad Sci U S A* **104**(28), 11766–71. eng.

Drucker, D. J.: 2007, The role of gut hormones in glucose homeostasis, *J Clin Invest* **117**(1), 24–32. eng.

Duivenvoorden, I., Teusink, B., Rensen, P. C., Romijn, J. A., Havekes, L. M. and Voshol, P. J.: 2005, Apolipoprotein c3 deficiency results in diet-induced obesity and aggravated insulin resistance in mice, *Diabetes* **54**(3), 664–71. TNO Prevention and Health, Gambius Laboratory, Leiden, Netherlands.

Durgan, D. J., Smith, J. K., Hotze, M. A., Egbejimi, O., Cuthbert, K. D., Zaha, V. G., Dyck, J. R. B., Abel, E. D. and Young, M. E.: 2006, Distinct transcriptional regulation of long-chain acyl-coa synthetase isoforms and cytosolic thioesterase 1 in the rodent heart by fatty acids and insulin., *Am J Physiol Heart Circ Physiol* **290**(6), H2480–H2497.

Dutton, S. and Trayhurn, P.: 2008, Regulation of angiopoietin-like protein 4/fasting-induced adipose factor (angptl4/fiaf) expression in mouse white adipose tissue and 3t3-l1 adipocytes, *Br J Nutr* **100**(1), 18–26. eng.

Edwards, M. G., Sarkar, D., Klopp, R., Morrow, J. D., Weindruch, R. and Prolla, T. A.: 2003, Age-related impairment of the transcriptional responses to oxidative stress in the mouse heart., *Physiol Genomics* **13**(2), 119–127.

Endo, Y., Matsushita, M. and Fujita, T.: 2007, Role of ficolin in innate immunity and its molecular basis., *Immunobiology* **212**(4-5), 371–379.

Endo, Y., Takahashi, M. and Fujita, T.: 2006, Lectin complement system and pattern recognition., *Immunobiology* **211**(4), 283–293.

Finck, B. N., Lehman, J. J., Leone, T. C., Welch, M. J., Bennett, M. J., Kovacs, A., Han, X., Gross, R. W., Kozak, R., Lopaschuk, G. D. and Kelly, D. P.: 2002, The cardiac phenotype induced by pparalpha overexpression mimics that caused by diabetes mellitus., *J Clin Invest* **109**(1), 121–130.

Folsom, A. R., Peacock, J. M., Demerath, E. and Boerwinkle, E.: 2008, Variation in angptl4 and risk of coronary heart disease: the atherosclerosis risk in communities study, *Metabolism* **57**(11), 1591–6. eng.

Fugier, C., Tousaint, J. J., Prieur, X., Plateroti, M., Samarut, J. and Delerive, P.: 2006, The lipoprotein lipase inhibitor angptl3 is negatively regulated by thyroid hormone, *J Biol Chem* **281**(17), 11553–9. eng.

Fujimoto, K., Koishi, R., Shimizugawa, T. and Ando, Y.: 2006, Angptl3-null mice show low plasma lipid concentrations by enhanced lipoprotein lipase activity, *Exp Anim* **55**(1), 27–34. eng.

Galaup, A., Cazes, A., Le Jan, S., Philippe, J., Connault, E., Le Coz, E., Mekid, H., Mir, L. M., Opolon, P., Corvol, P., Monnot, C. and Germain, S.: 2006, Angiopoietin-like 4 prevents metastasis through inhibition of vascular permeability and tumor cell motility and invasiveness, *Proc Natl Acad Sci U S A* **103**(49), 18721–6. eng.

Gale, N. W., Thurston, G., Hackett, S. F., Renard, R., Wang, Q., McClain, J., Martin, C., Witte, C., Witte, M. H., Jackson, D., Suri, C., Campochiaro, P. A., Wiegand, S. J. and Yancopoulos, G. D.: 2002, Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by angiopoietin-1., *Dev Cell* **3**(3), 411–423.

Ge, H., Cha, J. Y., Gopal, H., Harp, C., Yu, X., Repa, J. J. and Li, C.: 2005, Differential regulation and properties of angiopoietin-like proteins 3 and 4, *J Lipid Res* **46**(7), 1484–90. eng.

Ge, H., Yang, G., Huang, L., Motola, D. L., Pourbahrami, T. and Li, C.: 2004, Oligomerization and regulated proteolytic processing of angiopoietin-like protein 4., *J Biol Chem* **279**(3), 2038–2045.

Ge, H., Yang, G., Yu, X., Pourbahrami, T. and Li, C.: 2004, Oligomerization state-dependent hyperlipidemic effect of angiopoietin-like protein 4, *J Lipid Res* **45**(11), 2071–9. Touchstone Center for Diabetes Research, University of Texas Southwestern Medical Center, Dallas, TX 75390-8854, USA.

Gealekman, O., Burkart, A., Chouinard, M., Nicoloro, S. M., Straubhaar, J. and Corvera, S.: 2008, Enhanced angiogenesis in obesity and in response to ppargamma activators through adipocyte vegf and angptl4 production, *Am J Physiol Endocrinol Metab* **295**(5), E1056–64. eng.

Gilde, A. J., van der Lee, K. A. J. M., Willemsen, P. H. M., Chinetti, G., van der Leij, F. R., van der Vusse, G. J., Staels, B. and van Bilsen, M.: 2003, Peroxisome proliferator-activated receptor (ppar) alpha and pparbeta/delta, but not ppargamma, modulate the expression of genes involved in cardiac lipid metabolism., *Circ Res* **92**(5), 518–524.

Gin, P., Beigneux, A. P., Davies, B., Young, M. F., Ryan, R. O., Bensadoun, A., Fong, L. G. and Young, S. G.: 2007, Normal binding of lipoprotein lipase, chylomicrons, and apo-av to gpihbp1 containing a g56r amino acid substitution, *Biochim Biophys Acta* **1771**(12), 1464–8. eng.

Goudriaan, J. R., Dahlmans, V. E., Febbraio, M., Teusink, B., Romijn, J. A., Havekes, L. M. and Voshol, P. J.: 2002, Intestinal lipid absorption is not affected in cd36 deficient mice, *Mol Cell Biochem* **239**(1-2), 199–202. eng.

Gustavsson, M., Mallard, C., Vannucci, S. J., Wilson, M. A., Johnston, M. V. and Hagberg, H.: 2007, Vascular response to hypoxic preconditioning in the immature brain, *J Cereb Blood Flow Metab* **27**(5), 928–38. eng.

Harvey, N. L., Srinivasan, R. S., Dillard, M. E., Johnson, N. C., Witte, M. H., Boyd, K., Sleeman, M. W. and Oliver, G.: 2005, Lymphatic vascular defects promoted by prox1 haploinsufficiency cause adult-onset obesity., *Nat Genet* **37**(10), 1072–1081.

