Aspects involved in the (patho)physiology of the metabolic syndrome

Duivenvoorden, I.

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Chapter 1

General Introduction

In Western society the metabolic syndrome scores high in health risk tables. This syndrome is characterized by a number of metabolic abnormalities like obesity, insulin resistance, dyslipidemia and cardiovascular disease[1-3]. The combination of large amounts of carbohydrates and fats in the Western-type diets and the sedentary life-style are responsible for the fact that in Western society energy intake often exceeds energy expenditure. The excess in lipids and carbohydrates consumed is stored, which in turn can lead to obesity and tissue insulin resistance. The most important dietary nutrients in this respect are cholesterol, triglycerides (TG) and glucose.

Cholesterol is a lipid essential for biosynthesis of cellular membranes, steroid hormones and bile acids. However, high plasma cholesterol levels (hypercholesterolemia) are a risk factor for cardiovascular disease. TG, and their metabolites fatty acids (FA), are lipids that are mainly used for energy. In addition, FA have an important function in regulating gene expression, but in their free form FA are toxic to cells. TG are the form in which FA can be stored in the cell or be transported in the circulation. Especially cardiac and skeletal muscle are greatly dependent on this form of energy. High plasma levels of TG (hypertriglyceridemia) are a risk factor for the metabolic syndrome and, eventually, cardiovascular disease. Glucose is a small carbohydrate, which can be quickly converted into energy. Especially brain and muscle use glucose for energy. Plasma glucose levels are strictly regulated for normal function of the body. Both high and low blood glucose levels can have severe health implications.

These nutrients are all essential in the human body, however their levels need to be kept within certain ranges.

Lipid metabolism

Lipoprotein metabolism

Cholesterol and TG are transported in the circulation in the form of water-soluble spherical particles, called lipoproteins. Lipoproteins have a hydrophobic lipid-rich core, containing mainly TG and esterified cholesterol, surrounded by a polar surface monolayer, which is composed of phospholipids, free cholesterol and several proteins, termed apolipoproteins (apo)[4-6]. Lipoproteins can be divided into five major classes, which differ in origin, density, size and (apolipoprotein) composition[5,7]. As shown in **Table 1** these lipoprotein classes are: chylomicrons (CM), very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoproteins (HDL)[7].

The various apolipoproteins found in different combinations on lipoproteins have several distinct functions: they either stabilize the lipoprotein particles, serve as ligands for lipoprotein receptors or are cofactors/inhibitors of enzymes involved in lipoprotein metabolism such as lipoprotein lipase (LPL)[7,8].

The metabolism of lipoproteins can be divided in three pathways: the (nutrition-related) exogenous pathway, the endogenous pathway and the reversed cholesterol

transport pathway. In **Figure 1** these pathways are successively presented in a simple form.

Table 1. Human plasma lipoproteins: physical properties and composition

	CM	VLDL	IDL	LDL	HDL
Physical properties					
Source	Intestine	Liver	VLDL	VLDL/IDL	Liver and Intestine
Diameter (nm)	75 - 1200	30 – 80	25 - 35	18 - 25	5 - 12
Density (g/ml)	< 0.96	0.96 - 1.006	1.006 - 1.019	1.019 - 1.063	1.063 - 1.21
Composition					
Total lipid (%)	98 - 99	90 – 94	89	79	45 - 55
Protein (%)	1 - 2	6 – 10	11	21	45 - 55
Lipid Composition (% of total lipid)					
Triglycerides	88	56	29	13	15
Cholesterol esters	3	15	34	48	30
Free cholesterol	1	8	9	10	10
Phospholipids	8	20	26	28	45
Apolipoprotein Content					
Apo	A1,A4,B48, C1,C2,C3,E	B100,C1,C2, C3,E	B100,C1,C2, C3,E	B100	A1,A2,A4,C1, C2,C3,E

Apo apolipoprotein, CM chylomicron, HDL high density lipoprotein, IDL intermediate density lipoprotein, LDL low density lipoprotein, VLDL very low density lipoprotein

Exogenous pathway

Intestinal absorption of dietary lipids is facilitated by intestinal bile acids, lipases and proteases, which are supplied by bile and pancreatic juices released into the small intestinal lumen[9]. TG digestion, by lipases, leads to two free FA and a glycerol-FA. These molecules and cholesterol are absorbed by the epithelial cells of the small intestine (enterocytes). Inside the enterocytes, the FA are re-esterified into TG and are packaged into CM. CM are very large lipoprotein particles containing mostly TG but also cholesterol, phospholipids, and several proteins (apoB48, A1, and A4)[10]. The intestinal epithelial cells secrete the CM into the lymph, which drains into the circulation[11]. Upon entering the bloodstream, the particles acquire apoE, C1, C2 and C3. In the bloodstream the TG are hydrolyzed into free FA and glycerol by endothelium-bound LPL[12,13], allowing the delivery of FA, to the adjacent tissues like muscle (for energy) and adipose tissue (for storage). Upon hydrolysis of the core lipids, the lipoprotein particle becomes smaller and is called CM-remnant. Part of the excess surface material, such as phospholipids, free cholesterol and apolipoproteins, is transferred to HDL particles. The CM-remnant, relatively enriched in cholesterol, is rapidly cleared by the liver via apoE-mediated binding to specific lipoprotein receptors *i.e.*, the LDL receptor (LDLR) and LDLR-related protein (LRP)[12,14].

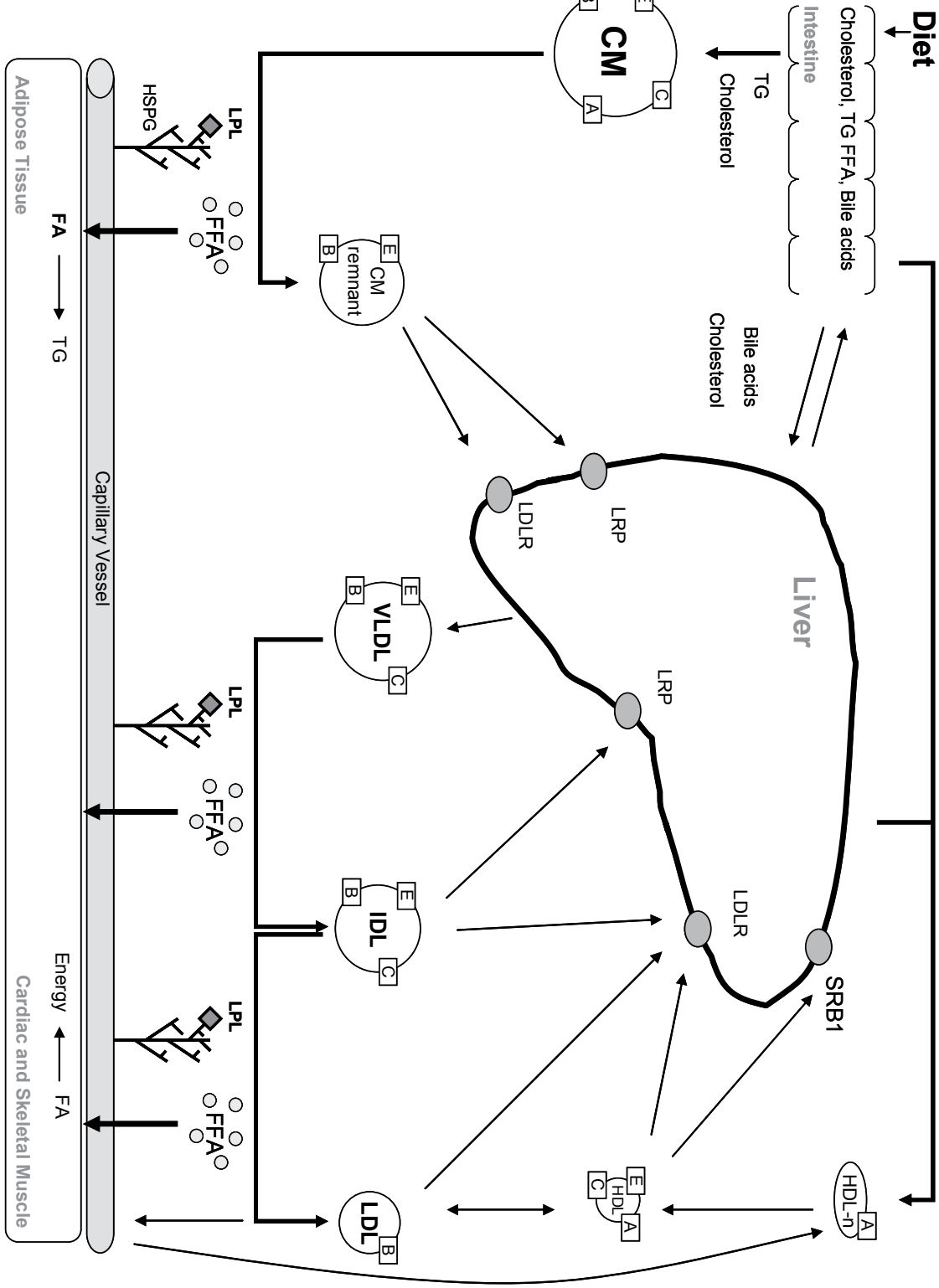

Figure 1. Schematic illustration of the pathways in lipid metabolism: exogenous, endogenous and the reverse cholesterol pathway
CM chylomicron, FA fatty acid, FFA free FA, (n-)HDL (nascent-) high density lipoprotein, HSPG heparin sulphate proteoglycans, IDL intermediate density lipoprotein, LDL low density lipoprotein, LDLR LDL receptor, LPL lipoprotein lipase, LRP LDLR-related protein, SRB1 scavenger receptor B1, TG triglyceride, VLDL very low density lipoprotein

Endogenous Pathway

The liver plays a major role in lipid metabolism. It takes up CM-remnant particles containing mainly cholesterol and it secretes VLDL-particles. VLDL consist of a core of TG and cholesterol, which are newly synthesized by the liver or derived from incoming CM-remnants, IDL, LDL and HDL. In the process of VLDL formation the major structural apolipoprotein of VLDL, apoB100, associates with the core lipids catalyzed by microsomal triacylglycerol transfer protein (MTP). Thereafter, the particle fuses with a lipid droplet to become a mature VLDL particle, which, with help of apoE[15], can be secreted into the blood[16,17]. Upon entering the circulation, the particle is enriched with apoE, and apoC. Like CM, VLDL particles serve as TG transporters to supply the periphery with energy in the form of FA. In a similar way to CM, hydrolysis of VLDL-TG by LPL results in smaller particles called IDL, which can either be taken up by the liver via the LDLR or LRP, or be further hydrolyzed by LPL and hepatic lipase into LDL. LDL is a small lipoprotein particle that has lost most of the apolipoproteins. ApoB100 remains associated with the particle and serves as a ligand for the uptake of LDL via the LDLR present on the liver and peripheral tissues[14,18]. In the human circulation LDL is the most abundant lipoprotein.

Reverse cholesterol pathway

Through the uptake of LDL particles by the vessel wall, cholesterol is present in the subendothelial space. There it is used, or is transported back to the liver, by nascent-HDL (n-HDL) via the so called reverse cholesterol transport pathway. n-HDL is a very small lipoprotein and contains apoA1 as its major apolipoprotein. n-HDL is synthesized by the liver and the small intestine and incorporates redundant surface lipids and apolipoproteins freed during lipolysis of TG-rich lipoproteins[19,20]. Through interaction with the ATP-binding cassette transporter A1 (ABCA1), the cellular cholesterol is taken up in the core of n-HDL in the circulation[21]. By this process, n-HDL is converted into mature (filled) spherical HDL. The cholesterol esters in the mature HDL are taken up by the liver via the scavenger receptor B1 (SR-B1) either directly or transferred from HDL to VLDL and LDL in exchange for TG. The cholesterol esters are then taken up via the classical LDLR- and LRP-mediated pathway.

Cholesterol metabolism

The liver plays a major role in the whole body cholesterol homeostasis. It takes up cholesterol from CM- and VLDL-remnants, from LDL and from HDL. The liver uses cholesterol for the synthesis of VLDL, but the major portion of liver cholesterol is used for the production of bile salts for excretion, together with biliary cholesterol, into the intestine. Approximately 95% of the bile acids are reabsorbed in the terminal ileum and return to the liver, where they are actively taken up. Biliary cholesterol delivers a large amount of the daily intestinal cholesterol and about 50% of the intestinal cholesterol is reabsorbed. This pathway, which is called the enterohepatic circulation, contributes greatly to plasma cholesterol levels[22,23]. Although bile acid and biliary cholesterol excretion is the only

quantitatively significant cholesterol removal pathway, recent work indicates that there might be a bile-independent cholesterol efflux towards the intestine (personal communication Dr. AK Groen, AMC, Amsterdam).

The *de novo* bile acid biosynthesis is initiated by the enzymes 7α-hydroxylase or sterol 27-hydroxylase. After excretion in the intestine, bile acids play an important role in the solubilization of fats, cholesterol and other lipophillic compounds such as drugs and vitamins A, D, E and K, enhancing their uptake by enterocytes.

In humans, approximately 1 gram of cholesterol is needed per day to maintain the enterohepatic circulation. The lipoprotein-derived cholesterol uptake by the liver from in the circulation is not sufficient for this pathway. Per day, the liver synthesizes approximately 700 mg cholesterol in order to maintain the enterohepatic circulation. The major rate-limiting enzyme in cholesterol production is 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. Inhibition of this enzyme *in vivo* by administering statins is currently the most commonly used approach for lowering plasma cholesterol.

Triglyceride and fatty acid metabolism

As depicted in **Figure 1**, dietary TG (exogenous TG) and TG synthesized in the liver (endogenous TG) are secreted into the circulation in the form of CM and VLDL, respectively. Plasma TG are lipolyzed and the FA generated are taken up by the adjacent tissues, *e.g.*, muscle for energy and adipose tissue for storage. For the lipolysis of TG the most crucial enzyme is LPL.

Lipoprotein lipase

LPL is an enzyme that belongs to a family of lipases, which also includes hepatic lipase and pancreatic lipase. LPL is synthesized and secreted by almost all tissues in the body, but most abundantly in skeletal and cardiac muscle and adipose tissue. Once secreted, it associates with the heparin sulphate proteoglycans (HSPG) of endothelial cells (**Figure 1**). LPL can not only interact with lipoproteins, it also interacts with lipoprotein receptors, thereby enhancing binding and internalization of lipoproteins.

Active LPL is a homodimer and its activity is influenced by many factors. Of the apolipoproteins residing on the lipoproteins, apoC2 is an essential cofactor for normal LPL function. ApoC1 and apoC3 are natural inhibitors of LPL, of which apoC3 is the most potent[24]. Research is ongoing on novel discovered apolipoproteins like apoA5[25,26], which has a stimulatory effect on LPL activity.

LPL activity is greatly regulated by the nutritional status. In the fed state LPL is highly expressed in adipose tissue under the influence of insulin. On the other hand, during fasting and exercise LPL activity is increased specifically in muscle[27]. Therefore, LPL is a key player in the partitioning of FA[27] and might have a large role in the aetiology of obesity[27-29].

Adipose tissue

Adipose tissue has long been regarded as a mere storage depot, but increasing evidence presents adipose tissue as a highly metabolically active tissue. Adipocytes are nowadays known to produce hormones (like leptin, adiponectin and resistine), cytokines (like TNFα, IL-6 and IL-10), growth factors, complement factors and prostacyclins, collectively called adipokines[30-32]. More molecules are added as research progresses. These adipokines play roles in whole-body insulin sensitivity and metabolic homeostasis. For instance, the hormone leptin regulates, among others, appetite and body weight, and adiponectine is known to be an insulin sensitizer. The cytokines are very important in inflammatory status. Dysregulation of cytokines or other adipokines can lead to great metabolic changes[30-32].

Upon lipolysis of CM- and VLDL-TG in the capillary bed by LPL, FA are taken up by the adipocytes and re-esterified in the form of TG. This process requires glucose for the formation of glycerol to which the FA are esterified (**Figure 2**). Vast amounts of TG are stored for later use, e.g., during fasting. Upon fasting, FA are liberated from the adipose tissue by the action of hormone sensitive lipase (HSL)[33] and the recently discovered adipose tissue TG lipase (ATGL)[33-35]. The free FA are released into the circulation, where they bind to albumin for transport.

Cellular fatty acid handling

The major portion of albumin-bound FA is transported to the liver. In the liver, these FA are re-esterified in TG and are used for production of VLDL. In the fasted state, muscle cells take up FA generated from the lipolysis of liver-derived VLDL (**Figure 1** and **2**). This FA uptake is mediated by several FA transporters as well as passive diffusion. The three main FA transporters are plasma membrane FA binding protein (FABPpm), FA translocase (FAT/CD36) and FA transport protein (FATP). Once inside the cell, cytosolic FABP (FABPc) binds the FA for transport through the watery environment of the cytosol toward the mitochondria. The FA must first be activated in the cytoplasm before they can be oxidized in the mitochondria for energy[36-38]. In the presence of adenosine triphosphate (ATP) and coenzyme A (CoA), this activation is catalyzed by fatty acyl-CoA synthase (FACS) resulting in an acyl-CoA (**Figure 3**)[39,40]. The acyl-CoA esters are directed to peroxisomes and mitochondria for β-oxidation or can serve as substrates for TG, phospholipids, and cholesterol ester synthesis. Under fasting conditions however, they are channeled primarily toward mitochondria for β-oxidation. Long-chain FA will not penetrate the inner membrane of mitochondria to be oxidized, unless they are converted to an acyl-carnitine intermediate. Therefore, carnitine palmitoyltransferase (CPT) I, localized in the outer mitochondrial membrane, couples the long-chain acyl-CoA to carnitine to form acyl-carnitine. This carnitine ester can be transported across the mitochondrial inner membrane by the carnitine/acylcarnitine translocase (CT). Inside the mitochondrial lumen, CPTII uncouples the acyl-CoA, which serves as a substrate for the β-oxidation spiral (**Figure 3**).

β-oxidation of the acyl-CoA involves successive cleavages releasing acetyl-CoA, by enzymes specific for the chain-length of the FA. Eventually, this reaction generates a large quantity of ATP, the energy-rich compound used for cellular reactions.

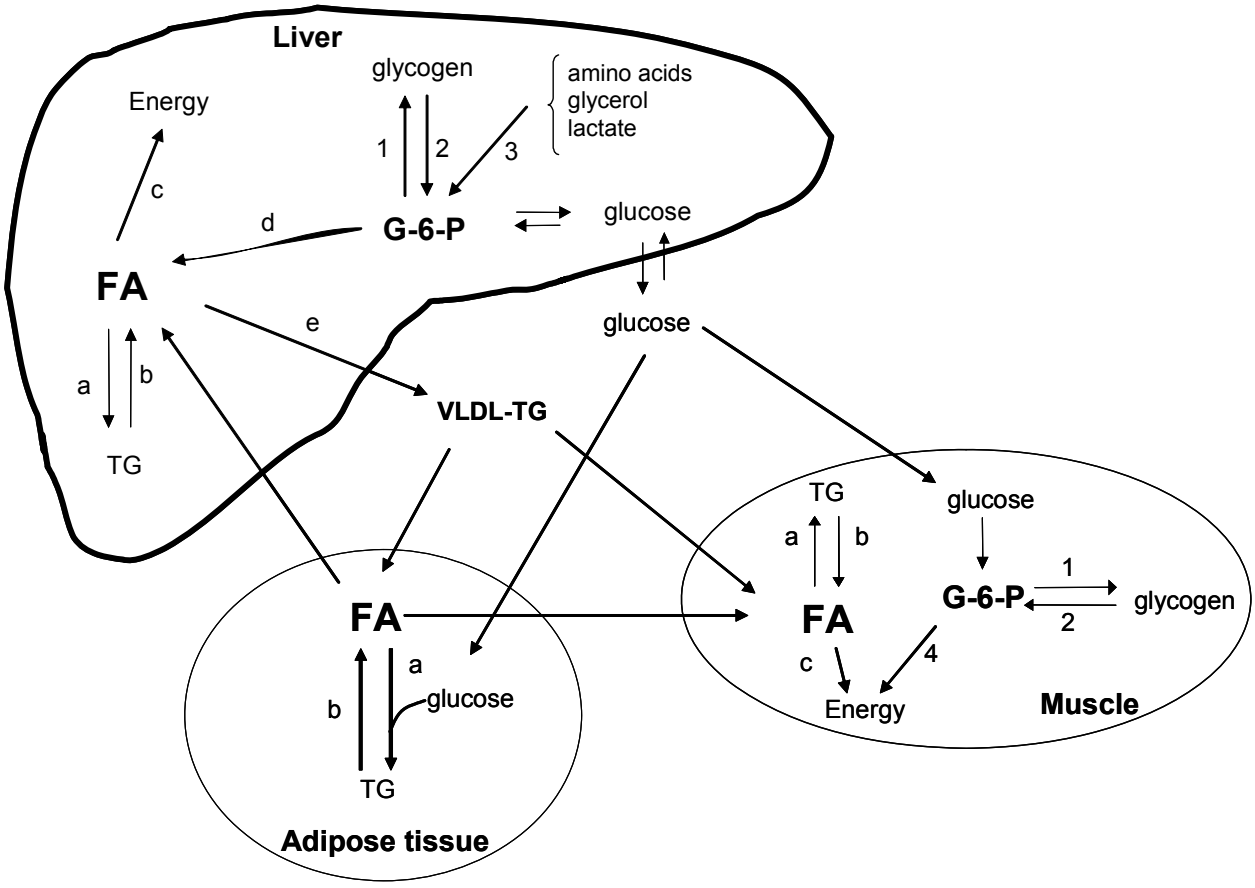

Figure 2. Schematic illustration of FA and glucose metabolism
Glucose metabolism: 1 glycogenesis, 2 glycogenolysis, 3 gluconeogenesis, 4 oxidation.
Lipid metabolism: a lipogenesis, b lipolysis, c oxidation, d *de novo* FA synthesis, e VLDL production.
FA fatty acid, TG triglycerides, G-6-P glucose-6-phosphate, VLDL very low density lipoprotein

Ketogenesis

During high rates of FA oxidation large amounts of acetyl-CoA are generated primarily in the liver. When the acetyl-CoA supply exceeds the energy needs of the liver itself, they are used for keton body (acetoacetate, β-hydroxybutyrate, and acetone) synthesis. Keton bodies can be used as fuel by tissues other than liver. In early stages of starvation, when the last remnants of fat are oxidized, heart and skeletal muscle will consume primarily keton bodies to preserve glucose for use by the brain, eventually the latter will use keton bodies as well.