Hata, A., Ridinger, D. N., Sutherland, S. D., Emi, M., Kwong, L. K., Shuhua, J., Lubbers, A., Guy-Grand, B., Basdevant, A. and Iverius, P. H.: 1992, Missense mutations in exon 5 of the human lipoprotein lipase gene. inactivation correlates with loss of dimerization., *J Biol Chem* **267**(28), 20132–20139.

Hatsuda, S., Shoji, T., Shinohara, K., Kimoto, E., Mori, K., Fukumoto, S., Koyama, H., Emoto, M. and Nishizawa, Y.: 2007, Association between plasma angiopoietin-like protein 3 and arterial wall thickness in healthy subjects, *J Vasc Res* **44**(1), 61–6. eng.

He, P., Borland, M. G., Zhu, B., Sharma, A. K., Amin, S., El-Bayoumy, K., Gonzalez, F. J. and Peters, J. M.: 2008, Effect of ligand activation of peroxisome proliferator-activated receptor-beta/delta (ppar-beta/delta) in human lung cancer cell lines, *Toxicology* **254**(1-2), 112–7. eng.

Heinaniemi, M., Uski, J. O., Degenhardt, T. and Carlberg, C.: 2007, Meta-analysis of primary target genes of peroxisome proliferator-activated receptors, *Genome Biol* **8**(7), R147. eng.

Hermann, L. M., Pinkerton, M., Jennings, K., Yang, L., Grom, A., Sowders, D., Kersten, S., Witte, D. P., Hirsch, R. and Thornton, S.: 2005, Angiopoietin-like-4 is a potential angiogenic mediator in arthritis, *Clin Immunol* **115**(1), 93–101. eng.

Herrmann, C., Goke, R., Richter, G., Fehmann, H. C., Arnold, R. and Goke, B.: 1995, Glucagon-like peptide-1 and glucose-dependent insulin-releasing polypeptide plasma levels in response to nutrients, *Digestion* **56**(2), 117–26. eng.

Hill, J. S., Davis, R. C., Yang, D., Wen, J., Philo, J. S., Poon, P. H., Phillips, M. L., Kempner, E. S. and Wong, H.: 1996, Human hepatic lipase subunit structure determination, *J Biol Chem* **271**(37), 22931–6. Lipid Research Laboratory, West Los Angeles VA Medical Center, Los Angeles, California 90073, USA.

Hoeks, J., Hesselink, M. K. C., Russell, A. P., Mensink, M., Saris, W. H. M., Mensink, R. P. and Schrauwen, P.: 2006, Peroxisome proliferator-activated receptor-gamma coactivator-1 and insulin resistance: acute effect of fatty acids., *Diabetologia* **49**(10), 2419–2426.

Hoeks, J., van Baak, M. A., Hesselink, M. K. C., Hul, G. B., Vidal, H., Saris, W. H. M. and Schrauwen, P.: 2003, Effect of beta1- and beta2-adrenergic stimulation on energy expenditure, substrate oxidation, and ucp3 expression in humans., *Am J Physiol Endocrinol Metab* **285**(4), E775–E782.

Honor, C., Hummelshoj, T., Hansen, B. E., Madsen, H. O., Eggleton, P. and Garred, P.: 2007, The innate immune component ficolin 3 (hakata antigen) mediates the clearance of late apoptotic cells., *Arthritis Rheum* **56**(5), 1598–1607.

Hukshorn, C. J., Westerterp-Plantenga, M. S. and Saris, W. H. M.: 2003, Pegylated human recombinant leptin (peg-ob) causes additional weight loss in severely energy-restricted, overweight men., *Am J Clin Nutr* **77**(4), 771–776.

Hwang, D.: 2001, Modulation of the expression of cyclooxygenase-2 by fatty acids mediated through toll-like receptor 4-derived signaling pathways, *Faseb J* **15**(14), 2556–64. eng.

Inaba, T., Matsuda, M., Shimamura, M., Takei, N., Terasaka, N., Ando, Y., Yasumo, H., Koishi, R., Makishima, M. and Shimomura, I.: 2003, Angiopoietin-like protein 3 mediates hypertriglyceridemia induced by the liver x receptor, *J Biol Chem* **278**(24), 21344–51. eng.

Inukai, K., Nakashima, Y., Watanabe, M., Kurihara, S., Awata, T., Katagiri, H., Oka, Y. and Katayama, S.: 2004, Angptl3 is increased in both insulin-deficient and -resistant diabetic states, *Biochem Biophys Res Commun* **317**(4), 1075–9. eng.

Ioka, R. X., Kang, M. J., Kamiyama, S., Kim, D. H., Magoori, K., Kamataki, A., Ito, Y., Takei, Y. A., Sasaki, M., Suzuki, T., Sasano, H., Takahashi, S., Sakai, J., Fujino, T. and Yamamoto, T. T.: 2003, Expression cloning and characterization of a novel glycosylphosphatidylinositol-anchored high density lipoprotein-binding protein, gpi-hbp1, *J Biol Chem* **278**(9), 7344–9. eng.

Ito, Y., Oike, Y., Yasunaga, K., Hamada, K., Miyata, K., Matsumoto, S., Sugano, S., Tanihara, H., Masuho, Y. and Suda, T.: 2003, Inhibition of angiogenesis and vascular leakiness by angiopoietin-related protein 4, *Cancer Res* **63**(20), 6651–7. eng.

Jaye, M., Lynch, K. J., Krawiec, J., Marchadier, D., Maugeais, C., Doan, K., South, V., Amin, D., Perrone, M. and Rader, D. J.: 1999, A novel endothelial-derived lipase that modulates hdl metabolism., *Nat Genet* **21**(4), 424–428.

Jin, W., Wang, X., Millar, J. S., Quertermous, T., Rothblat, G. H., Glick, J. M. and Rader, D. J.: 2007, Hepatic proprotein convertases modulate hdl metabolism, *Cell Metab* **6**(2), 129–36. eng.

Jong, M. C., Hofker, M. H. and Havekes, L. M.: 1999, Role of apocs in lipoprotein metabolism: functional differences between apoc1, apoc2, and apoc3, *Arterioscler Thromb Vasc Biol* **19**(3), 472–84. TNO-Prevention and Health, Gaubius Laboratory, MGC-Department of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands. mc.jong@pg.tno.nl.

Joosten, M. M., Beulens, J. W. J., Kersten, S. and Hendriks, H. F. J.: 2008, Moderate alcohol consumption increases insulin sensitivity and adipoq expression in postmenopausal women: a randomised, crossover trial., *Diabetologia* **51**(8), 1375–1381.

Juge-Aubry, C., Pernin, A., Favez, T., Burger, A. G., Wahli, W., Meier, C. A. and Desvergne, B.: 1997, Dna binding properties of peroxisome proliferator-activated receptor subtypes on various natural peroxisome proliferator response elements. importance of the 5'-flanking region., *J Biol Chem* **272**(40), 25252–25259.