In diabetes mellitus patients, high levels of FA oxidation may lead to deadly diabetic ketoacidosis.

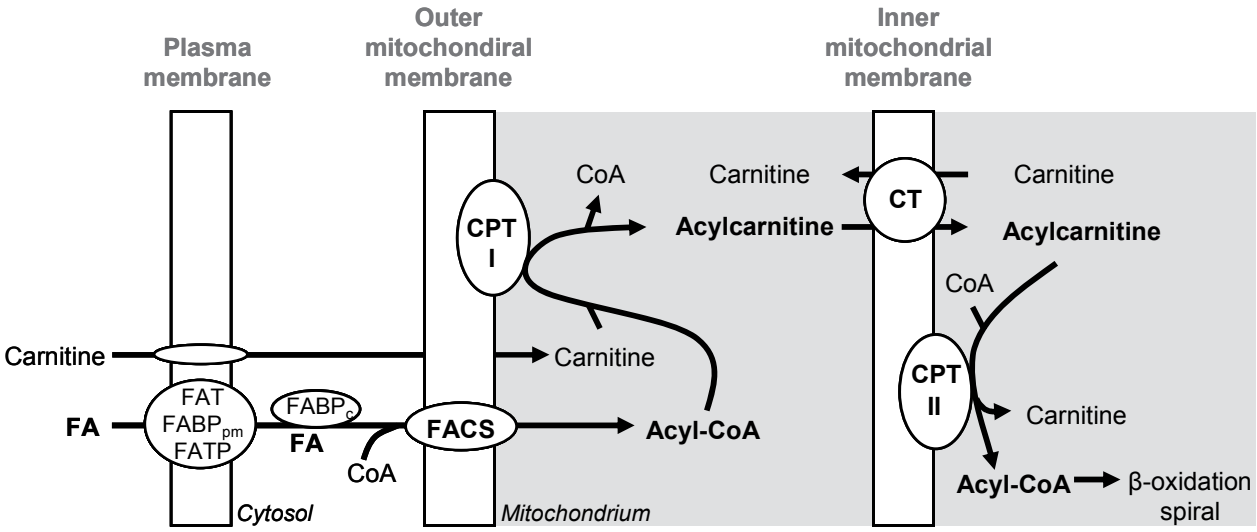

Figure 3. Schematic illustration of FA transport and handling in cells
CoA coenzyme A, CPT carnitine palmitoyltransferase, CT carnitine/acylcarnitine translocase, FA fatty acid, FABP FA binding protein, $FABP_c$ cytosolic FABP, $FABP_{pm}$ plasma membrane FABP, FACS fatty acyl-CoA synthase, FAT FA translocase, FATP FA transport protein

Glucose metabolism

Next to lipids, glucose is an important constituent of our diet and is a major source of energy. Metabolically active tissues like adipose tissue, liver and muscle need at least a small amount of glucose to maintain their basal functions. For some organs, like the brain, glucose is essential for proper functionality. Next to uptake via the diet, glucose can also be synthesized by the liver (and to lesser extent by the kidneys). Besides being oxidized for energy, glucose can also be stored in the form of glycogen. Blood glucose levels need to be strictly regulated to maintain proper physiology. Too low levels (hypoglycemia) and too high levels (hyperglycemia) can give rise to many complications.

Regulation of the blood glucose

After a meal, glucose and other carbohydrates are absorbed in the intestine and secreted into the blood via the portal vein. Via the glucose transporter GLUT4 the adipose and muscle tissues are able to take up glucose under the influence of insulin[41,42]. In the cell, glucose is used as fuel or is stored in the form of glycogen (in muscle) for later use. It can also be used for *de novo* lipogenesis.

Hepatic glucose metabolism includes uptake of glucose from the portal circulation via insulin-independent transporters like GLUT2 and *de novo* synthesis. The liver is capable of storing considerable amounts of glucose in the form of glycogen. During fasting, plasma glucose levels are maintained by the liver. First by glycogenolysis, and later by gluconeogenesis[43] (**Figure 2**). Under normal conditions, plasma glucose is kept within a strict range by the use of several hormones and nervous signals. This strict regulation is

important since too low levels of glucose prohibit normal function of brain (and some other tissues) resulting in loss of consciousness and in severe cases even in coma. On the other hand raised blood glucose levels (>7 mmol/l in fasted state) induce thirst, polyuria and in the long run macro- and microvasculair damage *e.g.*, atherosclerosis, nefro-, neuro- and retinopathy[44-46].

The hormone insulin is the most important player in the regulation of blood glucose levels and is the only glucose-lowering hormone. Other pancreatic hormones, like glucagon and somatostatin, are able to increase plasma glucose levels. Insulin is synthesized in the β-cells of the pancreas and is released in the blood in response to increasing plasma glucose levels after a meal. Insulin interacts with insulin receptors on the muscle and adipose tissue and stimulates these tissues to take up glucose by increasing the number of GLUT4 transporters. As a result, blood glucose levels and insulin secretion will decrease. Next to increasing body glucose uptake, insulin stimulates liver glycogen synthesis and decreases the hepatic glucose production and the VLDL-secretion. Furthermore, in the fed state, in adipose tissue HSL is inhibited by increased insulin levels, which leads to decreased TG lipolysis and FA secretion, as well as, increased esterification of FA in adipose tissue. The latter process needs glucose for glycerol production. The uptake of glucose by the adipose tissue is stimulated by the increased insulin levels. In this way after a meal, the insulin level ensures that: 1) dietary glucose is taken up by muscle (for energy) and adipose tissue (for FA esterification), 2) hepatic output of glucose is inhibited, concomitant with an increased conversion of hepatic glucose into glycogen and 3) lipolysis of CM- and VLDL-TG is enhanced at the adipose tissue, whereas this is decreased in muscle.

Thus, although the major physiological function of insulin is the maintenance of plasma glucose homeostasis, insulin also plays an important role in lipid metabolism[43,47]. In summary: Insulin ensures glucose and FA uptake from the diet whereafter these two fuels are used for energy and storage, respectively.

Hepatic glucose metabolism

As mentioned above glucose can be synthesized in the body by the liver. The rate of hepatic glucose production is an important determinant of blood glucose levels. In the fed state glucose enters the hepatocyte and is readily converted into glucose-6-phosphate by glucokinase (**Figure 2**). Glucose-6-phosphate is an important regulator of hepatic glucose metabolism. It can be oxidized via glycolysis, leading to formation of pyruvate, acetyl-CoA and finally energy. Glucose-6-phosphate can also be converted and stored in the form of glycogen.

During the fasting state, when energy supplies from dietary sources become limited, glucose can rapidly be mobilized from the hepatic glycogen stores (glycogenolysis). During glycogenolysis, glycogen is converted into glucose-6-phosphate and subsequently into glucose, which is released into the circulation. When hepatic glycogen reserves become

depleted, after 12 hours of fasting in the human situation, the process of glucose synthesis (gluconeogenesis) becomes accelerated.

Major precursors for glucose synthesis are lactate (the end product of anaerobe dissimilation of glucose in red blood cells and muscle) and glycerol (from lipolysis of TG in adipose tissue). In the fed state, when insulin levels are high and glucagon levels are low, glucose-6-phosphate is prone to glycolysis and glycogen synthesis. Activity of key enzymes involved in these processes, respectively phosphofructokinase-1 and glycogen synthase, is increased. This results in diminished glucose production.

During fasting, when insulin levels are low and glucagon levels are high, the processes that capture glucose-6-phosphate within the liver are low in activity. Under these circumstances, the activity of key enzymes involved in the process of glycogenolysis (glycogen phosphorylase) and glucogenesis (phosphoenol pyruvate carboxykinase - PEPCK) is increased. This results in enhanced glucose release by the liver.

Transcription factors

Much attention in research is focused on transcription factors, since they are known to be able to regulate important genes in several pathways involved in lipid and glucose homeostasis. These factors are differentially expressed in the cells of tissues. In the nucleus the transcription factors (or complexes of transcriptions factors heterodimerized with retinoid X receptor (RXR)[48]) bind to a specific responsive element in the promoter region of the target gene. Upon activation of the transcription factor or RXR the expression of the target gene is modulated.

Peroxisome proliferator-activated receptor

Many studies have been performed on the transcription factors named peroxisome proliferator-activated receptors (PPARs). Three PPAR isotypes have been identified namely: PPARα, PPARβ and PPARγ[49,50]. PPARs heterodimerize with RXR. The natural ligands for PPARs seem to be long-chain unsaturated FA such as linoleic acid, phytanic acid, conjugated linoleic acid and eicosanoids. Since PPARα and PPARγ have been shown to regulate genes involved in the FA oxidation pathways, focus is maintained on these two isotypes of the PPARs.

PPARα is highly expressed in adipose tissue and liver, and to a lower extent in kidney, heart and skeletal muscle. Next to FA, fasting conditions and fibrates are able to activate PPARα. The target genes of PPARα are a relatively homogenous group of genes involved in lipid catabolism, such as FAT and FATP, liver-FABP, FACS, CPTII, LPL and apoC3.

PPARγ is mainly expressed in adipose tissue, and to a lesser extent in liver, skeletal muscle, colon, the immune system and the retina[49]. PPARγ controls cellular differentiation, TG synthesis and lipid storage, and modulates the actions of insulin

(*i.e.,* by upregulating GLUT4)[50]. Insulin sensitivity can be improved using glitazones (anti-diabetic drugs) that are high affinity ligands for PPARγ[51].

Liver X Receptor

The Liver X receptors (LXRs), LXRα and LXRβ, are also transcription factors involved in the regulation of lipid metabolism. LXRα is highly abundant in liver and other tissues involved in lipid metabolism, whereas LXRβ is ubiquitously expressed. Both LXRα and LXRβ form obligate heterodimers with RXR and can be activated by lipids, *i.e.*, oxysterols, which are intermediates in cholesterol metabolism in the liver, adrenal glands and brain. Studies in mice have shown that ABC transporters and CYP7A1 (the 7α-hydroxylase gene) are target genes of LXR. Furthermore, an overlap between LXR and PPAR signaling pathways has been suggested, as well as activation of SREBP1c (see below) by LXR, indicating that LXRs are important and very complex factors in lipid homeostasis[52].

Farnesoid X Receptor

The farnesoid X receptors (FXRs) control bile acid as well as lipid metabolism and recent observations indicate even a role in carbohydrate metabolism[53]. They modulate the expression of a wide variety of target genes by binding either as a monomer or as a heterodimer with RXR. FXR is highly expressed in liver, intestine, kidney and the adrenal glands with lower levels in fat and heart. Bile acids have been identified as natural ligands for FXRα[54].

Sterol Regulatory Element Binding Protein

SREBPs (sterol regulatory element binding proteins) are transcription factors that can directly activate the expression of genes involved in synthesis and uptake of cholesterol, FA, TG and phospholipids. Two isotypes have been identified, SREBP1 (among which SREBP1a and SREBP1c) and SREBP2. SREBP1c preferentially regulates FA-biosynthetic pathways and SREBP2 favors cholesterol synthesis. SREBP1a is able to activate both pathways. Important target genes of SREBPs are fatty acid synthase (FAS), LDLR, LPL and HMGCoA synthetase and reductase[55,56].

Disorders in lipid and glucose metabolism

Glucose and lipid metabolism are interconnected complex processes designed to maintain energy homeostasis. Many different factors can lead to dysregulation of these processes with various serious consequences. Below, a number of metabolic abnormalities are briefly summarized: obesity, insulin resistance, type 2 diabetes, dyslipidemia and atherosclerosis. Together, these interconnected metabolic dysregulations represent the various aspects of the metabolic syndrome. During the last decades, the incidence of the metabolic syndrome has taken epidemic proportions. Whereas obesity, type 2 diabetes and dyslipidemia were long thought to be separate diseases, now they have been classified under the metabolic syndrome, or syndrome X. Debates are held on what exact definition is to be used. The world health organization (WHO) definition is: occurrence of diabetes or impaired fasting

glucose or impaired glucose tolerance or insulin resistance, plus two or more of the following: obesity (body mass index > 30), dyslipidemia (hypercholesterolemia, hypertriglyceridemia, low HDL levels), hypertension, microalbuminuria[1]. Since this syndrome is affecting more and more people, much research is being performed on revealing the connections between the different symptoms. Patients presenting one of the symptoms need to be checked for other features of the metabolic syndrome, since it is very unlikely they suffer from only a single disorder[1-3].

Obesity

Obesity, nowadays a common phenotype, is characterized by increased amounts of adipose tissue. The body mass index (BMI) is the most common measure to determine if a person is considered obese. Generally a BMI (calculated by body mass in kg/square of the height in m) over 30 is indicative of health-impairing obesity. Unfortunately, BMI is a crude measurement and other techniques are available to determine the severity of the obesity.

Next to psychosocial problems, obesity leads to painful joints, elevated plasma free FA, insulin resistance, hypertention, heart disease and many other metabolic dysregulations and diseases.

The distribution of the excess fat needs to be considered since body fat pads are roughly divided in visceral adipose tissue (abdominal or central fat), and subcutaneous adipose tissue (peripheral fat), which are metabolically different tissues. Visceral fat is more sensitive to the lipolytic effect of catecholamines and less sensitive to the antilipolytic and TG-storing effect of insulin compared to subcutaneous fat[57,58]. This difference, and the fact that visceral fat directly drains into the portal vein, leads to a relatively high exposure of the liver to visceral adipose tissue-derived free FA (see dyslipidemia) and/or adipokines[59]. Increased levels of leptin, resistine and cytokines, and decreased levels of adiponectin have been described for obese patients, generating metabolic dysregulation and an increased inflammatory state[30-32].

Although obesity can be the result of both genetic and environmental factors, the Western world diet and sedentary life-style are the predominant causes of obesity. Life-style changes and medication have proven to be effective in decreasing obesity in patients[57].

Insulin resistance

Obesity is often accompanied with TG accumulation in other tissues. TG accumulation in turn, often leads to insulin resistance in the respective tissues. Insulin resistance is characterized by unresponsive of the tissue to the actions of insulin. Therefore, more insulin is needed to maintain proper glucose homeostasis in the cells and plasma.

Several conditions are known to induce insulin resistance in tissues, especially TG storage in tissues other than adipose tissue. The liver is able to store rather large amounts of TG for later use. However, it has been recognized that excessive liver lipid accumulation (hepatic steatosis) is linked to hepatic insulin resistance[60-63], leading to increased hepatic

glucose production and VLDL production. Impaired hepatic insulin signaling seems to underlie this steatosis-induced insulin resistance in the liver[61].

In muscle, excess TG storage is also known to induce insulin resistance, although underlying mechanisms are still under debate[60,63-65].

Type 2 diabetes

The disease type 2 diabetes was known as a disease of the aging, since the pancreatic β-cells lose their ability to produce insulin over time. In the last decades patient numbers have explosively increased in all age-groups. This complex disease is a feature of the metabolic syndrome and is often associated with obesity. In type 2 diabetes, the pancreas usually is able to secrete insulin, however there is an imbalance between the capacity for insulin production and the responsiveness of tissues to insulin. When tissues are insulin resistant, the pancreatic production is increased in order to counteract this unresponsiveness, leading to hyperinsulinemia. In time, the pancreatic β-cells are unable to cope with the increased demand, which leads to their destruction. During the different stages of type 2 diabetes different treatment strategies are available, ranging from life-style adjustments, to oral medication (like thiazolidinediones or metformin). Insulin supplementation, however is usually inevitable in time[66].

Growing evidence over recent years supports a potential role for cytokine-associated, subacute inflammation in the pathogenesis of insulin resistance and type 2 diabetes. For example, the cytokine NFκB induces the expression of hepatocyte-specific target genes involved in the pathogenesis of type 2 diabetes (insulin resistance, increased VLDL-TG levels, and hepatic steatosis)[67-69].

Dyslipidemia

Disturbances in plasma lipid levels are known to lead to metabolic problems. Increased free FA levels are associated with obesity, hepatic steatosis and insulin resistance. In addition, increased plasma FA levels are often associated with hypertriglyceridemia[70]. Several genetic forms of dyslipidemia have been described[71]. However, increased plasma lipid levels may be the result of increased dietary intake, altered handling, or increased endogenous production. In hypertriglyceridemia the increased plasma TG levels are usually confined to the VLDL lipoprotein fraction caused by either increased VLDL-TG production or decreased VLDL-TG lipolysis and clearance.

The VLDL production rate is dependent on the hepatic lipid pool. This pool is the net result of hepatic FA uptake and lipogenesis on one hand, and FA oxidation and ketogenesis on the other hand. Also, VLDL production is suppressed by insulin, thus in insulin resistant states the regulation (suppression) of hepatic VLDL production by insulin is diminished. Several studies found a correlation between hepatic VLDL production and insulin resistance and type 2 diabetes[72,73].

In obese people, portal FA flux toward the liver from visceral adipose tissue is increased. In combination with hepatic insulin resistance this leads to increased lipogenesis and decreased FA oxidation giving rise to increased amounts of hepatic TG.

Additionally, it has been postulated that there is decreased LPL activity in insulin resistant tissue. Therefore, lipoproteins are less efficiently lipolyzed, resulting in larger lipoprotein-remnants. Due to the larger particle size, the uptake of these particles is decreased, also adding to the hypertriglyceridemia.

Atherosclerosis

Hyperlipidemia is the major cause of atherosclerosis and, eventually, cardiovascular disease. Increased levels of cholesterol-rich particles (CM- and VLDL-remnants and LDL) in the plasma results in increased penetration of these lipoproteins into the vessel wall. Modification of these lipoproteins in the subendothelial space or intima leads to excessive accumulation of cholesterol in the residing macrophages and conversion of these cells into foam cells. The formation of foam cells in the intima is commonly considered as the very initial step in atherosclerotic plaque formation. Foam cells in the intima produce inflammatory cytokines and chemotatic molecules. Inflammatory cells are recruited, leading to further growth of the lesion/plaque in the arterial wall. The bloodflow in the artery will become impaired. Moreover, rupture of the lesion may cause thrombus formation, leading to cardiovascular events, such as myocardial infarction and stroke[74,75].

Outline of the Thesis

The studies described in this thesis are aimed at unraveling the metabolic relationship between various aspects of the metabolic syndrome, like obesity, insulin resistance, hepatic steatosis and dyslipidemia.

In **chapter 2** our aim was to study whether the absence of apoC3, a strong inhibitor of LPL, accelerates the development of obesity and, consequently, insulin resistance. We hypothesized that the redistribution of plasma TG in *apoc3*$^{-/-}$ mice on a high-fat diet leads to weight gain. In these mice and wild type littermates we followed the development of features of the metabolic syndrome, *e.g.,* levels of plasma lipid, glucose and insulin, obesity and body composition, and tissue-specific insulin resistance.

Hepatic VLDL and glucose production is enhanced in type 2 diabetes and is associated with hepatic steatosis. In **chapter 3** we used methyl palmoxirate to acutely inhibit hepatic FA oxidation, and investigated whether changes in hepatic β-oxidation influence VLDL production/secretion, and whether this would affect hepatic steatosis and glucose production *in vivo*.

Dietary FA have profound impact on the occurrence of hyperlipidemia and/or hepatic steatosis, but mechanisms are not fully understood. In **chapter 4** we studied the effects of a saturated-fat diet, supplemented with fish oil, *trans*10*,cis*12-conjugated linoleic acid (CLA), or elaidic acid, on lipid and glucose metabolism in APOE*3Leiden mice. In

addition, by using the proteomic approach we measured numerous liver proteins of these mice to increase insight in the biochemical pathways underlying the metabolic relationship between dietary FA and hepatic lipid and glucose metabolism.