Kahn, S. E., Hull, R. L. and Utzschneider, K. M.: 2006, Mechanisms linking obesity to insulin resistance and type 2 diabetes, *Nature* **444**(7121), 840–6. Division of Metabolism, Endocrinology and Nutrition, Department of Medicine, VA Puget Sound Health Care System and University of Washington, 1660 South Columbian Way, Seattle, Washington 98108, USA. skahn@u.washington.edu.

Kaplan, R., Zhang, T., Hernandez, M., Gan, F. X., Wright, S. D., Waters, M. G. and Cai, T. Q.: 2003, Regulation of the angiopoietin-like protein 3 gene by lxr, *J Lipid Res* **44**(1), 136–43. eng.

Kathiresan, S., Willer, C. J., Peloso, G. M., Demissie, S., Musunuru, K., Schadt, E. E., Kaplan, L., Bennett, D., Li, Y., Tanaka, T., Voight, B. F., Bonnycastle, L. L., Jackson, A. U., Crawford, G., Surti, A., Guiducci, C., Burtt, N. P., Parish, S., Clarke, R., Zelenika, D., Kubalanza, K. A., Morken, M. A., Scott, L. J., Stringham, H. M., Galan, P., Swift, A. J., Kuusisto, J., Bergman, R. N., Sundvall, J., Laakso, M., Ferrucci, L., Scheet, P., Sanna, S., Uda, M., Yang, Q., Lunetta, K. L., Dupuis, J., de Bakker, P. I., O'Donnell, C. J., Chambers, J. C., Kooner, J. S., Hercberg, S., Meneton, P., Lakatta, E. G., Scuteri, A., Schlessinger, D., Tuomilehto, J., Collins, F. S., Groop, L., Altshuler, D., Collins, R., Lathrop, G. M., Melander, O., Salomaa, V., Peltonen, L., Orho-Melander, M., Ordovas, J. M., Boehnke, M., Abecasis, G. R., Mohlke, K. L. and Cupples, L. A.: 2009, Common variants at 30 loci contribute to polygenic dyslipidemia, *Nat Genet* **41**(1), 56–65. eng.

Kersten, S.: 2005, Regulation of lipid metabolism via angiopoietin-like proteins, *Biochem Soc Trans* **33**(Pt 5), 1059–62. Nutrition, Metabolism and Genomics group, Division of Human Nutrition, Wageningen University, PO BOX 8129, 6700 EV, Wageningen, The Netherlands. sander.kersten@wur.nl.

Kersten, S., Lichtenstein, L., Steenbergen, E., Mudde, K., Hendriks, H. F., Hesselink, M. K., Schrauwen, P. and Muller, M.: 2009, Caloric restriction and exercise increase plasma angptl4 levels in humans via elevated free fatty acids, *Arterioscler Thromb Vasc Biol* **29**(6), 969–74. eng.

Kersten, S., Mandard, S., Tan, N. S., Escher, P., Metzger, D., Chambon, P., Gonzalez, F. J., Desvergne, B. and Wahli, W.: 2000, Characterization of the fasting-induced adipose factor fiaf, a novel peroxisome proliferator-activated receptor target gene, *J Biol Chem* **275**(37), 28488–93.

Kim, I., Kim, H. G., Kim, H., Kim, H. H., Park, S. K., Uhm, C. S., Lee, Z. H. and Koh, G. Y.: 2000, Hepatic expression, synthesis and secretion of a novel fibrinogen/angiopoietin-related protein that prevents endothelial-cell apoptosis, *Biochem J* **346 Pt 3**, 603–10. eng.

Koishi, R., Ando, Y., Ono, M., Shimamura, M., Yasumo, H., Fujiwara, T., Horikoshi, H. and Furukawa, H.: 2002, Angptl3 regulates lipid metabolism in mice, *Nat Genet* **30**(2), 151–7. Biomedical Research Laboratories, Sankyo Co., Ltd., 1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140-8710, Japan. koishi@shina.sankyo.co.jp.

Koster, A., Chao, Y. B., Mosior, M., Ford, A., Gonzalez-DeWhitt, P. A., Hale, J. E., Li, D., Qiu, Y., Fraser, C. C., Yang, D. D., Heuer, J. G., Jaskunas, S. R. and Eacho, P.: 2005, Transgenic angiopoietin-like (angptl)4 overexpression and targeted disruption of angptl4 and angptl3: regulation of triglyceride metabolism, *Endocrinology* **146**(11), 4943–50.

Lacy-Hulbert, A., Smith, A. M., Tissire, H., Barry, M., Crowley, D., Bronson, R. T., Roes, J. T., Savill, J. S. and Hynes, R. O.: 2007, Ulcerative colitis and autoimmunity induced by loss of myeloid alphav integrins, *Proc Natl Acad Sci U S A* **104**(40), 15823–8. eng.

Le Jan, S., Amy, C., Cazes, A., Monnot, C., Lamande, N., Favier, J., Philippe, J., Sibony, M., Gasc, J. M., Corvol, P. and Germain, S.: 2003, Angiopoietin-like 4 is a proangiogenic factor produced during ischemia and in conventional renal cell carcinoma, *Am J Pathol* **162**(5), 1521–8. eng.

Lee, J. Y., Sohn, K. H., Rhee, S. H. and Hwang, D.: 2001, Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through toll-like receptor 4, *J Biol Chem* **276**(20), 16683–9. eng.

Lee, J. Y., Ye, J., Gao, Z., Youn, H. S., Lee, W. H., Zhao, L., Sizemore, N. and Hwang, D. H.: 2003, Reciprocal modulation of toll-like receptor-4 signaling pathways involving myd88 and phosphatidylinositol 3-kinase/akt by saturated and polyunsaturated fatty acids, *J Biol Chem* **278**(39), 37041–51. eng.

Lee, K. A. V. D., Willemsen, P. H., Vusse, G. J. V. D. and Bilsen, M. V.: 2000, Effects of fatty acids on uncoupling protein-2 expression in the rat heart., *FASEB J* **14**(3), 495–502.

Levin, M., Franklin, E. C., Frangione, B. and Pras, M.: 1972, The amino acid sequence of a major nonimmunoglobulin component of some amyloid fibrils., *J Clin Invest* **51**(10), 2773–2776.

Ley, R. E., Bckhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D. and Gordon, J. I.: 2005, Obesity alters gut microbial ecology., *Proc Natl Acad Sci U S A* **102**(31), 11070–11075.

Ley, R. E., Turnbaugh, P. J., Klein, S. and Gordon, J. I.: 2006, Microbial ecology: human gut microbes associated with obesity., *Nature* **444**(7122), 1022–1023.

Li, L., Beauchamp, M. C. and Renier, G.: 2002, Peroxisome proliferator-activated receptor alpha and gamma agonists upregulate human macrophage lipoprotein lipase expression, *Atherosclerosis* **165**(1), 101–10. eng.