Sphingolipids are lipids found as membrane constituents in plants, yeasts and animals and are present in our daily diet. The sphingolipid sphingomyelin has been shown to decrease plasma cholesterol in rats. In **chapter 5** we questioned whether various sphingolipids supplemented to the Western-type diet decrease plasma cholesterol and/or TG in hyperlipidemic APOE3*Leiden mice. More specifically, we wondered if sphingolipids added to the diet could be used to treat the dyslipidemia and the related abnormal hepatic lipid homeostasis, characteristic of the metabolic syndrome.

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Chapter 2

Apolipoprotein C3-deficiency results in diet-induced obesity and aggravated insulin resistance in mice

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Abstract

Our aim was to study whether the absence of apoC3, a strong inhibitor of lipoprotein lipase (LPL), accelerates the development of obesity and consequently insulin resistance.

Apoc3$^{-/-}$ mice and wild-type littermates were fed a high-fat (46 energy %) diet for 20 weeks. After 20 weeks of high-fat feeding, *apoc3*$^{-/-}$ mice showed decreased plasma triglyceride (TG) levels (0.11 ± 0.02 vs. 0.29 ± 0.04 mmol/l; $P < 0.05$), and were more obese (42.8 ± 3.2 vs. 35.2 ± 3.3 g; $P < 0.05$) compared with wild-type littermates. This increase in body weight was entirely explained by increased body lipid mass (16.2 ± 5.9 vs. 10.0 ± 1.8 g; $P < 0.05$). LPL-dependent uptake of TG-derived fatty acids by adipose tissue was significantly higher in *apoc3*$^{-/-}$ mice. LPL-independent uptake of albumin-bound fatty acids did not differ. Interestingly, whole-body insulin sensitivity using hyperinsulinemic-euglycemic clamps was decreased by 43% and suppression of endogenous glucose production was decreased by 25% in *apoc3*$^{-/-}$ mice compared with control mice. Absence of apoC3, the natural LPL inhibitor, enhances fatty acid uptake from plasma TG in adipose tissue, which leads to higher susceptibility to diet-induced obesity followed by more severe development of insulin resistance. Therefore, apoC3 is a potential target for treatment of obesity and insulin resistance.

Introduction

Lipoprotein lipase (LPL) hydrolyzes plasma triglycerides (TG) contained in circulating very low density lipoprotein (VLDL) particles and chylomicrons. Subsequently, these TG-derived fatty acids (FA) are taken up by the underlying tissues[1,2]. LPL activity is an important determinant of the rate of FA storage into white adipose tissue (WAT) and other tissues. For instance, overexpression of LPL in muscle leads to enhanced TG storage in muscle[3-5], whereas adipose tissue-specific LPL deficiency prevents excessive adipose tissue TG-storage in leptin-deficient mice[6]. The latter observation indicates a link between adipose tissue-specific LPL activity and obesity. Inhibition of LPL activity therefore may be an effective strategy for prevention of obesity. This concept is further confirmed by mouse models such as VLDL-receptor knockout and human apolipoprotein (apo) C1 overexpressing mice. These mice show decreased *in vivo* VLDL-TG lipolysis and, as a consequence, are protected from diet- and genetically-induced obesity[7-9], as well as insulin resistance.

These data suggest that overall reduction of the LPL activity can protect against obesity. It is unclear, however, whether the effect of LPL modulation acts in both directions, *i.e.*, whether *activation* of LPL can also lead to *enhanced* susceptibility to diet-induced obesity followed by aggravated development of insulin resistance. Adipose tissue-specific overexpression of LPL seemed to result in a relatively mild (20%) increase in fat pad weight[10]. This mild effect could be related to the fact that natural inhibitors of LPL, *e.g.*, apoC3, are still present to regulate *in vivo* adipose tissue LPL activity. Alternatively, it

might relate to the LPL activity ratio between adipose tissue and muscle tissue as discussed by Preiss-Landl et al.[11].

To elucidate the effect of deletion of the main endogenous LPL inhibitor apoC3 on diet-induced obesity and insulin resistance *in vivo*, we have used apoC3 knockout mice[12]. ApoC3 is mainly produced by the liver and is a well-known inhibitor of LPL activity[13]. *Apoc3*$^{-/-}$ mice have greatly enhanced *in vivo* VLDL-TG clearance, as caused by the absence of the endogenous block on LPL activity[14], which is reflected by a total absence of a postprandial TG response after a fat load[12,14]. The present study indeed showed that *apoc3*$^{-/-}$ mice are more sensitive to diet-induced obesity followed by a more aggravated development of insulin resistance compared with their control littermates. ApoC3, therefore, may be a potential therapeutic target for the treatment of obesity and insulin resistance.

Materials and methods

Animals and diet

Male and female *apoc3*$^{-/-}$ mice and their wild-type (WT) littermates (C57Bl/6 background) were originally obtained from The Jackson Laboratories (Bar Harbor, ME, USA) and further bred in our institution. The 4 to 5 month old animals (n=15) were individually housed, allowed free access to food and water, and were kept on a 12 h light cycle (lights on at 7.00 A.M.), under standard conditions. After a standard rat-mouse chow diet (Standard Diet Services, Essex, UK), the mice were given a high-fat corn oil diet (Hope Farms, Woerden, the Netherlands) until the end of the experimental period. This diet contained 24% corn oil, 24% casein, 20% cerelose, 18% corn starch and 6% cellulose by weight, resulting in 46.2% of calories derived from corn oil. Body weight and food intake were followed through the duration of the experiment. Food intake was assessed by determining the difference in food weight during a 7-day period to ensure reliable measurements. Food intake was assessed as food weight (g) per mouse per day. From these data, the “feed efficiency” was calculated as total body weight gained per week divided by the total amount of food consumed per week. All experiments were approved by the animal care committee of TNO Quality of Life (Leiden, the Netherlands).

Plasma parameters

Plasma levels of cholesterol, free fatty acids (FFA), TG (without free glycerol), glucose, keton bodies (β-hydroxybutyrate), insulin, and leptin were determined after an overnight fast in *apoc3*$^{-/-}$ and WT littermates after 0 and 20 weeks of high-fat diet feeding. Blood samples were taken from the tail vein in paraoxon-coated capillaries to prevent lipolysis[15]. The plasma was collected via centrifugation, and plasma cholesterol, TG, glucose, keton body, and FFA levels were determined using standard commercial kits, according to the manufacturer’s instructions (Roche Diagnostics, Mannheim, Germany; Triglyceride GPO-

Trinder, glucose Trinder 500 and β-hydroxybutyrate, Sigma Diagnostics, St. Louis, MO, USA, and NEFA-C, Wako chemicals, Neuss, Germany, respectively). Plasma insulin and leptin levels were measured by radioimmunoassay (RIAs), using rat insulin standards, that show 100% cross-reaction with mouse and human insulin, or mouse leptin standards (sensitive rat insulin RIA kit, mouse leptin RIA kit, Linco Research, St. Charles, MO, USA).

Body mass composition analysis

Mouse carcasses (wet weight) were dehydrated at 65°C until a constant mass was achieved (dry weight). The bodies were hydrolyzed in 100 ml ethanolic potassium hydroxide (3 M in 65% ethanol) for determination of body lipid, using enzymatic measurement of glycerol (Sigma Diagnostics), and body protein content by the Lowry assay[16]. Total water content was calculated as wet weight minus dry weight, and lean body mass (LBM) was calculated as wet weight minus total lipid weight.

WAT histology

Pieces of WAT from reproductive fat pads were fixed in formalin and embedded in paraffin. Sections of 3 µm were cut and stained with hematoxylin-phloxine-saffron. Adipocyte size was quantified using Leica Qwin v1.0 (Leica Micro systems, Wetzlar, Germany).

Tissue-specific FFA uptake from plasma TG

To exclude obesity-induced differences in adipose tissue FA uptake, we used body weight-matched *apoc3*$^{-/-}$ and WT mice in the fed state, which had been treated with the high-fat diet for 2 weeks. The mice were sedated by i.p. injection of hypnorm (0.5 ml/kg; Janssen Pharmaceutical, Tilburg, The Netherlands) and midazolam (12.5 mg/kg; Roche Netherlands, Mijdrecht, The Netherlands) and equipped with a catheter for tail-vein i.v. infusion. Large (150 nm) glycerol [^{3}H]triolein-labeled chylomicron-like particles, prepared as described by Rensen et al.[17], were mixed with a trace amount of [^{14}C]oleic acid complexed to bovine serum albumin (BSA) (both isotopes obtained from Amersham, Little Chalfont, UK) and continuously infused for 2 h into the animals as described earlier[18]. Blood samples were taken using chilled paraoxon-coated capillaries by tailbleeding at 1.5 and 2 h of infusion to quantify steady-state conditions. Subsequently, mice were sacrificed and organs were quickly harvested and snap-frozen in liquid nitrogen. Analyses and calculations were performed as described by Teusink et al.[18].

Total plasma and tissue LPL level

For determining the total LPL activity level, body weight-matched *apoc3*$^{-/-}$ and WT mice, treated with the high-fat diet for 1 week, were fasted for 4 h and received an i.p. injection of heparin (1 U/g body weight; Leo Pharmaceutical Products, Weesp, The Netherlands). Blood samples were taken after 20 min. Plasmas of these samples were snap-frozen and stored at -80°C until analysis. One week later, the animals in the fed state were sacrificed, and liver, heart, skeletal muscle (quadriceps), and WAT samples were collected. The

organ samples were cut into small pieces and put in 1 ml 2% BSA-containing DMEM medium. Heparin (2 U) was added, and samples were shaken at 37°C for 60 minutes. After centrifugation (10 min at 13,000 rpm), the supernatants were taken and snap-frozen until analysis. Total LPL activity of all samples was determined as modified from Zechner[19]. In short, the lipolytic activity of plasma or tissue supernatant was assessed by determination of [^{3}H]oleate production upon incubation of plasma or tissue supernatant with a mix containing an excess of both [^{3}H]triolein, heat-inactivated human plasma as source of the LPL coactivator apoC2, and FA-free BSA as FFA acceptor. Hepatic lipase (HL) and LPL activities were distinguished in the presence of 1 M NaCl, which specifically blocks LPL.

Modulated plasma LPL activity

To allow for studying the effect of apoC3-deficiency on the modulated LPL activity in plasma, post-heparin mouse plasma (as a source of LPL, apoC2, and apoC3) was incubated with a mix of [^{3}H]triolein-labeled 75 nm-sized VLDL mimicking protein-free emulsion particles[20] (0.25 mg TG/ml) and excess FA-free BSA (60 mg/ml). HL and LPL activities were distinguished as described above, and the LPL activity was calculated as the amount of FFA released per min per ml.

Hyperinsulinemic-euglycemic clamp

After 20 weeks of high-fat feeding and an overnight fast, the animals were anesthetized, as described earlier, and basal rates of glucose turnover were determined followed by a hyperinsulinemic-euglycemic phase (plasma glucose at ~ 7.5 mmol/l) as described previously[5,21]. After the final blood sample, mice were sacrificed, and liver, cardiac muscle, skeletal muscle (quadriceps), and adipose tissue samples were immediately frozen in liquid nitrogen and stored at -20°C for subsequent analysis. Carcasses were stored at -20°C until body mass composition was analyzed. Whole-body insulin-mediated glucose uptake and insulin-mediated suppression of endogenous glucose production were calculated on the basis of LBM. The whole-body insulin sensitivity index was expressed as the ratio between insulin-induced whole-body glucose disposal and hyperinsulinemic plasma insulin concentration. The endogenous glucose production insulin sensitivity index was expressed as ratio between insulin-mediated inhibition of endogenous glucose production and hyperinsulinemic plasma insulin concentration. Insulin clearance (ml/min) was calculated from steady-state insulin concentrations and insulin infusion rates:

$$\frac{\text{Insulin infusion (ng/min)}}{\text{Insulin concentration [Hyperinsulinemic] - [Basal] (ng/ml)}}$$

Tissue lipid levels

Tissues were homogenized in phosphate buffered saline (PBS) (~ 10% wet weight/vol), and samples were taken to measure protein content by the Lowry assay[16]. Lipid content was determined by extracting lipids using the Bligh and Dyer method[22] and by separating the lipids using high-performance thin-layer chromatography (HPTLC) on silica gel plates as described before[23], followed by TINA2.09 software analysis[24] (Raytest Isotopen meβgeräte, Straubenhardt, Germany).

Hepatic VLDL-TG Production

To determine the effect of apoC3-deficiency on the hepatic VLDL-TG production rate, animals that were fed the high-fat diet for 2 weeks were fasted for 4 h and anesthetized, followed by an i.v. injection of 10% Triton WR1339 (500 mg/kg body weight; Tyloxapol, Sigma Chemicals, Steinheim, Germany) to inhibit lipolysis and hepatic uptake of VLDL-TG. Blood samples were drawn at 0, 15, 30, 60, and 90 min after Triton injection and TG concentrations were determined in the plasma as described above.

Statistical analysis

The Mann-Whitney U test was used to determine differences between *apoc3*$^{-/-}$ and WT mice. The criterion for significance was set at $P < 0.05$. All data are presented as mean ± SD. Statistical analyses were performed using SPSS11.0 (SPSS, Chicago, IL, USA).

Results

High-fat feeding increased body weight in apoc3$^{-/-}$ mice as a result of an increase in body fat content

Male *apoc3*$^{-/-}$ and WT littermate mice were put on the high-fat diet for a period of 20 weeks. Already after 2 weeks, *apoc3*$^{-/-}$ mice showed a significant increase in body weight on high-fat diet compared with littermate controls, leading to a 22% higher body weight in *apoc3*$^{-/-}$ mice compared with WT mice at the end of the experiment (week 20), as shown in **Figure 1A**. A significant increase in body weight was also observed in female *apoc3*$^{-/-}$ mice compared with WT littermates, although less extreme (data not shown).

Food intake of male *apoc3*$^{-/-}$ and WT mice was comparable during the first 11 weeks. After 11 weeks until the end of the experiment the food intake of *apoc3*$^{-/-}$ mice was increased 5-15% compared with that of WT littermates (**Figure 1B**). The calculated feed efficiency (**Figure 1C**) was significantly increased in the *apoc3*$^{-/-}$ mice compared with WT littermates. The greatest difference in feed efficiency between the genotypes was seen at week 4 of high-fat feeding (0.048 ± 0.009 vs. 0.019 ± 0.007 g weight gain/g food consumed, for *apoc3*$^{-/-}$ and WT mice, $P < 0.05$). This difference between the groups gradually decreased towards the end of the experiment, although *apoc3*$^{-/-}$ mice still had a higher feed efficiency at 20 weeks of high-fat feeding compared with WT littermates (0.031 ± 0.006 vs. 0.019 ± 0.007 g weight gain/g food consumed, respectively, $P < 0.05$).

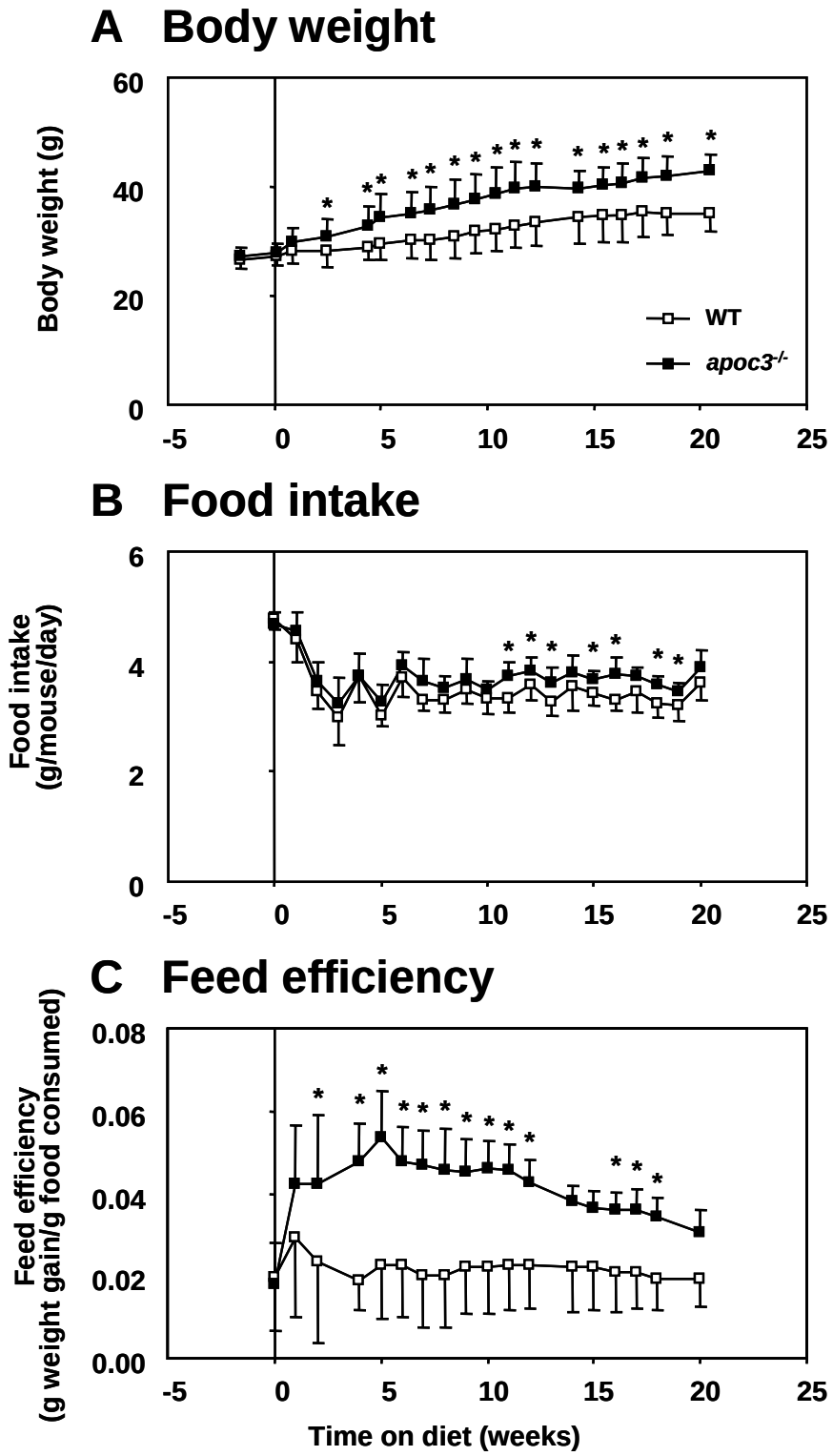

Figure 1. Growth (A), food intake (B) and feed efficiency (C) curves of *apoc3*$^{-/-}$ (closed squares) and WT (open squares) mice during a 20-week period of high-fat feeding

Body weight and food intake were measured periodically over the course of the experiment. Feed efficiency was calculated as the total weight gain divided by the total amount of food consumed during the experiment. Values represent the mean ± SD of 10 *apoc3*$^{-/-}$ and 13 WT mice. *P < 0.05, using nonparametric Mann-Whitney U tests

To investigate alterations in body composition, we analyzed mouse carcasses after 20 weeks of high-fat diet. Body weight, LBM, and the proportion of water, protein and lipid of *apoc3*$^{-/-}$ mice and WT littermates are shown in **Table 1**. Although body weight was ~ 7 g higher in *apoc3*$^{-/-}$ mice compared with WT mice, the LBM was comparable for both groups of mice. The absolute amount of body lipid in *apoc3*$^{-/-}$ mice was ~ 6 g higher compared with WT mice. No differences were found in protein content and amount of body water between *apoc3*$^{-/-}$ mice and WT littermates. Analysis of adipocyte size in the reproductive fat pads revealed that after 20 weeks of high-fat feeding, adipocytes of *apoc3*$^{-/-}$ mice and of WT mice are comparable in size (**Table 1**).

Table 1. Body mass composition and adipocyte size of *apoc3*$^{-/-}$ and WT mice after 20 weeks of high-fat feeding and an overnight fast

Genotype	Body Weight (g)	LBM (g)	Protein (g)	Water (g)	Lipid (g)	Adipocyte size (µm²)
WT	32.4 ± 5.5	23.9 ± 4.5	3.4 ± 0.4	17.9 ± 0.9	10.0 ± 1.8	6035 ± 761
apoc3$^{-/-}$	39.5 ± 3.4*	23.3 ± 3.3	3.9 ± 0.5	18.3 ± 0.8	16.2 ± 5.9*	5771 ± 413

Values represent the mean ± SD of 6 *apoc3*$^{-/-}$ and 10 WT mice. Lean body mass (LBM) and body total protein, water, and lipid content were determined as described in the materials and methods section. Reproductive fat pads were used for freeze sectioning, and subsequent staining and adipocyte size was measured as described.*$P < 0.05$, using nonparametric Mann-Whitney U tests

Apoc3$^{-/-}$ mice showed increased plasma TG-derived FA uptake by adipose tissue

To show that indeed the increased adipose tissue mass was due to increased LPL-dependent TG-derived FA uptake, we determined the tissue-specific uptake of FA derived from either plasma TG or albumin in several tissues of non-fasted, body weight-matched *apoc3*$^{-/-}$ and WT mice that were fed the high-fat diet for 2 weeks (**Figure 2**). The small difference in body weight after only 2 weeks of high-fat diet feeding ensured the availability of body weight-matched *apoc3*$^{-/-}$ and WT mice. We observed no differences in uptake of albumin-bound FA in liver, heart, muscle, and adipose tissue between *apoc3*$^{-/-}$ and WT littermates. Interestingly, TG-derived FA uptake was significantly increased in visceral, subcutaneous and reproductive fat pads from *apoc3*$^{-/-}$ mice compared with WT mice. No differences were found in TG-derived FA uptake in liver, heart, and skeletal muscle in *apoc3*$^{-/-}$ mice compared with littermates.

Apoc3$^{-/-}$ mice showed increased modulated plasma LPL activity

Because LPL-mediated TG-derived FA clearance was increased in WAT of *apoc3*$^{-/-}$ mice, we determined the total plasma and tissue LPL activity in body weight-matched *apoc3*$^{-/-}$

mice and littermate controls that were fed the high-fat diet for 1 week (plasma LPL) and 2 weeks (tissue LPL).

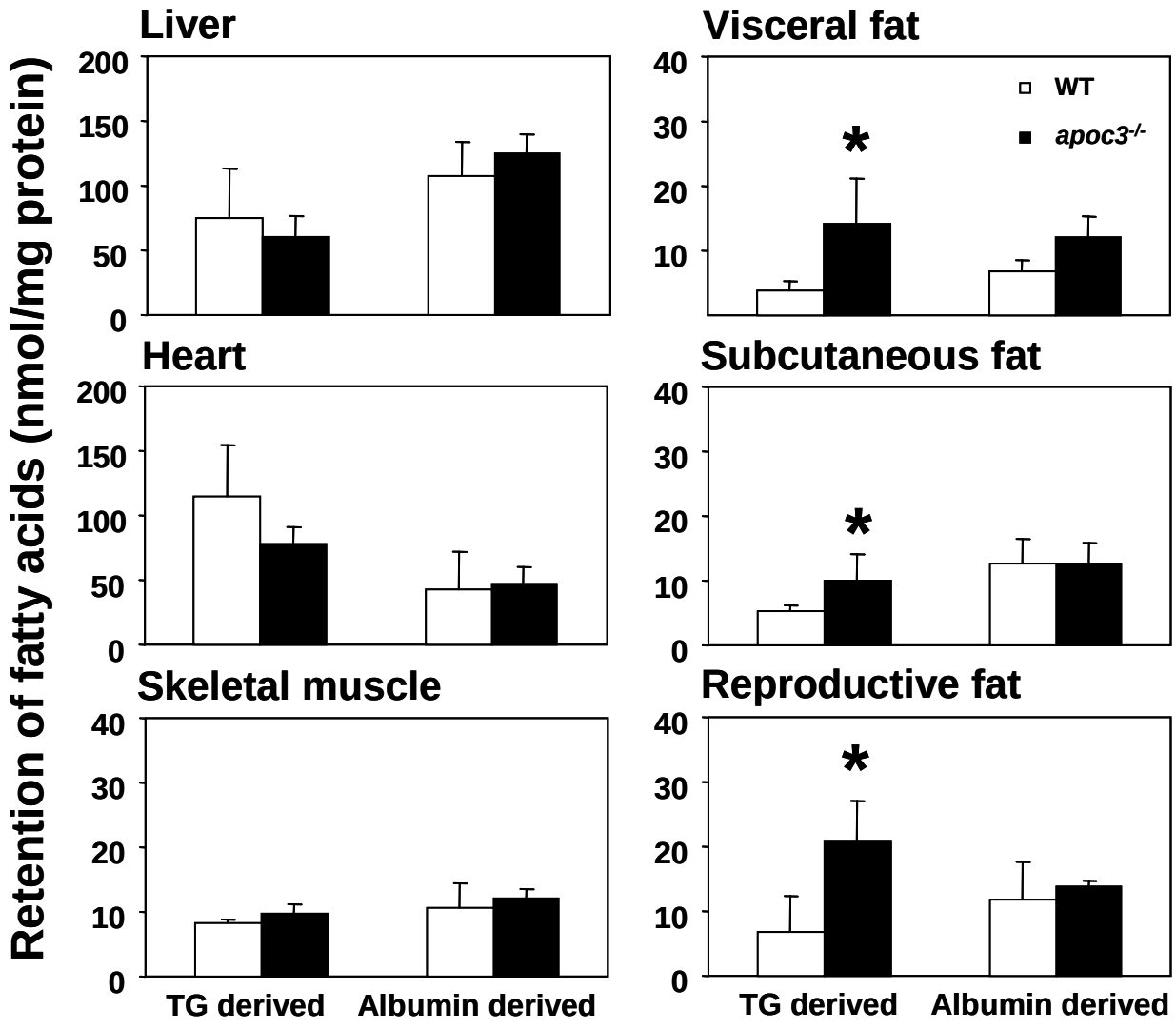

Figure 2. Retention of [^{3}H]TG and [^{14}C]FA label in tissues of *apoc3*$^{-/-}$ and WT mice
After 2 weeks on a high-fat diet, body weight-matched, fed, male *apoc3*$^{-/-}$ (closed bars) and WT (open bars) mice were infused for 2h with a solution containing [^{3}H]TG chylomicron-sized particles and albumin-bound [^{14}C]FA. Label content in lipids was measured and corrected for specific activities in plasma of [^{3}H]TG and [^{14}C]FA, respectively, in liver, heart, skeletal muscle tissue, and visceral, subcutaneous, and reproductive fat pads. Values represent the mean ± SD of n = 4 mice per group. *P < 0.05, using nonparametric Mann-Whitney U tests

Total post-heparin plasma LPL activity was similar in *apoc3*$^{-/-}$ and WT mice (7.0 ± 5.6 vs. 5.4 ± 3.2 µmol FFA/h/ml, respectively), demonstrating that the absence of apoC3 does not affect LPL expression. Likewise, tissue-specific LPL activity measured in liver, heart, skeletal muscle, and visceral, subcutaneous, and reproductive fat pads was not different between *apoc3*$^{-/-}$ and control mice (**Figure 3**). We next studied the LPL activity in post-heparin plasma in absence of excess heat-inactivated human plasma, and in presence of limited amounts of VLDL-like emulsion particles rather than excess solubilized TG.

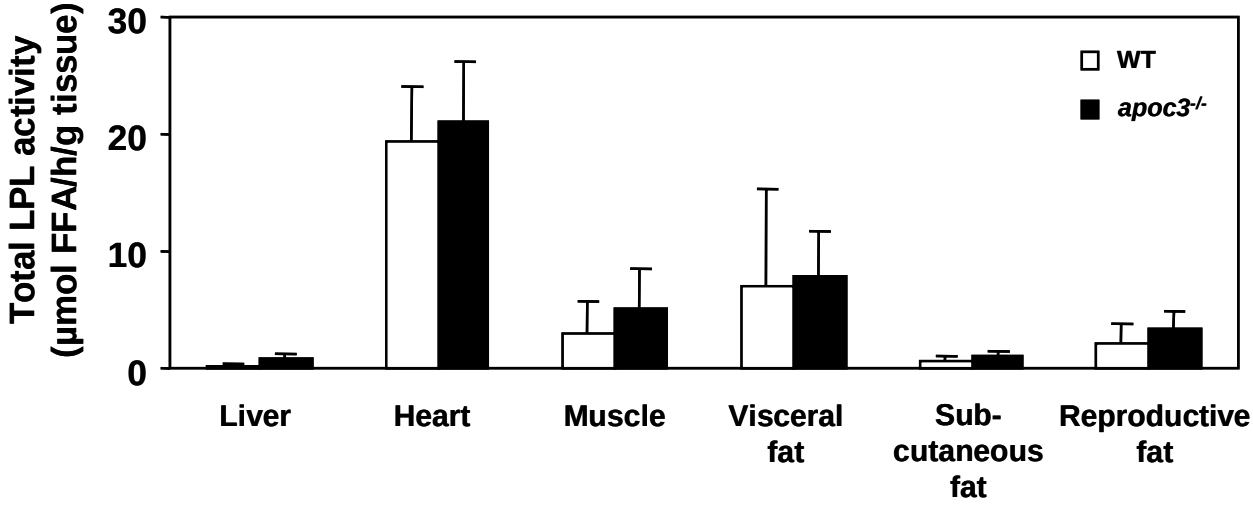

Figure 3. Tissue LPL activity levels in *apoc3*$^{-/-}$ (closed bars) and WT (open bars) mice
Fed body weight-matched *apoc3*$^{-/-}$ and WT mice were sacrificed, and liver, heart, skeletal muscle tissue, and visceral, subcutaneous, and reproductive fat pad samples were added to DMEM medium containing 2 U heparin. Supernatant was assayed with [^{3}H]triolein containing substrate mixture in absence or presence of 1 M NaCl to determine LPL activity based on generation of [^{3}H]oleate. Values represent the mean ± SD for n= 4 per group. *P < 0.05, using nonparametric Mann-Whitney U tests

Under these conditions, the LPL activity as modulated by endogenous mouse plasma factors (*e.g.*, apoC3) can be studied. Indeed, *apoc3*$^{-/-}$ mouse plasma showed 78% increased TG hydrolase activity compared with WT littermates judging from [^{3}H]oleate production (1.33 ± 0.20 vs. 0.75 ± 0.17 nmol oleate/ml/min, respectively, P < 0.05).

Collectively, these data clearly show that apoC3 modulates LPL activity by interfering with the interaction between LPL and TG-rich lipoproteins (*i.e.*, VLDL and chylomicrons), rather than by affecting total LPL levels.

Apoc3$^{-/-}$ mice had increased plasma glucose levels and strongly decreased whole-body insulin sensitivity

High-fat feeding induced increased total plasma cholesterol in both groups (**Table 2**). In *apoc3*$^{-/-}$ mice, plasma glucose, keton body, and leptin were also increased after 20 weeks high-fat compared with t=0 (chow diet). Plasma total cholesterol and FFA were comparable between the mice that were on chow and on the high-fat diet (**Table 2**). After 20 weeks of high-fat feeding plasma keton body and leptin levels were increased in *apoc3*$^{-/-}$ mice compared with littermates. Plasma TG levels were significantly lower in *apoc3*$^{-/-}$ mice compared with WT mice as reported earlier[12]. No significant differences were found with respect to plasma glucose and insulin levels in *apoc3*$^{-/-}$ versus WT animals before high-fat feeding (**Table 2**). At the end of the 20-week period of high-fat feeding, *apoc3*$^{-/-}$ mice showed significantly higher plasma glucose and slightly but not significantly increased plasma insulin levels compared with control littermates (**Table 2**).

Table 2. Plasma parameters determined in overnight-fasted *apoc3*$^{-/-}$ and WT mice at 0 and 20 weeks of high-fat diet exposure

	t=0 weeks		t=20 weeks	
	WT	*apoc3*$^{-/-}$	WT	*apoc3*$^{-/-}$
Total Cholesterol (mmol/l)	2.18 ± 0.87	2.09 ± 0.28	3.73 ± 1.09‡	3.36 ± 0.63‡
Triglycerides (mmol/l)	0.28 ± 0.09	0.15 ± 0.03*	0.29 ± 0.04	0.11 ± 0.02‡*
Free fatty acids (mmol/l)	1.08 ± 0.23	1.10 ± 0.25	1.07 ± 0.14	0.92 ± 0.15
Glucose (mmol/l)	4.65 ± 0.67	4.74 ± 0.46	5.69 ± 0.92	7.25 ± 1.21‡*
Keton body (mmol/l)	1.10 ± 0.31	1.21 ± 0.33	1.29 ± 0.19	1.75 ± 0.23‡*
Insulin (pmol/l)	80 ± 59	38 ± 20	161 ± 60	317 ± 155
Leptin (ng/ml)	4.68 ± 2.89	3.54 ± 2.41	6.41 ± 1.55	15.99 ± 5.11‡*

Values represent the mean ± SD of 7 *apoc3*$^{-/-}$ and 10 WT mice. Total cholesterol, triglycerides, free fatty acids, glucose, keton body, insulin, and leptin levels were determined in plasma obtained from the mice via tail-tip incision. *P < 0.05 vs. WT, ‡P < 0.05 vs. t=0, using nonparametric Mann-Whitney U tests

Insulin sensitivity was tested using hyperinsulinemic-euglycemic clamp analyses (**Table 3**). Under fasted conditions, plasma glucose levels were higher in *apoc3*$^{-/-}$ mice compared with WT littermates. During hyperinsulinemic conditions, no significant differences were observed in plasma glucose. Insulin levels were increased in *apoc3*$^{-/-}$ mice as compared with WT mice (**Table 3**). This increase in plasma insulin levels was explained by decreased insulin clearance in *apoc3*$^{-/-}$ mice (**Table 3**).

Table 3. Clamp conditions in *apoc3*$^{-/-}$ and WT mice at 20 weeks of high-fat feeding

Genotype	LBM (g)	Basal conditions		Hyperinsulinemic conditions		
		Glucose (mmol/l)	Insulin (pmol/l)	Glucose (mmol/l)	Insulin (pmol/l)	Insulin clearance (ml/min)
WT	23.9 ± 4.5	5.7 ± 0.9	161 ± 60	7.1 ± 1.2	234 ± 75	480 ± 133
apoc3$^{-/-}$	23.3 ± 3.3	7.3 ± 1.2*	317 ± 155	7.8 ± 0.8	524 ± 149*	110 ± 48*

Values represent the mean ± SD of n = 4 per group. Lean body mass (LBM) and concentrations of plasma glucose and insulin measured before (basal) and during the hyperinsulinemic-euglycemic clamp in *apoc3*$^{-/-}$ and WT mice. Insulin clearance was calculated using steady-state insulin concentrations and insulin infusion rates. Animals were fed high-fat diet for 20 weeks and were fasted overnight before the clamp experiment. *P < 0.05, using nonparametric Mann-Whitney U tests

The clamp results revealed that insulin-mediated whole-body glucose uptake was significantly lower in *apoc3*$^{-/-}$ mice compared with littermate controls (15 ± 5 vs. 35 ± 9%, respectively, P < 0.05; **Figure 4A**). Moreover, the endogenous glucose production was only slightly suppressed under hyperinsulinemic conditions in *apoc3*$^{-/-}$ mice as compared with WT mice (7 ± 1 vs. 27 ± 10% respectively, P < 0.05; **Figure 4B**).

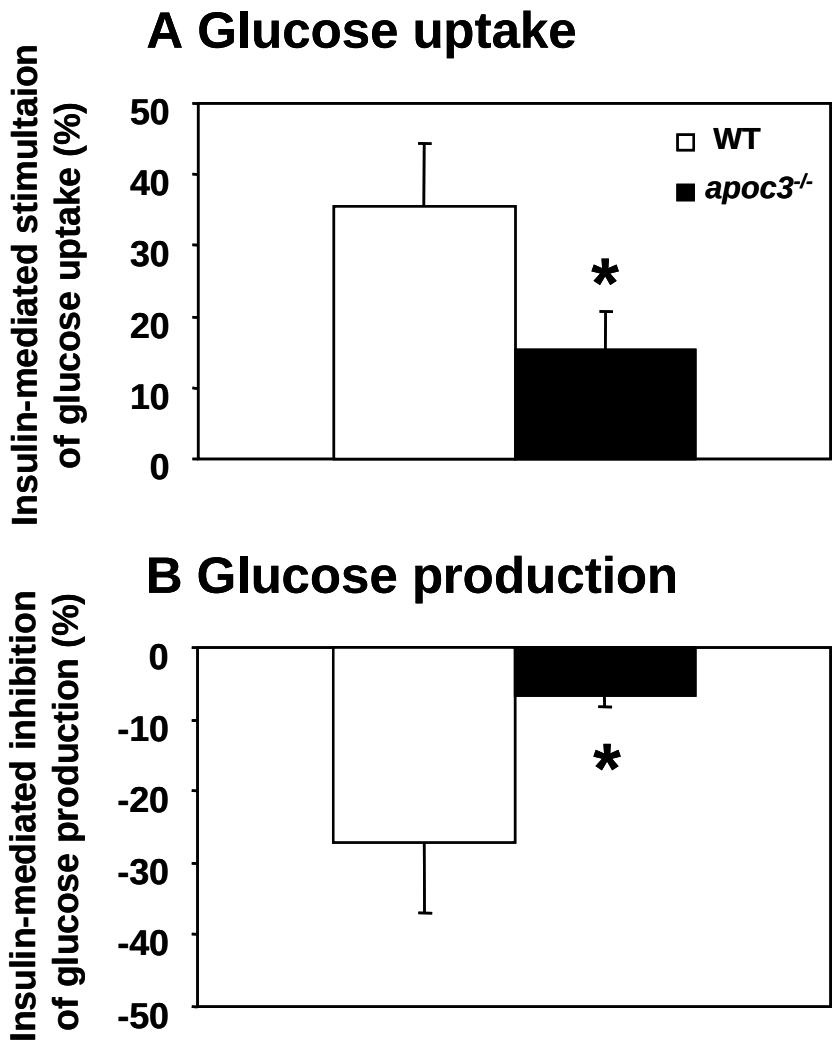

Figure 4. Hyperinsulinemic-euglycemic clamp results of *apoc3*$^{-/-}$ (closed bars) and WT (open bars) mice after 20 weeks of high-fat feeding

Whole-body insulin-mediated glucose uptake and insulin-mediated suppression of endogenous glucose production were calculated on the basis of lean body mass. The insulin-mediated stimulation of whole-body glucose uptake (A) was expressed as the ratio between insulin-induced whole-body glucose disposal and hyperinsulinemic plasma insulin concentration. Insulin-mediated inhibition of endogenous glucose production (B) was expressed as ratio between insulin-mediated inhibition of endogenous glucose production and hyperinsulinemic plasma insulin concentration. Values represent the mean ± SD for n= 4 per group.
*P < 0.05, using nonparametric Mann-Whitney U tests

Because *apoc3*$^{-/-}$ mice were insulin resistant compared with WT littermates, we analyzed liver and muscle lipid content. In *apoc3*$^{-/-}$ mice, hepatic lipid content was significantly higher compared with that in WT littermates (**Figure 5A**). Skeletal and cardiac muscle TG content did not significantly differ between both mouse groups.

Apoc3$^{-/-}$ mice showed unaltered VLDL-TG production rates

Since increased liver TG levels were observed in *apoc3*$^{-/-}$ mice, the ability of the liver to secrete VLDL-TG was investigated. Mice that were fed the high-fat diet for 2 weeks were

fasted for 4 h and received an injection of Triton WR1339. The accumulation of endogenous VLDL-TG in plasma was determined over time (**Figure 5B**). The VLDL-TG production rate as determined from the slope of the curve was unchanged in *apoc3*$^{-/-}$ mice compared with WT littermates (3.89 ± 0.54 vs. 3.73 ± 0.58 mmol TG/h, respectively). Therefore, the hepatic TG accumulation as observed in *apoc3*$^{-/-}$ mice is not caused by reduced VLDL secretion.