Lichtenstein, L., Berbee, J. F., van Dijk, S. J., van Dijk, K. W., Bensadoun, A., Kema, I. P., Voshol, P. J., Muller, M., Rensen, P. C. and Kersten, S.: 2007, Angptl4 upregulates cholesterol synthesis in liver via inhibition of lpl- and hl-dependent hepatic cholesterol uptake, *Arterioscler Thromb Vasc Biol* **27**(11), 2420–7. eng.

Mandard, S., Muller, M. and Kersten, S.: 2004, Peroxisome proliferator-activated receptor alpha target genes., *Cell Mol Life Sci* **61**(4), 393–416.

Mandard, S., Zandbergen, F., Tan, N., Escher, P., Patsouris, D., Koenig, W., Kleemann, R., Bakker, A., Veenman, F., Wahli, W., Muller, M. and Kersten, S.: 2004, The direct peroxisome proliferator-activated receptor target fasting-induced adipose factor (fiaf/pgar/angptl4) is present in blood plasma as a truncated protein that is increased by fenofibrate treatment, *J Biol Chem* **279**(33), 34411–20.

Mandard, S., Zandbergen, F., van Straten, E., Wahli, W., Kuipers, F., Muller, M. and Kersten, S.: 2006, The fasting-induced adipose factor/angiopoietin-like protein 4 is physically associated with lipoproteins and governs plasma lipid levels and adiposity, *J Biol Chem* **281**(2), 934–44.

Mann, J. I.: 2002, Diet and risk of coronary heart disease and type 2 diabetes, *Lancet* **360**(9335), 783–9. eng.

Matsusue, K., Miyoshi, A., Yamano, S. and Gonzalez, F. J.: 2006, Ligand-activated pparbeta efficiently represses the induction of lxr-dependent promoter activity through competition with rxr, *Mol Cell Endocrinol* **256**(1-2), 23–33. eng.

Merkel, M., Eckel, R. H. and Goldberg, I. J.: 2002, Lipoprotein lipase: genetics, lipid uptake, and regulation, *J Lipid Res* **43**(12), 1997–2006. eng.

Moon, H. D., Nakajima, K., Kamiyama, K., Takanashi, K., Sakurabayashi, I. and Nagamine, T.: 2008, Higher frequency of abnormal serum angiopoietin-like protein 3 than abnormal cholesteryl ester transfer protein in japanese hyperalphalipoproteinemic subjects, *Clin Chim Acta* **398**(1-2), 99–104. eng.

Murata, M., Yudo, K., Nakamura, H., Chiba, J., Okamoto, K., Suematsu, N., Nishioka, K., Beppu, M., Inoue, K., Kato, T. and Masuko, K.: 2009, Hypoxia upregulates the expression of angiopoietin-like-4 in human articular chondrocytes: role of angiopoietin-like-4 in the expression of matrix metalloproteinases and cartilage degradation, *J Orthop Res* **27**(1), 50–7. eng.

Nadra, K., Anghel, S. I., Joye, E., Tan, N. S., Basu-Modak, S., Trono, D., Wahli, W. and Desvergne, B.: 2006, Differentiation of trophoblast giant cells and their metabolic functions are dependent on peroxisome proliferator-activated receptor beta/delta., *Mol Cell Biol* **26**(8), 3266–3281.

Nettleton, J. A., Volcik, K. A., Demerath, E. W., Boerwinkle, E. and Folsom, A. R.: 2008, Longitudinal changes in triglycerides according to angptl4[e40k] genotype and longitudinal body weight change in the atherosclerosis risk in communities study, *Ann Epidemiol* **18**(11), 842–6. eng.

Olivecrona, T., Bergo, M., Hultin, M. and Olivecrona, G.: 1995, Nutritional regulation of lipoprotein lipase, *Can J Cardiol* **11 Suppl G**, 73G–78G. Department of Medical Biochemistry and Biophysics, University of Umea, Sweden.

Ono, M., Shimizugawa, T., Shimamura, M., Yoshida, K., Noji-Sakikawa, C., Ando, Y., Koishi, R. and Furukawa, H.: 2003, Protein region important for regulation of lipid metabolism in angiopoietin-like 3 (angptl3): Angptl3 is cleaved and activated in vivo, *J Biol Chem* **278**(43), 41804–9. eng.

Osborne, J. C., Bengtsson-Olivecrona, G., Lee, N. S. and Olivecrona, T.: 1985, Studies on inactivation of lipoprotein lipase: role of the dimer to monomer dissociation., *Biochemistry* **24**(20), 5606–5611.

Padua, D., Zhang, X. H., Wang, Q., Nadal, C., Gerald, W. L., Gomis, R. R. and Massague, J.: 2008, Tgfbeta primes breast tumors for lung metastasis seeding through angiopoietin-like 4, *Cell* **133**(1), 66–77. eng.

Palstra, R.-J., de Laat, W. and Grosveld, F.: 2008, Beta-globin regulation and long-range interactions., *Adv Genet* **61**, 107–142.

Park, T.-S., Yamashita, H., Blaner, W. S. and Goldberg, I. J.: 2007, Lipids in the heart: a source of fuel and a source of toxins., *Curr Opin Lipidol* **18**(3), 277–282.

Patsouris, D., Mandard, S., Voshol, P. J., Escher, P., Tan, N. S., Havekes, L. M., Koenig, W., Mrz, W., Tafuri, S., Wahli, W., Mller, M. and Kersten, S.: 2004, Pparalpha governs glycerol metabolism., *J Clin Invest* **114**(1), 94–103.

Pterfy, M., Ben-Zeev, O., Mao, H. Z., Weissglas-Volkov, D., Aouizerat, B. E., Pullinger, C. R., Frost, P. H., Kane, J. P., Malloy, M. J., Reue, K., Pajukanta, P. and Doolittle, M. H.: 2007, Mutations in lmf1 cause combined lipase deficiency and severe hypertriglyceridemia., *Nat Genet* **39**(12), 1483–1487.

Rakhshandehroo, M., Sanderson, L. M., Matilainen, M., Stienstra, R., Carlberg, C., de Groot, P. J., Mller, M. and Kersten, S.: 2007, Comprehensive analysis of pparalpha-dependent regulation of hepatic lipid metabolism by expression profiling., *PPAR Res* **2007**, 26839.

Rensen, P. C., Herijgers, N., Netscher, M. H., Meskers, S. C., van Eck, M. and van Berkel, T. J.: 1997, Particle size determines the specificity of apolipoprotein e-containing triglyceride-rich emulsions for the ldl receptor versus hepatic remnant receptor in vivo, *J Lipid Res* **38**(6), 1070–84. Division of Biopharmaceutics, Leiden-Amsterdam Center for Drug Research, University of Leiden, Sylvius Laboratories, The Netherlands.