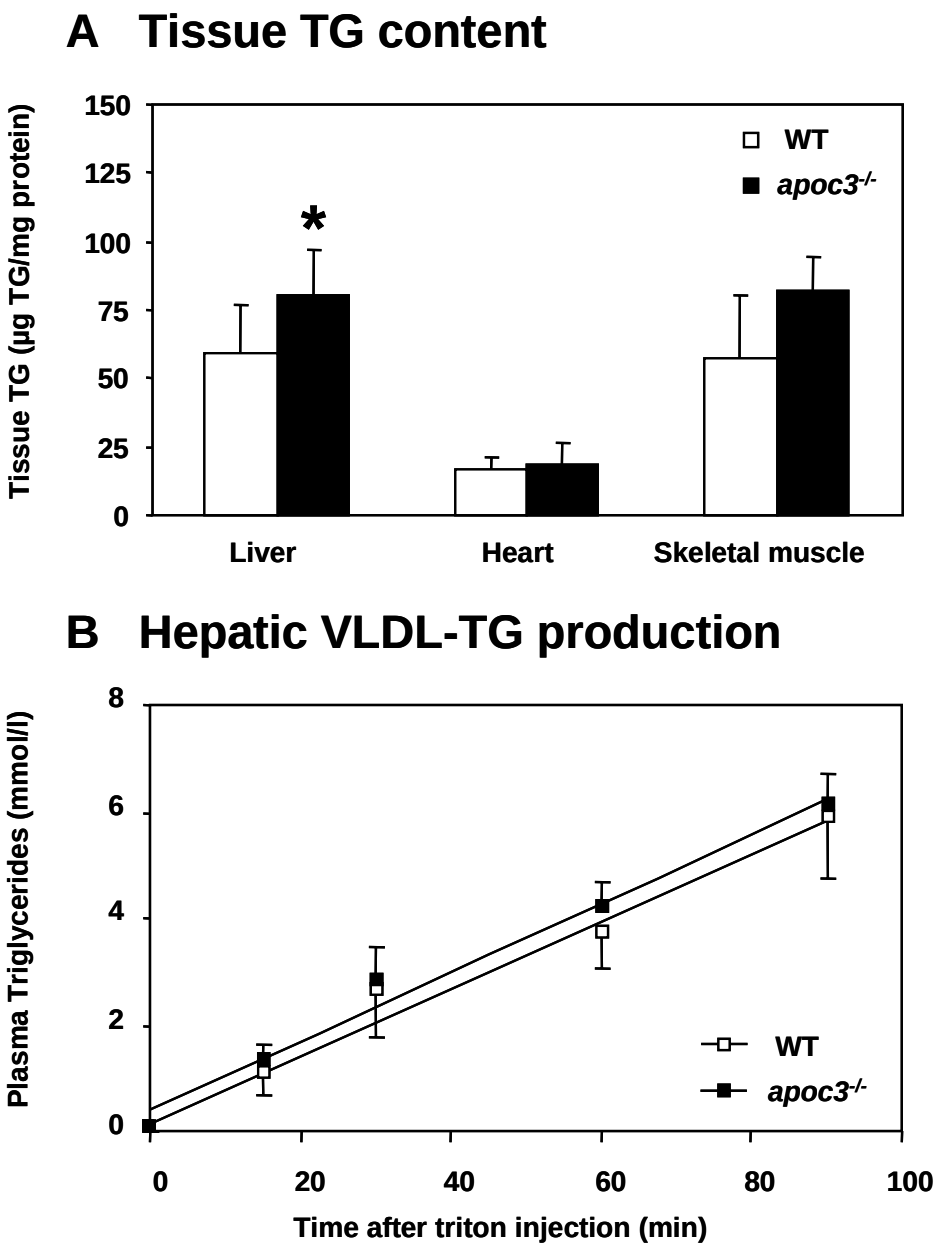

Figure 5. Tissue TG levels (A) and hepatic VLDL-TG production (B)
Hepatic, cardiac, and skeletal muscle TG levels were determined in *apoc3*$^{-/-}$ (closed bars) and WT (open bars) mice after 20 weeks of high-fat feeding. Lipids were extracted from tissue homogenates by the Bligh and Dyer method and subsequently separated on thin-layer silica gel plates (A). Hepatic VLDL-TG production was assessed by Triton WR1339 injection and plasma TG analyses in *apoc3*$^{-/-}$ (closed squares) and WT (open squares) mice after 2 weeks of high-fat feeding and a 4 h fast (B). Values represent the mean ± SD for n=5 per group. *P < 0.05, using nonparametric Mann-Whitney U tests

Discussion

LPL plays an important role in the delivery of FA into peripheral tissues. Several mouse studies indicate that decreased LPL activity in adipose tissue decreases the propensity to develop obesity[6-9,25]. However, it is unclear whether the opposite is true as well, *i.e.*, whether activation of LPL leads to an enhanced susceptibility to diet-induced obesity and associated insulin resistance. Adipose tissue-specific LPL overexpression leads to only a slight increase in fat pad weight[10]. However, this relatively minor effect can result from an altered muscle versus adipose tissue LPL activity ratio and/or the presence of natural regulators of *in vivo* LPL activity[11]. ApoC3 is a known inhibitor of LPL activity, and disruption of the gene in mice increases TG clearance[12,14].

In this study, we investigated the possible effects of enhanced whole-body LPL activity (resulting from deletion of the LPL inhibitor apoC3) on development of obesity and insulin resistance making use of the apoC3 knockout mouse (*apoc3*$^{-/-}$). The data show that *apoc3*$^{-/-}$ mice developed a higher adipose tissue mass during high-fat feeding compared with WT littermates. The higher adipose tissue mass in these mice was caused by a higher uptake of FA derived from plasma TG, leading to insulin resistance of whole-body glucose uptake and production. These data indicate that increased LPL activity, resulting from the absence of apoC3-dependent attenuation of the LPL activity, indeed profoundly increases the propensity to develop obesity and insulin resistance on a high-fat diet.

Apoc3$^{-/-}$ and WT littermates were fed a high-fat, high-caloric diet. Within 2 weeks of high-fat feeding, male *apoc3*$^{-/-}$ mice developed a significantly higher body weight. Body composition analysis, after 20 weeks of high-fat feeding, revealed that the increase in body weight in *apoc3*$^{-/-}$ mice, compared with WT littermates was completely explained by the increase in body fat mass. LBM, body protein, and body water were not different between the two mouse groups (**Table 1**). Up to a period of 11 weeks, no difference in daily food intake was observed between *apoc3*$^{-/-}$ and WT mice, although body weight already differed after a relative short period of two weeks. This suggests that the increase in body weight and fat mass is primarily due to the enhanced LPL activity and not due to a higher food intake. As a consequence, feed efficiency, as expressed as total weight gained per week divided by the total amount of food consumed per week, was clearly higher in *apoc3*$^{-/-}$ mice (**Figure 1C**). The unchanged adipocyte cell size found in *apoc3*$^{-/-}$ mice after 20 weeks of a high-fat diet suggests that more adipocytes should be present (**Table 1**). One might in fact expect the opposite, and we can only speculate on the effects of increased FA flux to adipose tissue and adipocyte differentiation. One clue seems to come from studies using peroxisome proliferator-activated receptor (PPAR) γ agonists. Treatment with PPARγ agonists leads to increased adipose tissue FA flux and accumulation, interestingly with increased adipose tissue LPL expression[26]. Consequently, more adipocytes occur with similar or even smaller size[27].

The observation that apoC3-deficiency has no effect on the uptake of albumin-bound FA by the liver, heart, muscle or adipose tissue (**Figure 2**) indicates that the effect of apoC3 on FA uptake in the respective tissues occurs solely via the modulation of LPL activity. We showed that TG-derived FA uptake in the various tissues is dependent on LPL activity in those tissues[18]. In the fasted state, skeletal muscle is relatively enriched in LPL, whereas adipose tissue is relatively rich in LPL in the fed state[2,6,28-31]. Thus, in the fasted state relatively more TG-derived FA is transported to the muscle, whereas in the fed state, transport of TG-derived FA to the adipose tissue is more pronounced. In the current study, we show that apoC3-deficiency does lead to increased uptake of TG-derived FA by adipose tissue. This implicates, therefore, that the effect of apoC3 on LPL-mediated FA uptake is relevant only in the (high-fat) fed condition. Despite that LPL activity is the highest in the heart (**Figure 3**), we did not observe an effect of apoC3-deficiency on TG-derived FFA uptake by this organ (**Figure 2**). These data are in agreement with our previous observations that either reduced expression of LPL, as seen in VLDLreceptor-deficient mice[32], or increased LPL activity by overexpression of the LPL activator apoA5[33] did not modulate the uptake of TG-derived FFA by the heart. Apparently, the cardiac uptake of TG-derived FFA is irrespective of LPL modulation, as related to its continuous need of vast amounts of fuel.

Apolipoproteins can alter LPL activity by interfering with the (physical) interaction between LPL and TG-rich lipoproteins such as VLDL and chylomicrons, without regulating the LPL protein and/or gene expression in tissues. To exclude an effect of apoC3-deficiency on total LPL levels, we analyzed post-heparin plasma and tissue-specific LPL levels in *apoc3*$^{-/-}$ and WT littermates. In this assay, plasma and tissue samples are diluted into a very large pool of solubilized TG and heat-inactivated human plasma, thereby abolishing the interaction of LPL with potential modulators of LPL activity (*e.g.*, apoC3) contained in the samples. Indeed, no differences were observed in total plasma or tissue LPL activity between *apoc3*$^{-/-}$ and littermate controls (**Figure 3**). However, under conditions in which mouse plasma is added to limited amounts of VLDL-like particles in absence of excess human plasma, the TG hydrolase activity of plasma LPL from *apoc3*$^{-/-}$ mice was indeed 78% higher than that of WT mice. Our data thus clearly shows that the absence of apoC3 from lipoprotein particles is responsible for enhancing LPL activity *in vitro* as evident from increased TG-derived FFA liberation and *in vivo* as evident from increased TG-derived FA flux into adipose tissue of *apoc3*$^{-/-}$ mice. Furthermore, the plasma TG half-life was only 6 ± 3 min in *apoc3*$^{-/-}$ mice vs. 22 ± 6 min for littermate controls. This clearly demonstrates increased LPL-mediated TG clearance in *apoc3*$^{-/-}$ mice, which is in agreement with previous reports[12,14]. Therefore, it would be interesting to investigate whether human apoC3-overexpressing mice could be protected from diet-induced obesity.

Although we cannot exclude that altered adipose tissue lipid handling, *e.g.*, hormone sensitive lipase (HSL) activity, leads to a similar adipose tissue phenotype, the

absence of HSL leads to decreased adipose tissue mass instead of an increase, despite increased adipose tissue LPL activity[34].

Using hyperinsulinemic-euglycemic clamp analyses, we observed that the insulin-mediated stimulation of whole-body glucose uptake was strongly impaired in *apoc3*$^{-/-}$ mice as compared with WT littermates (**Figure 4A**), indicating decreased insulin sensitivity in *apoc3*$^{-/-}$ mice. Insulin sensitivity was found to be negatively correlated with muscle TG content in several studies[3-5]. However, we could not observe a statistically significant increase in muscle TG content in *apoc3*$^{-/-}$ mice when compared with WT littermates (**Figure 5A**), in line with the observation that there was no increase in muscle uptake of albumin-derived FA or TG-derived FA in fed mice. Presumably, apoC3-deficiency leads to insulin resistance as a result of increased adipose tissue mass. For instance, apoC3-deficiency might affect secretion of various endocrine factors by adipose tissue, such as leptin, resistin, and adiponectin. These hormones are known to affect insulin sensitivity and are correlated with adipose tissue mass[35-38]. In this study, we found that *apoc3*$^{-/-}$ mice have increased plasma leptin levels (**Table 2**), in accordance with the increase in adipose tissue mass (**Table 1**). However, it is likely that the hyperleptinemia observed in these mice is the consequence rather than the cause of insulin resistance, as has been observed earlier in humans[35,39-42].

Suppression of the endogenous glucose production (largest contribution by liver) by insulin in *apoc3*$^{-/-}$ mice on a high-fat diet was absent compared with WT littermates (**Figure 4B**). In the liver, TG content is inversely correlated with hepatic insulin sensitivity[4]. Indeed, the decreased hepatic insulin sensitivity in *apoc3*$^{-/-}$ mice coincides with an increased hepatic TG content in these mice (**Figure 5A**). The higher TG levels in livers of *apoc3*$^{-/-}$ mice seem to be unrelated to increased hepatic total FA uptake in these mice (**Figure 2**). Also, the hepatic VLDL-TG production was not affected in *apoc3*$^{-/-}$ mice on high-fat diet (**Figure 5B**), which is similar to our previous observations on chow diet[14]. Alterations in intrahepatic FA metabolism and/or other factors, *e.g.*, adipokines, might in fact be fundamental to the observed mild hepatic steatosis in these high-fat-fed *apoc3*$^{-/-}$ mice.

In conclusion, enhanced LPL activity in mice as a result of absence of the natural inhibitor of LPL, apoC3, leads to enhanced susceptibility to diet-induced obesity. This enhanced obesity seems to be fully explained by the increased TG-derived FA flux towards adipose tissue rather than by differences in food intake. The increased obesity and probably the altered FA partitioning result in severe development of peripheral and hepatic insulin resistance in *apoc3*$^{-/-}$ mice. Absence of hepatic production of apoC3 decreases plasma TG levels by enhancing adipose tissue TG-derived FA uptake. Since TG-rich lipoproteins are atherogenic, the decrease in plasma TG levels might be beneficial in the prevention of cardiovascular diseases. Adversely, the higher *in vivo* LPL activity in turn leads to higher susceptibility to diet-induced obesity and insulin resistance. Intriguingly, our results show that the liver can modulate plasma lipid levels and at the same time fat mass and insulin sensitivity through production of just one protein, apoC3. It warrants

further investigation, whether in humans, apoC3 is a potential therapeutic target for treatment of obesity and insulin resistance, and at the same time for prevention of cardiovascular risk.

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Chapter 3

Acute inhibition of hepatic β-oxidation in APOE*3Leiden mice does not affect hepatic VLDL secretion or insulin sensitivity

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Abstract

Hepatic very low density lipoprotein (VLDL) and glucose production are enhanced in type 2 diabetes and are associated with hepatic steatosis. Whether the derangements in hepatic metabolism are attributable to steatosis or to the increased availability of fatty acid (FA) metabolites is unknown. We used methyl palmoxirate (MP), an inhibitor of carnitine palmitoyl transferase I (CPTI), to acutely inhibit hepatic FA oxidation, and investigated whether the FA were rerouted into VLDL secretion and whether this would affect hepatic glucose production. After an overnight fast, male APOE3*Leiden transgenic mice received an oral dose of 10 mg/kg MP. Administration of MP led to an 83% reduction in plasma β-hydroxybutyrate (keton body) levels compared with vehicle-treated mice (0.47 ± 0.07 vs. 2.81 ± 0.16 mmol/l, respectively, $P < 0.01$), indicative of impaired ketogenesis. Plasma free FA levels were increased by 32% and cholesterol and insulin levels were decreased by 17% and 50%, respectively, in MP-treated mice compared with controls. MP treatment led to a 30% increase in liver triglyceride (TG) content. Surprisingly, no effect on hepatic VLDL-TG production was observed between the groups at 8 h after MP administration. In addition, the capacity of insulin to suppress endogenous glucose production was unaffected in MP-treated mice compared with controls. In conclusion, acute inhibition of FA oxidation increases hepatic lipid content but does not stimulate hepatic VLDL secretion or reduce insulin sensitivity.

Introduction

Hepatic very low density lipoprotein (VLDL) - triglyceride (TG) secretion is an important determinant of plasma lipid levels. The rate of VLDL secretion is generally believed to be substrate-driven (*i.e.*, the hepatic content of TG and fatty acids (FA) determines VLDL assembly and the rate of VLDL secretion)[1-3]. Indeed, addition of FA to hepatocytes *in vitro* leads to increased hepatic TG and eventually to enhanced VLDL secretion[4]. However, the hypothesis that VLDL secretion is substrate-driven *in vivo* has not been proven.

Hepatic TG content is the result of uptake and synthesis on the one hand and β-oxidation and VLDL-TG secretion on the other. The importance of β-oxidation is illustrated by the severe phenotype of humans and mice with impaired β-oxidation. Deficiency in hepatic β-oxidation enzymes results in severe plasma hypoketosis and fatty liver[5,6].

In β-oxidation the rate-limiting enzyme is carnitine palmitoyl transferase I (CPTI), which couples long-chain FA to carnitine for transportation into the mitochondria[7,8]. Methyl palmoxirate (MP) is a specific and irreversible inhibitor of CPTI and thereby inhibits β-oxidation[9,10].

In this study, we tested the effect of acute inhibition of β-oxidation *in vivo* by MP on hepatic VLDL production in fasted hyperlipidemic APOE3*Leiden transgenic mice. APOE3*Leiden mice are characterized by a human-like lipoprotein profile[11,12]. We and

others have shown that these mice provide a suitable model to study hepatic VLDL metabolism, since these mice have decreased VLDL-TG production and fatty liver comparable to human conditions[12-14].

We observed that acute inhibition of FA β-oxidation in the liver leads to increased hepatic TG content but neither increases hepatic VLDL secretion nor induces hepatic insulin resistance.

Materials and methods

Animals and diet

Male 3 to 4 month old APOE3*Leiden transgenic mice were fed a Western-type diet containing 15% cacao butter and 0.25% cholesterol (Hope Farms, Woerden, The Netherlands) for 8 weeks before the experiments. The animals were allowed free access to food and water and were kept on a normal diurnal rhythm under standard conditions. After an overnight fast, the animals were matched for body weight and received a dose of 10 mg/kg MP (McN-3716: methyl 2-tetradecyloxiranecarboxylate; kindly provided by Dr. Hegardt, Barcelona, Spain) by gavage in 0.05% methyl cellulose solution or methyl cellulose solution alone (vehicle) as described earlier[9]. Blood samples were taken by tail-tip bleeding every 2 h after MP or vehicle administration. After 8 h, the animals were sacrificed and liver samples were taken and snap-frozen in liquid nitrogen for lipid content and mRNA expression analysis. Parallel groups of mice were used either to study VLDL-TG production or to perform hyperinsulinemic clamp analyses (see below). The animal care committee of TNO Quality of Life (Leiden, The Netherlands) approved all experiments.

Plasma parameters

Blood samples were taken from the tail vein into chilled paraoxon-coated capillaries to prevent *ex vivo* lipolysis[15]. Plasma was collected via centrifugation and plasma cholesterol, TG (without free glycerol), glucose, β-hydroxybutyrate (β-HB) and FFA levels were determined using standard commercial kits, according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany; Triglyceride GPO-Trinder, glucose Trinder 500 and β-hydroxybutyrate, Sigma Diagnostics, St. Louis, MO, USA; NEFA-C, Wako chemicals, Neuss, Germany, respectively). Plasma insulin was measured by a radioimmunoassay using rat insulin standards, which shows 100% cross-reaction with mouse insulin (sensitive rat insulin RIA kit, Linco research, St. Charles, MO, USA).

Liver lipid levels

Liver samples taken from mice 8 h after administration of MP or vehicle were homogenized in phosphate buffered saline (PBS) (10% wet wt/vol), and samples were taken to measure protein content by the Lowry assay[16]. Lipid content was determined by extraction of lipids using the Bligh and Dyer method[17], followed by lipid separation using high performance thin layer chromatography (HPTLC) on silica gel plates as described

previously[18] and subsequent analysis by TINA2.09 software[19] (Raytest Isotopen Meβgeräte, Straubenhardt, Germany)[20].

Hepatic VLDL-TG Production

Hepatic VLDL-TG production, *de novo* apolipoprotein (apo) B secretion, and VLDL composition were determined in overnight-fasted APOE3*Leiden mice given a gavage of MP (10 mg/kg) or vehicle. After 6 h of MP or vehicle administration, the animals were anesthetized with 0.5 ml/kg Hypnorm (Janssen Pharmaceutica, Beerse, Belgium) and 12.5 mg/g Midazolam (Roche, Mijdrecht, The Netherlands) and were injected intravenously with 0.1 ml PBS containing 100 µCi Tran^{35}S-label™ (ICN Biomedicals, Irvine, CA, USA) to measure *de novo* total apoB synthesis. After 30 min, the animals received a 15% (by volume) Triton WR1339 injection (500 mg/kg body weight; Tyloxapol, Sigma Chemicals, Steinheim, Germany) to prevent systemic lipolysis of newly secreted hepatic VLDL-TG[21]. Blood samples were drawn at 0, 15, 30, 60, and 90 min after Triton WR1339 injection, and TG concentrations were determined in the plasma as described above. At 90 min, the animals were sacrificed and blood was collected by cardiac puncture for isolation of VLDL.

VLDL composition analyses

VLDL particles (d < 1.019) were separated from other lipoproteins in plasma by density gradient ultracentrifugation as described[22]. Protein content of the VLDL fraction was determined by the Lowry assay[16]. TG and total cholesterol were measured as in plasma (see above). Phospholipids and free cholesterol were determined using standard commercial kits according to the manufacturer's instructions (Wako Chemicals). ^{35}S-labelled total apoB content was measured in the VLDL fraction after precipitation with isopropanol as described previously[23,24].

Hyperinsulinemic clamp analysis

Insulin sensitivity was determined in a group of APOE3*Leiden mice, fed a Western-type diet, which were fasted overnight, body weight-matched and given either MP or vehicle. Basal and insulin-mediated suppression of endogenous (hepatic) glucose production was studied by hyperinsulinemic euglycemic clamp analysis using [^{3}H]D-glucose as a tracer. The clamp analysis and calculations were performed as described previously[25,26].

Hepatic mRNA expression

Livers were immediately removed from the mice and snap-frozen in liquid nitrogen. Total RNA was isolated as described by Chomczynski and Sacchi[27] by use of RNA-Bee™ (Campro Scientific, Berlin, Germany). cDNA synthesis was done according to Bloks et al.[28]. Real-time quantitative PCR[29] was performed using an Applied Biosystems 7700 Sequence Detector according to the manufacturer's instructions. Primers were obtained from Invitrogen (Paisley, UK), and fluorogenic probes, labeled with 6-carboxyfluorescein (6-FAM), and 6-carboxytetramethylrhodamine (6-TAMRA), were supplied by Eurogentec

(Seraing, Belgium). Primers and probes used in this experiment were described previously[30-32]. All expression data were subsequently standardized for cyclophylin RNA, which was analyzed in a separate run.

Statistical analysis

The Mann-Whitney U test was used to determine differences between MP- and vehicle-treated mice. The level of significance was set at $P < 0.05$. All data presented as mean ± SD. Analyses were performed using SPSS11.0 (SPSS, Chicago, IL, USA).