Rieck, M., Meissner, W., Ries, S., Muller-Brusselbach, S. and Muller, R.: 2008, Ligand-mediated regulation of peroxisome proliferator-activated receptor (ppar) beta/delta: a comparative analysis of ppar-selective agonists and all-trans retinoic acid, *Mol Pharmacol* **74**(5), 1269–77. eng.

Romeo, S., Pennacchio, L. A., Fu, Y., Boerwinkle, E., Tybjaerg-Hansen, A., Hobbs, H. H. and Cohen, J. C.: 2007, Population-based resequencing of angptl4 uncovers variations that reduce triglycerides and increase hdl, *Nat Genet* **39**(4), 513–6. eng.

Romeo, S., Yin, W., Kozlitina, J., Pennacchio, L. A., Boerwinkle, E., Hobbs, H. H. and Cohen, J. C.: 2009, Rare loss-of-function mutations in angptl family members contribute to plasma triglyceride levels in humans, *J Clin Invest* **119**(1), 70–9. eng.

Roudkenar, M. H., Kuwahara, Y., Baba, T., Roushandeh, A. M., Ebishima, S., Abe, S., Ohkubo, Y. and Fukumoto, M.: 2007, Oxidative stress induced lipocalin 2 gene expression: addressing its expression under the harmful conditions., *J Radiat Res (Tokyo)* **48**(1), 39–44.

Sanderson, L. M., de Groot, P. J., Hooiveld, G. J., Koppen, A., Kalkhoven, E., Muller, M. and Kersten, S.: 2008, Effect of synthetic dietary triglycerides: a novel research paradigm for nutrigenomics, *PLoS ONE* **3**(2), e1681. eng.

Sanger, G. J. and Lee, K.: 2008, Hormones of the gut-brain axis as targets for the treatment of upper gastrointestinal disorders, *Nat Rev Drug Discov* **7**(3), 241–54. eng.

Saramki, A., Diermeier, S., Kellner, R., Laitinen, H., Vasnen, S. and Carlberg, C.: 2009, Cyclical chromatin looping and transcription factor association on the regulatory regions of the p21 (cdkn1a) gene in response to 1alpha,25-dihydroxyvitamin d3., *J Biol Chem* **284**(12), 8073–8082.

Schaffer, J. E.: 2003, Lipotoxicity: when tissues overeat., *Curr Opin Lipidol* **14**(3), 281–287.

Scheja, L., Heese, B., Zitzer, H., Michael, M. D., Siesky, A. M., Pospisil, H., Beisiegel, U. and Seedorf, K.: 2008, Acute-phase serum amyloid a as a marker of insulin resistance in mice., *Exp Diabetes Res* **2008**, 230837.

Schmuth, M., Haqq, C. M., Cairns, W. J., Holder, J. C., Dorsam, S., Chang, S., Lau, P., Fowler, A. J., Chuang, G., Moser, A. H., Brown, B. E., Mao-Qiang, M., Uchida, Y., Schoonjans, K., Auwerx, J., Chambon, P., Willson, T. M., Elias, P. M. and Feingold, K. R.: 2004, Peroxisome proliferator-activated receptor (ppar)-beta/delta stimulates differentiation and lipid accumulation in keratinocytes., *J Invest Dermatol* **122**(4), 971–983.

Schoonjans, K., Peinado-Onsurbe, J., Lefebvre, A. M., Heyman, R. A., Briggs, M., Deeb, S., Staels, B. and Auwerx, J.: 1996, Pparalpha and ppargamma activators direct a distinct tissue-specific transcriptional response via a ppre in the lipoprotein lipase gene., *EMBO J* **15**(19), 5336–5348.

Schrauwen, P., Hesselink, M. K. C., Vaartjes, I., Kornips, E., Saris, W. H. M., Giacobino, J.-P. and Russell, A.: 2002, Effect of acute exercise on uncoupling protein 3 is a fat metabolism-mediated effect., *Am J Physiol Endocrinol Metab* **282**(1), E11–E17.

Schug, T. T., Berry, D. C., Shaw, N. S., Travis, S. N. and Noy, N.: 2007, Opposing effects of retinoic acid on cell growth result from alternate activation of two different nuclear receptors., *Cell* **129**(4), 723–733.

Sendak, R. A. and Bensadoun, A.: 1998, Identification of a heparin-binding domain in the distal carboxyl-terminal region of lipoprotein lipase by site-directed mutagenesis, *J Lipid Res* **39**(6), 1310–5. eng.

Shan, L., Yu, X. C., Liu, Z., Hu, Y., Sturgis, L. T., Miranda, M. L. and Liu, Q.: 2009, The angiopoietin-like proteins angptl3 and angptl4 inhibit lipoprotein lipase activity through distinct mechanisms, *J Biol Chem* **284**(3), 1419–24. eng.

Shi, H., Kokoeva, M. V., Inouye, K., Tzameli, I., Yin, H. and Flier, J. S.: 2006, Tlr4 links innate immunity and fatty acid-induced insulin resistance, *J Clin Invest* **116**(11), 3015–25. eng.

Shimamura, M., Matsuda, M., Ando, Y., Koishi, R., Yasumo, H., Furukawa, H. and Shimomura, I.: 2004, Leptin and insulin down-regulate angiopoietin-like protein 3, a plasma triglyceride-increasing factor, *Biochem Biophys Res Commun* **322**(3), 1080–5. eng.

Shimamura, M., Matsuda, M., Yasumo, H., Okazaki, M., Fujimoto, K., Kono, K., Shimizugawa, T., Ando, Y., Koishi, R., Kohama, T., Sakai, N., Kotani, K., Komuro, R., Ishida, T., Hirata, K., Yamashita, S., Furukawa, H. and Shimomura, I.: 2007, Angiopoietin-like protein3 regulates plasma hdl cholesterol through suppression of endothelial lipase, *Arterioscler Thromb Vasc Biol* **27**(2), 366–72. Department of Medicine and Pathophysiology, Graduate School of Frontier Bioscience, Osaka University, Osaka, Japan.

Shimizugawa, T., Ono, M., Shimamura, M., Yoshida, K., Ando, Y., Koishi, R., Ueda, K., Inaba, T., Minekura, H., Kohama, T. and Furukawa, H.: 2002, Angptl3 decreases very low density lipoprotein triglyceride clearance by inhibition of lipoprotein lipase, *J Biol Chem* **277**(37), 33742–8. eng.

Shoji, T., Hatsuda, S., Tsuchikura, S., Kimoto, E., Kakiya, R., Tahara, H., Koyama, H., Emoto, M., Tabata, T. and Nishizawa, Y.: 2009, Plasma angiopoietin-like protein 3 (angptl3) concentration is associated with uremic dyslipidemia, *Atherosclerosis* . Eng.