Results

MP decreases β-HB and increases FFA levels in plasma

Male APOE3*Leiden mice, fed a Western-type diet for 8 weeks, were fasted overnight and treated with 10 mg/kg MP or vehicle. Plasma β-HB (one of the keton bodies produced by the liver) was decreased by 83% 8 h after MP administration, confirming inhibition of the β-oxidation. A strong decrease in plasma β-HB was already apparent after 2 h, and levels remained constant up to at least 8 h, whereas the solvent had no effect (**Figure 1**). **Table 1** summarizes plasma parameters measured 8 h after MP or vehicle administration. Plasma FFA levels were increased by 32% upon MP treatment. Glucose and TG did not show significant differences, whereas plasma insulin and cholesterol levels were both decreased significantly, by 50% and 17%, respectively, in the MP-treated group versus controls.

Inhibition of β-oxidation increases lipid storage in liver without affecting VLDL production

Liver lipid analysis showed that MP-treated mice had 30% increased TG contents in liver compared with vehicle-treated animals (**Figure 2A**). To analyze whether the increased hepatic TG content was associated with altered hepatic VLDL-TG production, mice were injected with Triton WR1339 at 6 h after MP or vehicle gavage, and the accumulation of endogenous VLDL-TG in plasma was determined over time. Plasma β-HB levels in this subset of mice were decreased similarly, as shown in **Figure 1**. As evident from **Figure 2B**, the VLDL-TG production rate, as determined from the slope of the curve, was unchanged in MP-treated animals compared with controls (2.12 ± 0.58 vs. 2.51 ± 0.53 mmol/l TG/h, respectively). Furthermore, the composition of the VLDL particles (**Table 2**) as well as the rate of *de novo* total apoB production of newly synthesized VLDL particles did not differ between MP- and vehicle- treated mice (35 ± 3 vs. 36 ± 4 x 10^4 dpm/ml plasma/mg protein, respectively).

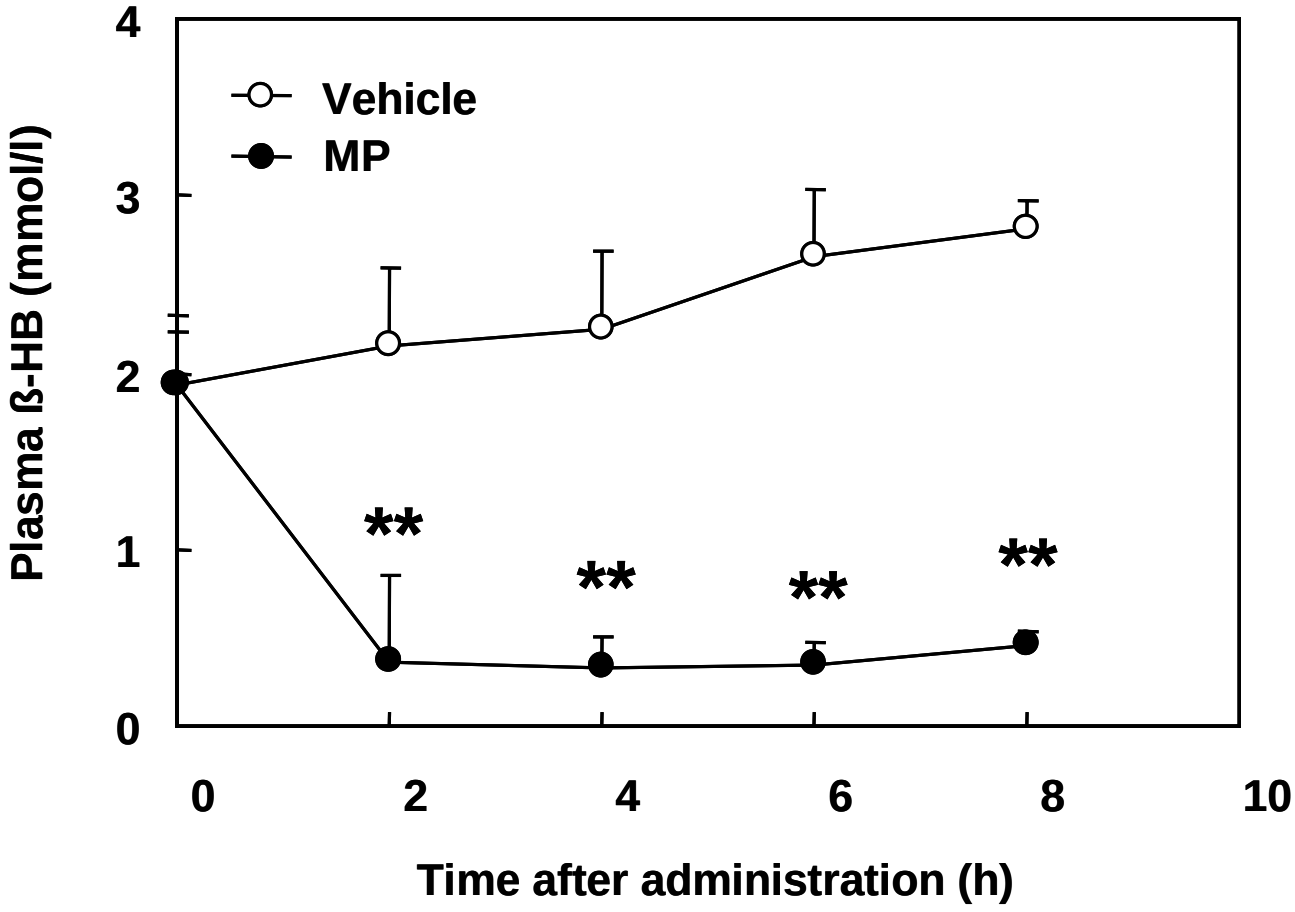

Figure 1. Time course effect of MP on β-HB levels
Plasma samples from overnight-fasted APOE*3Leiden mice, treated with MP (closed circles) or vehicle (open circles), were taken every 2 h to determine plasma β-HB levels. Values represent mean ± SD of 5 mice per group, **P < 0.01. MP methyl palmoxirate, β-HB β-hydroxybutyrate

Table 1. Plasma parameters determined in overnight-fasted APOE*3Leiden mice at 8 h after MP administration

	Vehicle	MP
β-Hydroxybutyrate (mmol/l)	2.81 ± 0.16	0.47 ± 0.07**
Triglycerides (mmol/l)	0.77 ± 0.19	0.85 ± 0.06
Total cholesterol (mmol/l)	5.00 ± 0.61	4.14 ± 0.82*
Free fatty acids (mmol/l)	1.17 ± 0.20	1.55 ± 0.26*
Glucose (mmol/l)	4.71 ± 1.14	4.13 ± 0.71
Insulin (pmol/l)	103.0 ± 37.9	50.7 ± 11.1*

Overnight-fasted APOE*3Leiden mice were administered 10 mg/kg body weight methyl palmoxirate (MP), 8 h later plasma was obtained from the mice via tail-tip incision. β-hydroxybutyrate, triglycerides, total cholesterol, free fatty acids, glucose, and insulin levels were determined in plasma. Values represent mean ± SD of 5 mice per group. *P < 0.05, **P < 0.01

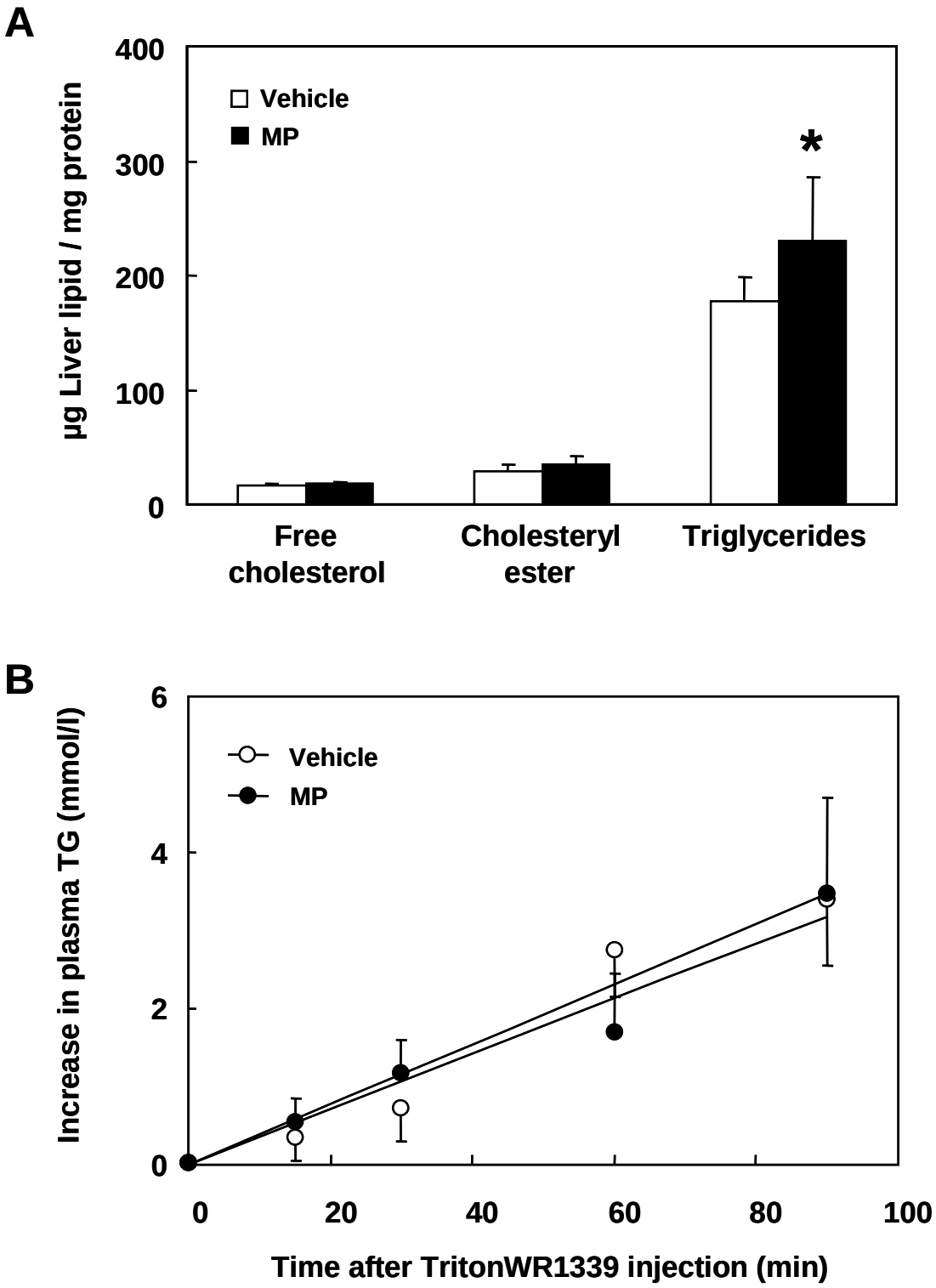

Figure 2. Effect of MP on hepatic lipid levels (A) and VLDL production (B)
Liver samples were taken 8 h after MP or vehicle treatment. Lipids were extracted from liver homogenates and subsequently separated on thin-layer silica gel plates as described. Liver free cholesterol, cholesteryl ester, and TG levels are depicted for MP-treated (closed bars) and vehicle-treated (open bars) mice (A). The increase in plasma TG levels in MP-treated (closed circles) and vehicle-treated (open circles) mice was measured in time after Triton WR1339 injection. (B). Values represent mean ± SD for 5 mice per group, *P < 0.05

Table 2. Composition of VLDL obtained from Triton WR1339-injected mice at 8 h after treatment with MP or vehicle

Parameter	Vehicle	MP
Triglycerides (% of total)	72.8 ± 2.1	69.0 ± 3.7
Free cholesterol (% of total)	2.4 ± 0.2	2.6 ± 0.3
Cholesteryl esters (% of total)	12.2 ± 2.3	14.1 ± 3.3
Phospholipids (% of total)	9.0 ± 1.3	10.3 ± 1.2
Protein (% of total)	3.7 ± 0.7	4.1 ± 0.8

VLDL was isolated using ultracentrifugation and triglycerides, free cholesterol, cholesteryl esters, phospholipids and protein contents were determined and expressed as percentages of total. Values represent mean ± SD for 5 mice per group

Acute inhibition of β-oxidation does not lead to hepatic insulin resistance.

Increased hepatic TG content is negatively associated with insulin sensitivity[33]. Since plasma insulin levels decreased in the MP-treated mice, we analyzed the insulin-mediated suppression of endogenous (hepatic) glucose production during a hyperinsulinemic clamp procedure. Although hepatic TG content was increased in the MP-treated mice, endogenous (hepatic) glucose production was equally suppressed by insulin in both groups. The glucose production decreased from 32.5 ± 6.5 to 19.1 ± 10.1 µmol/min/kg body weight (-40%) in MP-treated mice and from 28.7 ± 7.1 to 14.7 ± 8.8 µmol/min/kg body weight (-46%) in vehicle-treated mice. These results show that the livers of MP-treated mice displayed normal hepatic insulin sensitivity.

Hepatic mRNA levels

Hepatic expression of several genes was studied in mice at 8 h after treatment with MP or vehicle using RT-PCR. The expression of genes involved in β-oxidation (peroxisome proliferator-activated receptor α [*pparα*], *cpt1a*, medium-chain acyl-CoA [*mcad*], HMG-CoA synthase [*hmgs*]) were all 40-56% higher in MP-treated livers compared with controls, albeit that statistical significance was achieved for only *pparα* and *cpt1a* (**Table 3**). Regarding genes involved in VLDL production, microsomal TG transfer protein (*mttp*) expression was increased in MP-treated mice, whereas no differences were observed in acyl:diacylglycerol transferase 1 (*dgat1*), *dgat2*, *apob*, and *apoe* expression. Expression of the sterol regulatory element-binding protein 1c (*srebp1c*), which is involved in the activation of genes involved in the uptake and synthesis of FA, TG and cholesterol, was strongly decreased in MP-treated mice. In FA synthesis, gene expression levels of fatty acid synthase (*fas*) and acetyl-CoA carboxylase 1 (*acc1*) were not changed between the two groups. Also no changes were observed in gene expression levels of pyruvate kinase (*pk*) and phosphoenolpyruvate carboxykinase (*pepck*), which are involved in hepatic glucose metabolism.

Table 3. Hepatic mRNA expression levels in control and MP-treated mice as determined by RT-PCR and related to cyclophilin

mRNA source	Vehicle	MP	Change
β-oxidation			
pparα	100 ± 28 %	149 ± 17 %*	↑
cpt1a	100 ± 24 %	143 ± 18 %*	↑
mcad	100 ± 32 %	140 ± 9 %	NS
hmgs	100 ± 37 %	156 ± 18 %	NS
TG synthesis/VLDL production			
mttp	100 ± 24 %	159 ± 26 %*	↑
dgat1	100 ± 33 %	147 ± 11 %	NS
dgat2	100 ± 29 %	119 ± 15 %	NS
apob	100 ± 42 %	95 ± 11 %	NS
apoe	100 ± 21 %	104 ± 18 %	NS
Fatty acid synthesis			
srebp1c	100 ± 48 %	27 ± 13 %*	↓
fas	100 ± 49 %	79 ± 27 %	NS
acc1	100 ± 35 %	103 ± 18 %	NS
Glucose production			
pk	100 ± 42 %	84 ± 16 %	NS
pepck	100 ± 29 %	111 ± 26 %	NS

Values represent mean ± SD of 4 MP treated and 5 vehicle-treated mice per group, *P < 0.05, NS not significant. acc1 acetyl-coenzyme A carboxylase 1, apo apolipoprotein, dgat acyl:diacylglycerol transferase, cpt1a carnitine palmitoyl transferase 1a, fas fatty acid synthase, hmgs 3-hydroxy-3-methylglutaryl-coenzyme A synthase, mcad medium-chain acyl-coenzyme A, mttp microsomal triglyceride transfer protein, NS not significant, pepck phosphoenolpyruvate carboxykinase, pk pyruvate kinase, pparα paroxisome proliferators-activated receptor α, srebp1c sterol regulatory element binding protein 1c

Discussion

Hepatic FA and TG metabolism encompasses a number of interacting and dynamic processes, including uptake of plasma FFA and TG from lipoprotein remnants, storage in the form of TG, β-oxidation, and VLDL-TG formation/secretion. The rate of VLDL-TG secretion has long been thought to be substrate-driven[1-3]. We questioned whether an acute inhibition of hepatic β-oxidation would lead to a redirection of FA toward hepatic TG synthesis specifically directed to VLDL secretion. MP was used to irreversibly inhibit the crucial enzyme in β-oxidation, CPT1A[9,10]. Indeed, within 2 h after oral dosing of MP, plasma keton bodies (*i.e.*, β-HB) decreased and remained low for up to 8 h after gavage (**Figure 1**). Since plasma keton bodies are derived solely from hepatic β-oxidation, we concluded that hepatic β-oxidation of long-chain FA was almost completely inhibited by the applied dose of MP. It is known that, at low doses, MP inhibits mostly CPT1A (present in liver and heart), whereas higher doses also inhibit CPT1B (present in skeletal muscle and heart) in rat[7,9].

Several studies have demonstrated that oxirane carboxylates (such as etomoxir and MP) are effective at decreasing both keton body and glucose levels in rodents, dogs and humans[9,34-37]. However, in our overnight-fasted mice, plasma glucose levels were similar between MP-treated and control mice, suggesting that muscle β-oxidation was not

completely inhibited, in contrast to liver β-oxidation, as judged from the extremely low β-HB levels. The residual level of β-HB in MP-treated animals may be derived from short-chain FA oxidation that does not depend on CPT1A for transport into mitochondia.

We observed that the hepatic expression of genes involved in β-oxidation (*i.e., pparα, cpt1a, mcad, and hmgs)* was higher in MP-treated mice (**Table 3**), albeit statistically significant differences were only observed for *pparα* and *cpt1a*, as a result of the moderate sample sizes in combination with relatively large standard deviations in the control group. This increase of genes involved in β-oxidation may be an attempt of the liver to compensate for the strongly decreased hepatic β-oxidation.

Plasma FFA levels increased significantly at 8 h after MP treatment (**Table 2**). This increase was most likely caused by the decreased hepatic FA oxidation. Interestingly, we observed that acute inhibition of β-oxidation and/or the decrease of plasma keton bodies was associated with strongly decreased plasma insulin levels (**Table 2**). In agreement with this, Boden and Chen[38] showed that in humans there is a positive correlation between plasma β-HB concentrations and insulin secretion capacity.

As expected, inhibition of hepatic β-oxidation led to a significant accumulation of TG within the liver (**Figure 2A**). Hepatic TG accumulation is in agreement with the symptoms seen in patients and animal models with β-oxidation disorders[5,6]. Microscopic analysis of liver slices revealed a mixed micro/macrovesicular accumulation of lipid droplets, also called micro/macrovesicular steatosis (data not shown). This observation of microvesicular steatosis seems to be in agreement with the recent finding with cyclopropane carboxylic acid (CPCA), another β-oxidation inhibitor, in rats[39]. Although one might expect to observe increased liver mRNA levels of TG-synthesizing enzymes such as DGAT1, this was not observed in our experimental setting.

Interestingly, the increased TG accumulation in the liver was not associated with increased hepatic VLDL-TG production and/or changes in VLDL-composition (**Figure 2B, Table 2**). We did observe an increase in gene mRNA expression of *mttp* in the livers of MP-treated mice. We did not analyze microsomal transfer protein (MTP) activity, so we can only conclude that this increased *mttp* expression did not (yet) lead to increased MTP protein levels or activity. We cannot exclude the possibility that chronic, long-term inhibition of hepatic β-oxidation might induce hepatic VLDL-TG production.

Hepatic *srebp1c* expression was strongly decreased in MP-treated mice. Since insulin increases hepatic *srebp1c* expression[40], it is possible that the low plasma insulin levels caused this downregulation of liver *srebp1c* expression. However, this low *srebp1c* expression did not seem to affect RNA expression levels of enzymes involved in *de novo* lipogenesis, such as *fas* or *acc1*, at least at the examined time point.

Because TG accumulation is known to be associated with insulin resistance in the liver[33], we performed hyperinsulinemic clamp procedures to assess the insulin-mediated suppression of endogenous glucose production. Under normal conditions, the major part

of endogenous glucose production is derived from liver[38]. Our results clearly show that there was no significant difference in insulin-mediated suppression of endogenous (hepatic) glucose production between treated and untreated mice. Gene expression of enzymes involved in hepatic glucose production also did not show any differences between the two groups. Whole-body insulin-mediated glucose uptake was not significantly different between MP-treated and control mice (data not shown). This confirms, in part, that muscle FA β-oxidation must be still functional at the applied low dose of MP. It has been shown by Dobbins et al.[41] that chronic suppression of hepatic β-oxidation leads to insulin resistance in both liver and muscle in rats. We clearly show that acute inhibition of FA β-oxidation affects neither hepatic nor muscle insulin sensitivity *in vivo* in mice.

We conclude that acute inhibition of hepatic FA β-oxidation leads to hepatic micro/macrovesicular steatosis, but does not affect either hepatic VLDL secretion or hepatic insulin sensitivity.