Sonnenburg, W. K., Yu, D., Lee, E. C., Xiong, W., Gololobov, G., Key, B., Gay, J., Wilganowski, N., Hu, Y., Zhao, S., Schneider, M., Ding, Z. M., Zambrowicz, B. P., Landes, G., Powell, D. R. and Desai, U.: 2009, Glycosylphosphatidylinositol-anchored hdl-binding protein stabilizes lipoprotein lipase and prevents its inhibition by angiopoietin-like 3 and angiopoietin-like 4, *J Lipid Res* . Eng.

Staiger, H., Haas, C., Machann, J., Werner, R., Weisser, M., Schick, F., Machicao, F., Stefan, N., Fritsche, A. and Haring, H. U.: 2009, Muscle-derived angiopoietin-like protein 4 is induced by fatty acids via peroxisome proliferator-activated receptor (ppar)-delta and is of metabolic relevance in humans, *Diabetes* **58**(3), 579–89. eng.

Staiger, H., Machicao, F., Werner, R., Guirguis, A., Weisser, M., Stefan, N., Fritsche, A. and Haring, H. U.: 2008, Genetic variation within the angptl4 gene is not associated with metabolic traits in white subjects at an increased risk for type 2 diabetes mellitus, *Metabolism* **57**(5), 637–43. eng.

Stanley, W. C., Recchia, F. A. and Lopaschuk, G. D.: 2005, Myocardial substrate metabolism in the normal and failing heart., *Physiol Rev* **85**(3), 1093–1129.

Stejskal, D., Karpisek, M., Humenanska, V., Solichova, P. and Stejskal, P.: 2007, Angiopoietin-like protein 3: development, analytical characterization, and clinical testing of a new elisa, *Gen Physiol Biophys* **26**(3), 230–3. eng.

Suganami, T., Tanimoto-Koyama, K., Nishida, J., Itoh, M., Yuan, X., Mizuarai, S., Kotani, H., Yamaoka, S., Miyake, K., Aoe, S., Kamei, Y. and Ogawa, Y.: 2007, Role of the toll-like receptor 4/nf-kappab pathway in saturated fatty acid-induced inflammatory changes in the interaction between adipocytes and macrophages, *Arterioscler Thromb Vasc Biol* **27**(1), 84–91. eng.

Sukonina, V., Lookene, A., Olivecrona, T. and Olivecrona, G.: 2006, Angiopoietin-like protein 4 converts lipoprotein lipase to inactive monomers and modulates lipase activity in adipose tissue, *Proc Natl Acad Sci U S A* **103**(46), 17450–5. Department of Medical Biosciences, Umea University, SE-901 87 Umea, Sweden.

Talmud, P. J., Smart, M., Presswood, E., Cooper, J. A., Nicaud, V., Drenos, F., Palmen, J., Marmot, M. G., Boekholdt, S. M., Wareham, N. J., Khaw, K. T., Kumari, M. and Humphries, S. E.: 2008, Angptl4 e40k and t266m: effects on plasma triglyceride and hdl levels, postprandial responses, and chd risk, *Arterioscler Thromb Vasc Biol* **28**(12), 2319–25. eng.

Teusink, B., Voshol, P. J., Dahlmans, V. E. H., Rensen, P. C. N., Pijl, H., Romijn, J. A. and Havekes, L. M.: 2003, Contribution of fatty acids released from lipolysis of plasma triglycerides to total plasma fatty acid flux and tissue-specific fatty acid uptake., *Diabetes* **52**(3), 614–620.

Thompson, R. H. and Merola, G. V.: 1993, A simplified alternative to the aoac official method for cholesterol in multicomponent foods, *J AOAC Int* **76**(5), 1057–68. U.S. Department of Agriculture, Agricultural Research Service, BHNRC, Nutrient Composition Laboratory, Beltsville, MD 20705.

Tilg, H. and Moschen, A. R.: 2006, Adipocytokines: mediators linking adipose tissue, inflammation and immunity., *Nat Rev Immunol* **6**(10), 772–783.

Turnbaugh, P. J., Backhed, F., Fulton, L. and Gordon, J. I.: 2008, Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome, *Cell Host Microbe* **3**(4), 213–23. eng.

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R. and Gordon, J. I.: 2006, An obesity-associated gut microbiome with increased capacity for energy harvest., *Nature* **444**(7122), 1027–1031.

van der Lee, K. A., Vork, M. M., Vries, J. E. D., Willemsen, P. H., Glatz, J. F., Reneman, R. S., der Vusse, G. J. V. and Bilsen, M. V.: 2000, Long-chain fatty acid-induced changes in gene expression in neonatal cardiac myocytes., *J Lipid Res* **41**(1), 41–47.

van Dielen, F. M., van't Veer, C., Schols, A. M., Soeters, P. B., Buurman, W. A. and Greve, J. W.: 2001, Increased leptin concentrations correlate with increased concentrations of inflammatory markers in morbidly obese individuals., *Int J Obes Relat Metab Disord* **25**(12), 1759–1766.

van Dijk, K. W., Rensen, P. C., Voshol, P. J. and Havekes, L. M.: 2004, The role and mode of action of apolipoproteins ciii and av: synergistic actors in triglyceride metabolism?, *Curr Opin Lipidol* **15**(3), 239–46. Department of Human Genetics, Leiden University Medical Center, PO Box 9503, 2000 RA Leiden, The Netherlands. kowvd@lumc.nl.

van Vlijmen, B. J., Rohlmann, A., Page, S. T., Bensadoun, A., Bos, I. S., van Berkel, T. J., Havekes, L. M. and Herz, J.: 1999, An extrahepatic receptor-associated protein-sensitive mechanism is involved in the metabolism of triglyceride-rich lipoproteins, *J Biol Chem* **274**(49), 35219–26. Department of Biopharmaceutics, Leiden/Amsterdam Center for Drug Research, Leiden 2300 RA, The Netherlands.

Vidal-Puig, A. J., Grujic, D., Zhang, C. Y., Hagen, T., Boss, O., Ido, Y., Szczepanik, A., Wade, J., Mootha, V., Cortright, R., Muoio, D. M. and Lowell, B. B.: 2000, Energy metabolism in uncoupling protein 3 gene knockout mice., *J Biol Chem* **275**(21), 16258–16266.

Vilella, E., Joven, J., Fernandez, M., Vilaro, S., Brunzell, J. D., Olivecrona, T. and Bengtsson-Olivecrona, G.: 1993, Lipoprotein lipase in human plasma is mainly inactive and associated with cholesterol-rich lipoproteins, *J Lipid Res* **34**(9), 1555–64. Centre de Recerca Biomedica, Hospital de Sant Joan, Reus, Spain.

Voshol, P. J., Haemmerle, G., Ouwens, D. M., Zimmermann, R., Zechner, R., Teusink, B., Maassen, J. A., Havekes, L. M. and Romijn, J. A.: 2003, Increased hepatic insulin sensitivity together with decreased hepatic triglyceride stores in hormone-sensitive lipase-deficient mice, *Endocrinology* **144**(8), 3456–62. The Netherlands Organization for Applied Scientific Research-Prevention and Health, Leiden University Medical Centre, Department of Endocrinology and Metabolic Diseases, Leiden, The Netherlands. p.j.voshol@pg.tno.nl.

Wang, B., Wood, I. S. and Trayhurn, P.: 2007, Dysregulation of the expression and secretion of inflammation-related adipokines by hypoxia in human adipocytes, *Pflugers Arch* **455**(3), 479–92. eng.

Wang, J. and Hegele, R. A.: 2007, Homozygous missense mutation (g56r) in glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (gpi-hbp1) in two siblings with fasting chylomicronemia (mim 144650), *Lipids Health Dis* **6**, 23. eng.

Weinstein, M. M., Yin, L., Beigneux, A. P., Davies, B. S., Gin, P., Estrada, K., Melford, K., Bishop, J. R., Esko, J. D., Dallinga-Thie, G. M., Fong, L. G., Bensadoun, A. and Young, S. G.: 2008, Abnormal patterns of lipoprotein lipase release into the plasma in gpihbp1-deficient mice, *J Biol Chem* **283**(50), 34511–8. eng.

Weisberg, S. P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R. L. and Ferrante, A. W.: 2003, Obesity is associated with macrophage accumulation in adipose tissue., *J Clin Invest* **112**(12), 1796–1808.

Wiesner, G., Brown, R. E., Robertson, G. S., Imran, S. A., Ur, E. and Wilkinson, M.: 2006, Increased expression of the adipokine genes resistin and fasting-induced adipose factor in hypoxic/ischaemic mouse brain, *Neuroreport* **17**(11), 1195–8. eng.

Wiesner, G., Morash, B. A., Ur, E. and Wilkinson, M.: 2004, Food restriction regulates adipose-specific cytokines in pituitary gland but not in hypothalamus, *J Endocrinol* **180**(3), R1–6. eng.

Willer, C. J., Sanna, S., Jackson, A. U., Scuteri, A., Bonnycastle, L. L., Clarke, R., Heath, S. C., Timpson, N. J., Najjar, S. S., Stringham, H. M., Strait, J., Duren, W. L., Maschio, A., Busonero, F., Mulas, A., Albai, G., Swift, A. J., Morken, M. A., Narisu, N., Bennett, D., Parish, S., Shen, H., Galan, P., Meneton, P., Hercberg, S., Zelenika, D., Chen, W. M., Li, Y., Scott, L. J., Scheet, P. A., Sundvall, J., Watanabe, R. M., Nagaraja, R., Ebrahim, S., Lawlor, D. A., Ben-Shlomo, Y., Davey-Smith, G., Shuldiner, A. R., Collins, R., Bergman, R. N., Uda, M., Tuomilehto, J., Cao, A., Collins, F. S., Lakatta, E., Lathrop, G. M., Boehnke, M., Schlessinger, D., Mohlke, K. L. and Abecasis, G. R.: 2008,

Newly identified loci that influence lipid concentrations and risk of coronary artery disease, *Nat Genet* **40**(2), 161–9. eng.

Wong, H., Yang, D., Hill, J. S., Davis, R. C., Nikazy, J. and Schotz, M. C.: 1997, A molecular biology-based approach to resolve the subunit orientation of lipoprotein lipase., *Proc Natl Acad Sci U S A* **94**(11), 5594–5598.

Xu, A., Lam, M. C., Chan, K. W., Wang, Y., Zhang, J., Hoo, R. L., Xu, J. Y., Chen, B., Chow, W. S., Tso, A. W. and Lam, K. S.: 2005, Angiopoietin-like protein 4 decreases blood glucose and improves glucose tolerance but induces hyperlipidemia and hepatic steatosis in mice, *Proc Natl Acad Sci U S A* **102**(17), 6086–91. Department of Medicine, Research Center of Heart, Brain, Hormone, and Healthy Aging, and Genome Research Center, University of Hong Kong, Hong Kong, China. amxu@hkucc.hku.hk.

Yagyu, H., Chen, G., Yokoyama, M., Hirata, K., Augustus, A., Kako, Y., Seo, T., Hu, Y., Lutz, E. P., Merkel, M., Bensadoun, A., Homma, S. and Goldberg, I. J.: 2003, Lipoprotein lipase (lpl) on the surface of cardiomyocytes increases lipid uptake and produces a cardiomyopathy., *J Clin Invest* **111**(3), 419–426.

Yang, Y. H., Wang, Y., Lam, K. S., Yau, M. H., Cheng, K. K., Zhang, J., Zhu, W., Wu, D. and Xu, A.: 2008, Suppression of the raf/mek/erk signaling cascade and inhibition of angiogenesis by the carboxyl terminus of angiopoietin-like protein 4, *Arterioscler Thromb Vasc Biol* **28**(5), 835–40. eng.

Yau, M. H., Wang, Y., Lam, K. S., Zhang, J., Wu, D. and Xu, A.: 2009, A highly conserved motif within the nh2-terminal coiled-coil domain of angiopoietin-like protein 4 confers its inhibitory effects on lipoprotein lipase by disrupting the enzyme dimerization, *J Biol Chem* **284**(18), 11942–52. eng.

Yilmaz, Y., Ulukaya, E., Atug, O. and Dolar, E.: 2009, Serum concentrations of human angiopoietin-like protein 3 in patients with nonalcoholic fatty liver disease: association with insulin resistance, *Eur J Gastroenterol Hepatol* . Eng.

Yin, W., Romeo, S., Chang, S., Grishin, N. V., Hobbs, H. H. and Cohen, J. C.: 2009, Genetic variation in angptl4 provides insights into protein processing and function, *J Biol Chem* **284**(19), 13213–22. eng.

Yoon, J. C., Chickering, T. W., Rosen, E. D., Dussault, B., Qin, Y., Soukas, A., Friedman, J. M., Holmes, W. E. and Spiegelman, B. M.: 2000, Peroxisome proliferator-activated receptor gamma target gene encoding a novel angiopoietin-related protein associated with adipose differentiation, *Mol Cell Biol* **20**(14), 5343–9. Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115, USA.

Yoshida, K., Shimizugawa, T., Ono, M. and Furukawa, H.: 2002, Angiopoietin-like protein 4 is a potent hyperlipidemia-inducing factor in mice and inhibitor of lipoprotein lipase, *J Lipid Res* **43**(11), 1770–2. Biomedical Research Laboratories, Sankyo Co, Ltd, 1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140-8710, Japan.