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Chapter 4

Response of apolipoprotein E*3-Leiden transgenic mice to dietary fatty acids: combining liver proteomics with physio-logical data

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Abstract

Dietary fatty acids have a profound impact on atherosclerosis, but mechanisms are not well understood. We studied the effects of a saturated fat diet supplemented with fish oil, *trans*10,*cis*12 CLA (CLA), or elaidic acid on lipid and glucose metabolism and liver protein levels of APOE*3Leiden transgenic mice, a model for lipid metabolism and atherosclerosis. Fish oil lowered plasma and liver cholesterol and triglycerides, plasma free fatty acids, and glucose, but increased plasma insulin. CLA lowered plasma cholesterol but increased plasma and liver triglycerides, β-hydroxybutyrate, and insulin. Elaidic acid lowered plasma and liver cholesterol. Proteomics identified significant regulation of 65 cytosolic and 8 membrane proteins. Many of these proteins were related to lipid and glucose metabolism, and to oxidative stress. Principle component analysis revealed that fish oil had a major impact on cytosolic proteins, and elaidic acid on membrane proteins. Correlation analysis between physiological and protein data revealed novel clusters of correlated variables, among which a metabolic syndrome cluster. The combination of proteomics and physiology gave new insights in mechanisms by which these dietary fatty acids regulate lipid metabolism and related pathways, for example, by altering protein levels of long-chain acyl-CoA thioester hydrolase and adipophilin in the liver.

Introduction

Coronary heart disease (CHD) is one of the major causes of mortality in industrialized countries, with diet believed to play a major role in disease development. Several dietary fatty acids (FA) may contribute to, or decrease the risk of CHD, primarily because of their detrimental, or beneficial, effects on the lipoprotein profile[1]. Numerous controlled feeding studies in humans have established that saturated FA increase and polyunsaturated FA decrease total and low density lipoprotein (LDL) cholesterol[2]. *Trans-FA* have been shown to raise LDL cholesterol and lower high density lipoprotein (HDL) cholesterol relative to *cis*-unsaturated FA[3,4] and to increase triglycerides (TG)[5]. Conjugated linoleic acids (CLA), which structurally may be classed as *trans-FA*, protect against the development of atherosclerosis in rabbits, hamsters, and transgenic mice[6-8]. Nevertheless, *in vivo* data on possible hypolipidemic effects of CLA are conflicting[9].

The mechanisms by which the different dietary FA affect lipid metabolism and the development of CHD are often not completely understood, although some studies are available. Omega-3 FA (present in fish and fish oil) decrease TG levels by inhibition of FA synthesis in the liver and up-regulation of oxidation in liver and skeletal muscle[10]. The effects of CLA on lipid metabolism appear to be produced largely by the *t*10,*c*12 isomer of CLA. This isomer significantly reduced apolipoprotein (apo) B secretion from HepG2 cells[11] as well as hepatic stearoyl-CoA desaturase expression[12] and activity[13]. Although these results suggest a TG-lowering effect of *trans*10,*cis*12 (*t*10,*c*12) CLA mediated by

falls in VLDL-TG secretion and FA synthesis, respectively, *in vivo* data on the hypolipidemic effects of CLA are inconsistent[9]. *Trans*-FA adversely affect essential FA metabolism and prostaglandin balance by inhibiting the enzyme delta-6 desaturase[14] and they may promote insulin resistance[15].

The differences in study design and the conflicting results from different animal models make it difficult to assess the physiological, biochemical and molecular mechanisms by which these dietary FA exert their effect. Thus, studies are needed in which the effects of different dietary FA are studied in a single model that is sensitive to relatively mild perturbations in the diet. The APOE*3Leiden mouse model responds well to modulators of lipoprotein metabolism and atherosclerosis, such as cafestol and plant stanols[16-18]. In addition, APOE*3Leiden mice have proven to be responsive to fish oil[19]. APOE*3Leiden mice express the human APOE*3Leiden gene, resulting in a lipoprotein profile similar to that of humans. Hence, these mice easily develop diet-induced hyperlipidemia and atherosclerosis[20,21] and are therefore suitable for a comparative study on the effects of dietary FA.

In this study, we have compared the impact of dietary fish oil, *t*10,*c*12 CLA, and elaidic acid on lipoprotein metabolism and insulin levels. Both CLA and fish oil are suggested to act as peroxisome proliferator-activated receptor (PPAR) agonists[10,22], and therefore we also included the TG-lowering drug fenofibrate as a positive control. Fibrates are PPAR agonists known to stimulate cellular FA uptake, conversion of FA to acyl-CoA derivatives, and catabolism of FA via the β-oxidation pathways. Combined with a reduction in FA and TG synthesis, this results in a decrease in very low density lipoprotein (VLDL) production[23], an observation confirmed in the APOE*3Leiden mouse. Effects on FA, lipoprotein, and glucose metabolism were assessed by measuring metabolite concentrations in plasma. Impact on liver physiology was studied by liver lipid analysis and by 2-dimensional (2D) gel electrophoresis on both cytosolic and membrane proteins. Proteomics was used to identify potential pathways through which dietary FA may exert their specific effects on physiology and CHD.

Materials and Methods

This study was approved by the animal care committee of TNO Quality of Life, Leiden, The Netherlands.

Animals and diet

Female APOE*3Leiden transgenic mice, aged 5-6 months, were kept in groups of 4 animals under standard conditions with free access to food and water. Forty animals consumed a semi-synthetic high-fat/cholesterol (HFC) diet (Hope Farms, The Netherlands) containing cocoa butter (15% w/w) and cholesterol (0.25% w/w) during a 3-week run-in period. They were then randomly assigned to 5 treatment groups of 8 animals each that were matched for body weight and total cholesterol. During the intervention period of 3

weeks, one group continued to be given the HFC diet (control group). The second group (fish oil group) received the HFC diet supplemented with 3% (w/w) of a mixture of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), (Marinol^{TM}, Loders Croklaan, The Netherlands). The third group (CLA group) received the HFC diet supplemented with1% (w/w) of the *t*10,*c*12 isomer of CLA (Loders Croklaan), the fourth group (elaidic group) received the HFC diet supplemented with 3% (w/w) of elaidic acid (Loders Croklaan). The fifth group (fenofibrate group) received the HFC diet supplemented with 0.04% (w/w) of fenofibrate (Sigma, Dorset, UK). All supplements were added at the expense of cacao butter, leading to equal fat and energy contents per gram food in the different diets (**Table1**). The percentage of energy provided by fish oil (EPA+DHA), *t*10,*c*12 CLA, and elaidic acid was 2.3%, 1.7%, and 4.1% respectively. Adequate portions of the diets were replaced every other day. Body weight and food intake were monitored throughout the experiment.

Table 1. Fatty acid composition of the experimental diets

Fatty acid (FA)	Control	Fish oil	CLA	Elaidic acid	Fenofibrate
			g/100 g diet		
Saturated FA	10.6	10.3	10.6	9.4	11.1
Monounsaturated FA	5.9	5.7	5.8	6.5	6.1
Elaidic acid	0.0	0.0	0.0	0.9	0.0
Polyunsaturated FA	1.9	2.4	2.2	1.6	1.6
n-6 polyunsaturated FA	1.1	1.0	1.7	1.0	1.1
*t*10,*c*12 CLA	0.0	0.0	0.7	0.0	0.0
n-3 polyunsaturated FA	0.8	1.4	0.5	0.6	0.5
Eicosapentaenoic acid	0.3	0.7	0.2	0.3	0.2
Docosahexaenoic acid	0.0	0.4	0.0	0.0	0.0

The fatty acid composition of the experimental diets was analyzed as described in materials and methods. In addition to the fat, all diets contained 0.25% cholesterol, 40.5% sucrose, 10% corn starch, 5.95% cellulose, 20% casein, 1% choline chloride, 0.2% methionine, and 5.1% mineral mixture. The fenofibrate diet contained 0.04% (w/w) of fenofibrate. All percentages are in weight/weight.
CLA *trans*10,*cis*12 conjugated linoleic acid

Dietary lipid analysis

Total lipids from diet subsamples were extracted using the method of Folch[24] and total lipid was then converted to its methyl esters by direct *trans*-esterification using 1% sulphuric acid in methanol. The proportion of individual FA in the diet was determined with an Agilent 6890 gas chromatograph fitted with a 30 m DB23 capillary column (J&W Scientific, Folsom, CA, USA) with an inner diameter of 0.25 mm and 0.25 µm film thickness. Helium

(25 psi) was used as a carrier gas. The initial oven temperature was programmed at 800°C, then to rise to 1800°C at a rate of 250°C per min, then to rise to 2200°C at a rate of 10°C per min, and then kept constant. The temperature of the injector and the flame-ionization detector was set at 2500°C, while a split ratio of 50:1 was used. A standard mixture was used to identify the FA methyl esters by means of the retention times. Results were expressed as a proportion of total identified FA. Butylated hydroxy-toluene (0.005% w/v, Sigma) was added to all organic solvents to prevent oxidation of the polyunsaturated FA.

Plasma analyses

At week 0 and 3 of the intervention period, mice were fasted for 4 h, after which blood samples were obtained from the tail vain into chilled paraoxon-coated capillaries to prevent lipolysis[25]. Plasma was collected via centrifugation at 13000 rpm for 5 min for the measurement of plasma total cholesterol (Roche Diagnostics, Mannheim, Germany), TG without free glycerol (Triglyceride GPO-Trinder, Sigma Diagnostics, St. Louis, MO, USA), non-esterified fatty acids (Wako chemicals, Neuss, Germany), glucose (Trinder 500, Sigma Diagnostics), and β-hydroxybutyrate (Sigma Diagnostics) by standard commercial kits, according to the manufacturer's instructions. Plasma insulin was measured by radio-immunoassay, using rat insulin standards that have 100% cross-reaction with mouse and human insulin (sensitive rat insulin assay, Linco research, St. Charles, MO, USA).

For size fractionation of lipoproteins, 50 µl of pooled plasma was injected onto a Superose 6 column (3.2 x 300 mm, Äkta purifier, Amersham Pharmacia Biotech, Uppsala, Sweden) and eluted at a constant flow rate of 50 µl/min with phosphate buffered saline (PBS) (pH 7.4, containing 1 mmol/l EDTA). Fractions of 50 µl were collected and assayed for total cholesterol (as described above) and TG (Triglycerides GPO-PAP, Roche Diagnostics).

Liver lipid analyses

After the intervention period of 3 weeks, mice were fasted 4 h and then sacrificed to obtain liver tissue for lipid analyses and proteomics. The liver was perfused with ice-cold PBS, weighed and samples were snap-frozen in liquid nitrogen. Liver lipid content was analyzed by sample homogenization in PBS (+/-10% wet wt/vol). Protein content was determined by a Lowry assay[26], followed by extraction of lipids using the Bligh and Dyer method[27]. The lipids were separated using high-performance thin-layer chromatography (HPTLC) on silica gel plates as described before[28] and subsequent analysis was performed by TINA2.09 software[29] (Raytest Isotopen meβgeräte, Straubenhardt, Germany).

Proteomics

Preparation of cytoplasm protein fraction

A sample of frozen liver (± 125 mg) was added to 500 µl extraction buffer (pH 7.1) containing 50 mM Tris, 100 mM KCl, 20% glycerol, 1.4 µM pepstatin A, 1.0 mM PMSF and the protease inhibitor cocktail (Roche Diagnostics) Complete™ according to the manufacturer's instructions (Boehringer Mannheim). This sample was homogenized with an eppendorf homogenizer on ice for 30 sec, sonicated in ice water for 15 sec and again homogenized on ice for 30 sec. Thereafter, the homogenate was centrifuged (Beckman TL-100) for 30 min at 55000 rpm at 4°C. The resulting supernatant was withdrawn, and the pellet was weighed and re-homogenized in the extraction buffer as outlined above. After the second centrifugation step, the supernatant was added to the first fraction and the protein content of the combined supernatant fractions was measured using the Bradford assay[30].

Preparation of membrane protein fraction

The pellet was weighed and re-homogenized in CHAPS buffer (0.2 M KCl, 0.1 M sodium phosphate, 20% glycerol, 0.12M CHAPS and Complete™) according to the manufacturer's instructions at a ratio of 2 µl buffer per mg of pellet. The sample was homogenized and sonicated as for the preparation of cytoplasm proteins. Urea and DL-dithiothreitol (DTT) were added to obtain final concentrations of 9 M and 70 mM, respectively. When the additions were fully dissolved, the sample was centrifuged at 55000 rpm for 30 min at 20°C. The supernatant was carefully removed, after which 2% ampholytes 3-10 (Servalyt™, Serva Electrophoresis, Heidelberg, Germany) were added prior to 2D electrophoresis. The protein content of the membrane protein fraction was measured using the Bradford assay[30].

2D gel electrophoresis

For the cytoplasm protein fractions, four 2D electrophoresis gels were run per dietary treatment, each gel representing a pool of two randomly selected animals per group. For the membrane protein fraction, two 2D electrophoresis gels were run per treatment, each gel representing a pool of four randomly selected animals per group. BioRad immobilized pH gradient (IPG) strips (pI 5-8 for cytoplasm proteins and pI 3-10 for membrane proteins) were used for the separation of the proteins in the first dimension. Strips were rehydrated in 300 µg of protein sample at 20°C for one hour without applied voltage on a BioRad IEF cell. After 1 h, each lane was overlaid with mineral oil to prevent the strips from drying out. For another 16 h a voltage of 50 V per strip was applied, after which the strips were transferred to a clean tray and overlaid with mineral oil. The initial start up and ramping protocol were performed as recommended by BioRad. After 1 h, the strips were removed to a tray containing fresh wicks and were overlaid with mineral oil. The run proceeded until the

preset volthours had been reached, after which the voltage was held at 500 V total until the strips were ready to be transferred to the second dimension SDS-PAGE step.

SDS-PAGE

IPG strips were removed from the focusing tray and incubated in fresh equilibration buffer (6 M Urea; 2% SDS; 0.375 M Tris-HCl, pH 8.8: 20% glycerol; and 130 mM DTT) for 10-15 minutes at room temperature before transfer to a second equilibration buffer (6 M Urea; 2% SDS; 0.375 M Tris-HCl, pH 8.8; 20% glycerol; and 135 mM iodoacetamide) for 10-15 minutes at room temperature. The strip was then rinsed in tank buffer (24 mM Tris; 0.2 M glycine; and 0.1% SDS, pH 8.6) and applied to the top of an 18x18 cm gel cassette. The strip was fixed in position by overlaying with molten agarose (2% agarose in tank buffer with 2 mg/100 ml bromophenol blue). A 7.5 µl unstained BioRad precision standard was inserted in the well formed on the right of the cassette. Gels were run at 200 V for 9.5 h or until the bromophenol blue had reached the bottom of the gel. After the second dimension run, the gels were placed into a fixation solution of 50% ethanol, 2% ortho-phosphoric acid and 48% H_2O for a minimum of three hours. Gels were then washed for at least one hour with a couple of changes of H_2O after which they were shaken in a staining solution of 34% methanol, 2% ortho-phosphoric acid and 64% H_2O containing 17% $(NH_4)_2\ SO_4$ and 1 mg/ml Coomassie blue sprinkled on top of the staining solution.

2D electrophoresis gel comparisons

2D electrophoresis gels were analyzed using PDQuest software (BioRad). Spots with densities that significantly differed between treatments were excised from the SDS-PAGE gels using the BioRad spot cutter. Gel plugs were directly placed into a 96-well V-bottomed plate with 100 µl of water that was removed immediately before the trypsination process. The proteins were trypsinized using the MassPrep Station (Waters, Micromass, Manchester, UK) protocol, which includes sequentially: destain steps for Coomassie Blue removal, reduction of the protein with DTT, alkylation of the protein with iodoacetamide, removal of DTT and iodoacetamide, dehydration of the gel plug, incubation with trypsin, and extraction of the peptides. Of the extracted peptides 1 µl was applied to the target area of a 96 x 2 teflon MALDI target plate (Applied Biosystems, Warrington, UK) and allowed to dry to ~ 50% of the original volume. At this point, 0.5 µl of an α-cyano-4-hydroxycinnamic acid matrix solution (5 mg/ml in 70% acetonitrile/H_2O, 0.1% TFA) was applied to the target. The samples were dried in a stream of air before matrix-assisted laser desorption/ionisation (MALDI) mass spectrometric analysis.

MALDI-TOF mass spectrometric analysis

MALDI – time of flight (TOF) mass spectrometry was performed using an Applied Biosystems Voyager-DE PRO in reflectron mode. Each spectrum was obtained using 500 shots of the appropriate laser power and, where appropriate, spectra were accumulated and filed. A macro was applied, which allowed baseline correction and de-isotoping of the peptide mass peaks. A peptide mass list of the most intense peaks was generated automati-

cally, and the list was pasted into Matrix Science Mascot by using the MSDB database during the search. We set the following search criteria: allowance of 0 or 1 missed cleavage, trypsin as digestion enzyme, carbamidomethyl modification of cystein, methione oxidation as partial modification, and charge state as MH^+.

Statistical analysis

The Mann-Whitney U test was used to determine differences in responses during the intervention period between the control group and the other treatment groups. Thus, the effects of dietary fish oil, *t*10,*c*12 CLA, elaidic acid, and fenofibrate were compared with the effects of saturated fat. All data are presented as mean ± SD. Analyses were performed using SPSS11.0 (SPSS, Chicago, IL, USA). Principle component analysis was performed using PLS toolbox (Version 3.0, Eigenvector Research, Manson, WA, USA) working under Matlab (Version 6.5, The MathWorks, Natick, MA, USA). Analysis of correlations was done with Pearson correlation coefficients. Proteins with very low expression, specifically occurring in the fish-oil treatment, were removed from the set, as they led to many false-positive correlations between those proteins. The analysis of multiple hypotheses testing for many combinations of variables was done with the QVALUE tool[31], running under the statistical software package R (http://www.r-project.org/). The resulting *q*-values estimate the probability that a correlation that is called significant, is false positive. For example, a *q*-value of 0.05 would mean, that we should expect that 5 out of 100 associations that were tested significant, are in fact false positive.

Results

Physiological studies

Food intake and body weight

Both food intake and body weight remained constant in all treatment groups throughout the intervention period (data not shown).

Plasma lipids, glucose and insulin

After the 3-week intervention period, all diets significantly lowered plasma total cholesterol levels compared with the control diet (**Table 2**). Fish oil reduced plasma total cholesterol by 55%, *t*10,*c*12 CLA by 50%, elaidic acid by 25%, and fenofibrate by 55%, compared with the control group (all $P < 0.05$). Analysis of the lipoprotein profiles revealed that this decrease in cholesterol could be explained by a decrease in VLDL and IDL cholesterol levels compared with the control group. *t*10,*c*12 CLA appeared to cause a slight increase in LDL cholesterol compared with the control group (**Figure 1**).

Plasma levels of TG were significantly decreased by 67% in the fish oil group and by 82% in the fenofibrate group, as compared with the control group (both $P < 0.05$). Plasma levels of TG were significantly increased by 64% in the CLA group compared with the control group ($P < 0.05$). Plasma levels of TG in the elaidic acid group did not differ

significantly from the placebo group (**Table 2**). Changes in levels of total TG were due to either an increase or a decrease in the TG levels of predominantly VLDL and IDL lipoprotein particles (**Figure 1**).

Fish oil and fenofibrate treatment significantly decreased the amount of free FA in plasma to almost half the concentrations in the control group (both $P < 0.05$). *t*10,*c*12 CLA and elaidic acid did not affect plasma levels of free FA (**Table 2**). Plasma β-hydroxybutyrate, a keton body that is often used as an indicator of hepatic β-oxidation, was significantly increased by *t*10,*c*12 CLA, elaidic acid, and fenofibrate treatment as compared with the control group (**Table 2**; all $P < 0.05$). The fish oil group showed a trend toward a higher plasma level of β-hydroxybutyrate although this increase was not significant.

Plasma glucose was significantly lower in the fish oil group and in the fenofibrate group (both $P< 0.05$). Plasma insulin levels were increased 2.8 times in *t*10,*c*12 CLA-fed animals and also slightly increased (0.25 times) in fish oil-fed animals (**Table 2**; both $P < 0.05$).