Yu, X., Burgess, S. C., Ge, H., Wong, K. K., Nassem, R. H., Garry, D. J., Sherry, A. D., Malloy, C. R., Berger, J. P. and Li, C.: 2005, Inhibition of cardiac lipoprotein utilization by transgenic overexpression of angptl4 in the heart, *Proc Natl Acad Sci U S A* **102**(5), 1767–72. Department of Physiology, Touchstone Center for Diabetes Research, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390, USA.

Zandbergen, F., van Dijk, S., Muller, M. and Kersten, S.: 2006, Fasting-induced adipose factor/ angiopoietin-like protein 4: a potential target for dyslipidemia?, *Future Lipidology* **1**(2), 227–236. http://www.futuremedicine.com/doi/abs/10.2217/17460875.1.2.227.

Zhang, C. C., Kaba, M., Ge, G., Xie, K., Tong, W., Hug, C. and Lodish, H. F.: 2006, Angiopoietin-like proteins stimulate ex vivo expansion of hematopoietic stem cells, *Nat Med* **12**(2), 240–5. eng.

Zhang, L., Lookene, A., Wu, G. and Olivecrona, G.: 2005, Calcium triggers folding of lipoprotein lipase into active dimers, *J Biol Chem* **280**(52), 42580–91. eng.

Zhang, Y., Repa, J. J., Gauthier, K. and Mangelsdorf, D. J.: 2001, Regulation of lipoprotein lipase by the oxysterol receptors, lxralpha and lxrbeta, *J Biol Chem* **276**(46), 43018–24. eng.

Zhu, H., Li, J., Qin, W., Yang, Y., He, X., Wan, D. and Gu, J.: 2002, [cloning of a novel gene, angptl4 and the functional study in angiogenesis], *Zhonghua Yi Xue Za Zhi* **82**(2), 94–9. chi.

Ziouzenkova, O., Perrey, S., Asatryan, L., Hwang, J., MacNaul, K. L., Moller, D. E., Rader, D. J., Sevanian, A., Zechner, R., Hoefler, G. and Plutzky, J.: 2003, Lipolysis of triglyceride-rich lipoproteins generates ppar ligands: evidence for an antiinflammatory role for lipoprotein lipase, *Proc Natl Acad Sci U S A* **100**(5), 2730–5. Cardiovascular Division, Brigham and Women's Hospital, Harvard University, Boston, MA 02115, USA.

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Publications list

L. Lichtenstein, J.F.P. Berbée, S. van Dijk, K. Willems van Dijk, A. Bensadoun, I.P. Kema, P.J. Voshol, M. Müller, P.C.N. Rensen, S. Kersten, Angptl4 up-regulates cholesterol synthesis in liver via inhibition of LPL- and HL-dependent hepatic cholesterol uptake, **ATVB**, 27: 2420-2427, 2007.

S. Kersten, **L. Lichtenstein**, E. Steenbergen, K. Mudde, H.F.J. Hendriks, M.K. Hesselink, P. Schrauwen and M. Müller, Caloric restriction and exercice increase plasma ANGPTL4 levels in humans via elevated free fatty acids, **ATVB**, 29: 969-974, 2009.

A. Georgiadi, **L. Lichtenstein**, T. Degenhardt, M. Boekschoten, M. van Bilsen, B. Desvergne, M. Müller, S. Kersten, Induction of cardiac Angptl4 by dietary fatty acids is mediated by PPARβ/δ and protects against oxidative stress*, under revision.*

L. Lichtenstein, N.J. de Wit, G.J. Hooiveld, E. Oosterink, R. van der Meer, A. Köster, J.T. Tamsma, N. Soon Tan, M. Müller, S. Kersten, Angptl4 protects against severe pro-inflammatory effects of dietary saturated fat**,** *in preparation.*

L. Lichtenstein, S. Kersten, LiPoLysis revisited*, submitted.*

Girousse, G. Tvernier, C. Tiraby, **L. Lichtenstein**, J.S. Iacovoni, A. Mairal, F. Villarroya, D. Langin, Transcription of the human uncoupling protein 3 gene is governed by a complex interplay between the promotor and intronic sequences, **Diabetologia**, 52: 1638-1646, 2009.

About the author

Laeticia Lichtenstein was born the 9th of august 1981, in Saint Girons, France. After finishing her bachelor in cellular biology, she decided to acquire pharmacology knowledge, and participated to the research master in pharmacology proposed by the doctoral school of Paul Sabatier University, in Toulouse. At this occasion, she explored research within Obesity Research Unit INSERM U586 of Dr Dominique Langin, digging into the gene regulation of the amazing mitochondrial protein UCP3. The master diploma in one hand, she took her luggage full of sneakers, and came in Wageningen. For 4 years, she tried to combine pleasure to work, doing her PhD on "Functional characterization of Angptl4 protein", under the supervision of chef, a.k.a, Dr. Sander Kersten. Her project was funded by *TiFN* (Top Institute Food and Nutrition). Laeticia, missing intensely the *"Pink Town",* and the so-called *"Saucisse de Toulouse",* among other famous culinary specialties, decided to go back to the sun and mountains, to do a Post-doc. She will start working beginning 2010 with Laurent Martinez.

Overview of complete educational training

Specific activities

Gordon research conference on Lipoprotein Metabolism, Mount Holyoke College, South Hadley, USA, 2006.
NuGO week, Oxford, UK, 2006.
NuGO week, Montecatini, Italy, 2009.
Nwo-nutrition meeting. Deume, 2006, 2008 and 2009.
Netherlands Lipoprotein club, Leiden TNO, 2007- 2009.
5th Dutch Endo-Neuro-Psycho Meeting. Doorwerth, 2006.
8th Dutch Endo-Neuro-Psycho Meeting. Doorwerth, 2009.
49th International Conference on the Bioscience of the Lipids, Maastricht, 2008.
Bioinformatics – A User's Approach, Wageningen, 2007.
Epigenesis and Epigenetics – *Physiological consequences of perinatal programming*, Wageningen, 2008.

General Courses

Getting your message across- Media skills for scientific researchers, Wageningen University, Wageningen, 2006.
Techniques for writing and presenting a scientific paper, Wageningen University, Wageningen, 2006.
Networking workshop, TI Food and Nutrition, Wageningen, 2008
8th Masterclass Nutrigenomics – "*Regulation of energy homeostasis: from molecular nutrition to prevention of disease"*, Wageningen, 2009.
5th Masterclass Nutrigenomics – "*From molecular nutrition to prevention of disease: how to ask simple questions on complex problems?"*, Wageningen, 2005.
Vlag PhD week, 2006
NuGO PhD week 2006
International course on Laboratory Animal Science, Utrecht University, 2007.

Optional

Organization and participation PhD-study tour USA, Wageningen University, 2007.
Journal club, Division of Human Nutrition (every four weeks), Wageningen University.
Setting-up of NMG group Journal club (every two weeks), Wageningen University.
NMG scientific meetings (every week).
Nutrigenomics Consortium (NGC) Scientific Meetings (every two months).

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