Table 2. Plasma lipid, glucose, and insulin levels of APOE*3Leiden mice fed a high-fat/high-cholesterol diet (control), or this diet supplemented with fish oil, *trans*10,*cis*12 CLA, elaidic acid, or fenofibrate for 3 weeks

	Control	Fish oil	CLA	Elaidic acid	Fenofibrate
Total cholesterol (mmol/l)	13.74 ± 2.85	6.18 ± 0.53*	6.81 ± 0.85*	10.31 ± 1.93*	6.25 ± 0.70*
Triglycerides (mmol/l)	1.09 ± 0.33	0.36 ± 0.10*	1.79 ± 0.36*	1.32 ± 0.87	0.20 ± 0.05*
Free fatty acids (mmol/l)	1.31 ± 0.24	0.76 ± 0.12*	1.37 ± 0.23	1.06 ± 0.13	0.72 ± 0.06*
β-hydroxybutyrate (mmol/l)	0.36 ± 0.12	0.46 ± 0.21	0.75 ± 0.35*	0.56 ± 0.10*	0.75 ± 0.17*
Glucose (mmol/l)	11.41 ± 1.30	8.84 ± 1.00*	12.65 ± 1.57	10.9 ± 1.89	9.33 ± 1.94*
Insulin (pmol/l)	514 ± 41	639 ± 81*	1430 ± 653*	535 ± 82	582 ± 99

Blood samples were taken via tail-tip incision after a 4h-fasting period. Total cholesterol, triglycerides, free fatty acids, β-hydroxybutyrate, glucose, and insulin were measured as described in materials and methods. Values represent the mean ± SD for n=8 mice per group. *P < 0.05 vs. control group.
CLA *trans*10,*cis*12 conjugated linoleic acid

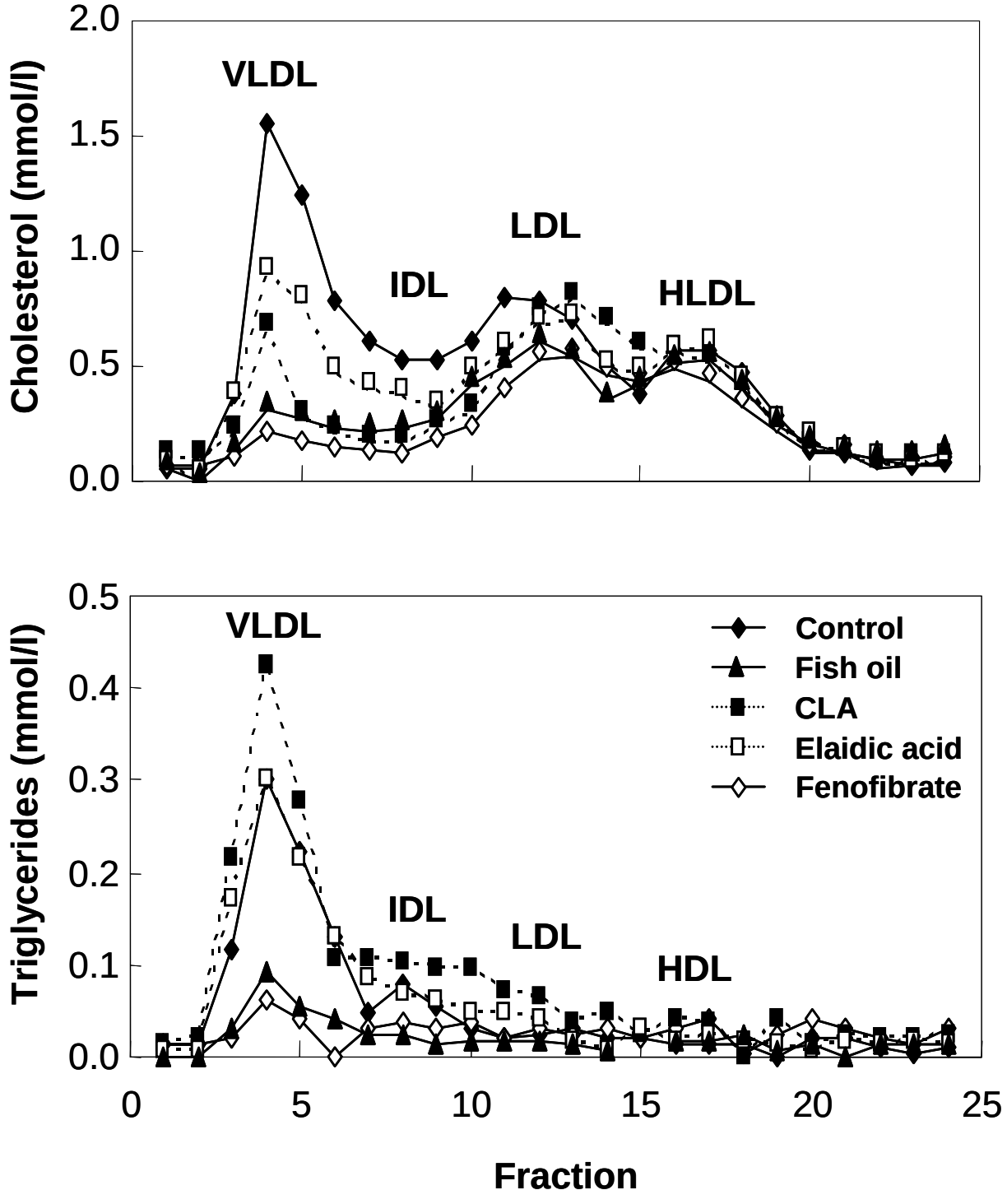


Figure 1. Cholesterol (upper panel) and triglyceride (lower panel) lipoprotein profiles
Plasmas of APOE*3Leiden mice, which had been fasting for 4 h, were pooled per dietary treatment group. Lipoprotein profiles were determined as described in materials and methods. CLA *trans*10,*cis*12 conjugated linoleic acid, HDL high density lipoprotein, IDL intermediate density lipoprotein, LDL low density lipoprotein, VLDL very low density lipoprotein

Liver weight and liver lipids

Consumption of fish oil led to a small but significant ($P < 0.05$) decrease in liver weight, whereas fenofibrate treatment caused a small but significant ($P < 0.05$) increase after 3 weeks of treatment (**Table 3**). Both treatments caused a significant ($P < 0.05$) decrease in the amount of free cholesterol, TG, and cholesteryl esters in the liver cells as compared with the control treatment. Consumption of *t*10,*c*12 CLA resulted in a two-fold increase in liver weight compared with the control group ($P< 0.05$). Liver cells contained significantly more TG and less cholesteryl esters after treatment with this dietary FA (**Table 3**).

Table 3. Liver weight and liver lipid levels determined in APOE*3Leiden mice fed a high-fat/high-cholesterol diet (control), or this diet supplemented with fish oil, *trans*10,*cis*12 CLA, elaidic acid, or fenofibrate for 3 weeks

Liver	Control	Fish oil	CLA	Elaidic acid	Fenofibrate
Weight (g)	1.15 ± 0.11	1.03 ± 0.10*	2.34 ± 0.09*	1.19 ± 0.13	1.70 ± 0.21*
Cholesteryl ester (µg /mg protein)	34.8 ± 3.2	25.2 ± 1.4*	22.9 ± 3.9*	37.0 ± 2.4	24.2 ± 2.3*
Free cholesterol (µg /mg protein)	13.4 ± 3.3	10.3 ± 1.1*	12.4 ± 1.0	13.8 ± 1.9	9.5 ± 1.2*
Triglycerides (µg /mg protein)	96.4 ± 18.0	67.6 ± 3.0*	144.6 ± 21.3*	109.0 ± 10.6	63.8 ± 4.4*

Hepatic levels of cholesteryl esters, free cholesterol, and triglycerides were determined as described in materials and methods. Values represent the mean ± SD for n=8 mice per group. *P < 0.05 vs. control. CLA *trans*10,*cis*12 conjugated linoleic acid

Proteomics

2D gel electrophoresis and MALDI-TOF mass spectrometry

When comparing the 2D gel electrophoresis gels, we found significant changes in the levels of 74 liver cytosolic proteins and 14 liver membrane proteins that were significantly up- or down-regulated by at least one of the dietary treatments compared with the control group. Of these, we could identify 65 liver cytosolic proteins and 8 liver membrane proteins using MALDI-TOF mass spectrometry (**Figure 2**). The cytosolic proteins were categorized according to their major functions to facilitate the study of pattern changes between treatments. These classifications are arbitrary to extent that they depend on currently recognized functions of proteins. However, clear effects could be seen on pathways that are involved with glucose and lipid metabolism, and in oxidation and aging. These pathways are also consistent with the physiological effects that were observed.

Principle component analysis

Liver cytosolic proteins

Principle component analysis of the log-transformed spot density values revealed that more than 47% of all variance in the dataset was accounted for by the first principle component (PC1) and nearly 21% of variance in the dataset was accounted for by the second principle component (PC2) (**Figure 3**, upper panel). The largest treatment effect on the first principle component (*i.e.*, the largest distance between the spots representing the saturated fat control group and the spots representing a dietary intervention group on the x-axis) was produced by fish oil. When considering the second principle component, fenofibrate treatment initiated a specific treatment effect, whereas all other treatments were situated much closer to the saturated fat control group. Treatment with elaidic acid produced the least treatment effect in relation to the saturated fat control group, both for PC1 and PC2. **Table 4a** summarizes the cytosolic proteins that provide the largest contribution to the dietary treatment effects in the principle component analysis.

Liver membrane proteins

Principle component analysis of the log-transformed spot density values revealed that more than 40% of all variance was accounted for by PC1 and more than 30% of variance was accounted for by PC2 (**Figure 3**, lower panel). The largest treatment effect was produced by elaidic acid, both on PC1 and PC2. The proteins that provided the largest contributions to the dietary treatment effects in the principle component analysis are outlined in **Table 4b**.

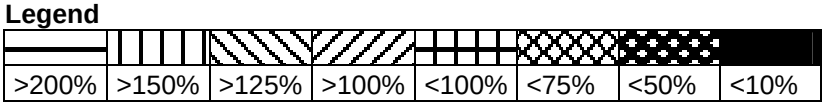
Pair-wise correlation analysis

In addition to PC analysis (PCA), we performed a pair-wise correlation analysis over the different treatments, including the physiological data on plasma lipid, glucose and insulin levels, and liver lipids (**Table 2** and **3**), as well as the data on protein levels. Such an analysis shows which parameters vary in a similar way throughout the different treatments. **Figure 4** shows a network of pair-wise interactions with a Pearson correlation higher than 0.8, a relating P-value of 0.0053 or lower, and a relating *q*-value 0.026 or lower, prepared using the software tool Cytoscape[32].

Figure 2 (below). Overview of cytoplasm and membrane proteins of which levels were significantly increased or decreased in one or more of the dietary intervention groups, as compared with the control group

Relative protein masses were calculated using the PDQuest software as described in materials and methods. The code indicates the percentage increase or decrease in protein mass in any dietary intervention group as compared with the control group. Proteins were identified using proteomics as described in materials and methods. CLA *trans*10,*cis*12 conjugated linoleic acid

Legend



Liver cytoplasm

		Fish Oil	CLA	Elaidic acid	Fenofibrate
Glucose metabolism	Galactokinase				
	Ketohexokinase				
	Fructose bisphosphatase 1				
	Malate dehydrogenase				
	Alpha enolase				
	Alpha enolase				
	Thriosephophate isomerase				
	Phosphoglycerate mutase				
	Isocitrate dehydrogenase				
	Adenosine kinase				
Lipid metabolism	Adenosine kinase				
	Long chain acetyl-CoA dehydrogenase				
	Long chain acyl-CoA thioester hydrolase				
	Long chain acyl-CoA thioester hydrolase				
	Mitochondrial long-chain acyl-CoA thioester				
	Acyl-CoA thioester hydrolase				
	Acyl-CoA thioesterase				
	Apolipoprotein E				
	Apolipoprotein A1 precursor				
	Adipophilin				
	2-hydroxyphytanoyl-Coa lyase				
	CTP:phosphocholine cytidyltransferase b2				
	Isovaleryl CoA dehydrogenase				
	Phosphatidylethanolamine-binding protein				
	Hypothetical GDSL-like Lipase/ acylhydrolase				
Protein metabolism	Annexin A5				
	Formimimo cyclodeaminase				
	Ornithine aminotransferase				
	Glutamine synthetase				
	Glutamine synthetase				
	Histidine ammonia-lyase				
	Arginase-1liver				
	3-hydroxyanthranilate 3,4-dioxygenase				
	Cysteine sulfinic acid decarboxylase				

Liver Cytoplasm continued

		Fish Oil	CLA	Elaidic acid	Fenofibrate
Oxidation and aging	Senescence marker protein 30				
	Selenium binding protein 56kDa				
	Selenium binding protein 56kDa				
	T-complex protein 1, beta subunit				
	Peroxyredoxin 6				
	Glutathione S-transferase Mu 2				
	Gluthatione S-transferase Mu 1				
	Heat shock protein 74 kDa				
	Epoxide hydrolase				
	Catalase				
	Aldehyde dehydrogenase				
	Aldehyde dehydrogenase				
	Aldehyde dehydrogenase				
	L-gulonolactone oxidase				
Homocysteine metabolism	Thioether S-methyltransferase				
	Adenosylhomocysteinase				
	Glycine-N-methyltransferase				
BH4 metabolism	Sepiapterin reductase				
	Phenylanaline-4-hydroxylase				
Haem biosynthesis	Delta-aminoevulinate dehydratase				
	Hydroxymethylbiliane				
Energy metabolism	Pyrophosphatase				
	Nicotinate-nucleotide pyrophosphorylase				
Enzymes	N-sulfotransferase				
	Proteasome beta subunit				
	Nucleoside diphosphate kinase B				
	Guanidinoacetate N-methyltransferase				
	Purine nucleoside phosphorylase				
	Ubiquitin / ribosomal protein CEP52				
Carcinogenesis marker	Tetranectin				
Structural proteins	Tubulin alpha-2 chain				

Liver Membrane

Lipid metabolism	Carnitine palmitoyltransferase isoenzyme				
Protein metabolism	Fumarylacetoacetase				
Oxidation	Aldehyde dehydrogenase				
	Cytochrome P450 2B10				
	Cytochrome B5				
Pyrimidine biosynthesis	CTP synthase				
Carcinogenesis marker	Tetranectin precursor				
Structural protein	Vimentin				

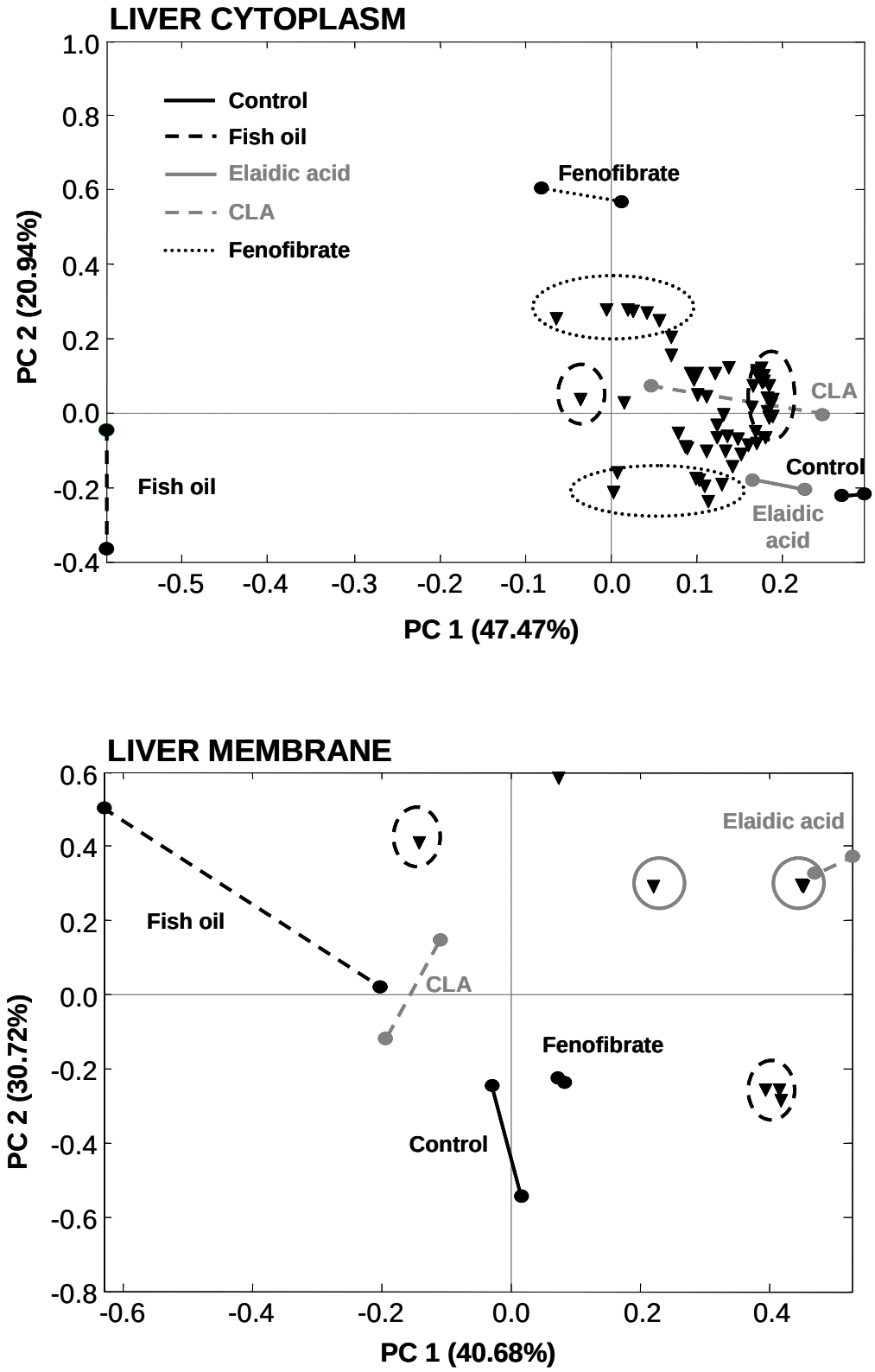


Figure 3. Scoreplot of an unsupervised principle component analyses of liver cytoplasm and membrane proteins that are significantly up- or down-regulated by fish oil, *trans*10,*cis*12 conjugated linoleic acid (CLA), elaidic acid and/or fenofibrate as compared with the control group

Table 4a. Liver cytoplasm proteins representing the highest positive and negative loadings towards the dietary treatment effects

Treatment	Proteins representing the highest positive loadings towards treatment	Proteins representing the highest negative loadings towards treatment
Fish oil	Annexin A5 Ornithine aminotransferase Ubiquitin/ribosomal protein CEP52 Selenium binding protein Triosephosphate isomerase Glutathione S-transferase Mu2 Epoxide hydrolase	Galactokinase Hydroxymethylbiliane synthase Isocitrate dehydrogenase Thioether S-methyltransferase Adipophilin GDSL-like lipase Nicotinate-nucleotide pyrophosphorylase T-complex protein 1, beta subunit Phosphatidyl ethanolamin-binding protein Apolipoprotein A1 precursor Guanidinoacetate N-methyltransferase Glutamine synthase Tubulin alpha 2 chain Purine nucleoside phosphorylase Glutatione S-transferase Mu1
CLA	Galactokinase Hydroxymethylbiliane synthase Isocitrate dehydrogenase Thioether S-methyltransferase Adipophilin Hypothetical GDSL-like lipase Nicotinate-nucleotide pyrophosphorylase T-complex protein 1, beta subunit Phosphatidyl ethanolamin-binding protein Apolipoprotein A1 precursor Guanidinoacetate N-methyltransferase Glutamine synthase Tubulin alpha 2 chain Purine nucleoside phosphorylase Glutatione S-transferase Mu1	Annexin A5 Ornithine aminotransferase Ubiquitin/ribosomal protein CEP52 Selenium binding protein Triosephosphate isomerase Glutathione S-transferase Mu2 Epoxide hydrolase
Fenofibrate	Acyl CoA thioester hydrolase Long chain acetyl-CoA dehydrogenase Long chain acyl-CoA thioester hydrolase Long chain acyl-CoA thioester hydrolase Acyl-CoA thioester hydrolase Cysteine sulfinic acid decarboxylase CTP:phosphocholine cytidyltransferase b2 Mitochondrial long chain acyl-CoA thioester hydrolase Catalase	Annexin A5 Ornithine aminotransferase Fructose biphophatase 1
Control + Elaidic acid	Adenosine kinase Phenylalanine-4-hydroxylase 3-Hydroxyanthranilate 3,4-dioxygenase Glutamine syntase Glycine-N-methyltransferase Fructose biphosphatase 1 Alpha enolase Senescence marker protein 30	Ubiquitin/ribosomal protein CEP52 Long chain acyl-CoA thioester hydrolase

Table 4b. Membrane proteins representing the highest positive and negative loadings towards the dietary treatment effects

Treatment	Proteins representing the highest positive loadings towards treatment	Proteins representing the highest negative loadings towards treatment
Fish oil		Fumarylacetoacetase Vimentin Cytochrome B5 precursor
CLA		Aldehyde dehydrogenase CTP synthase
Elaidic acid	Aldehyde dehydrogenase CTP synthase	
Fenofibrate +control		Carnitine palmitoyltransferase isoenzyme Cytochrome P450 2B10 Tetranectin precursor

Loadings were calculated using principle component analysis as described in materials and methods. CLA *trans*10,*cis*12 conjugated linoleic acid

Discussion

This study has been unique in two ways. First, it compared three important dietary FA known to regulate lipid, and possibly glucose metabolism, in a single well-validated animal model for lipid metabolism and atherosclerosis. Second, it combined physiological data on plasma and liver levels with a proteomic study of liver proteins. Such a combined approach allowed us to identify pathways and proteins that may underlie the changes in lipid and glucose metabolism under these dietary regimes. Conclusions from the results can first be derived by considering individual dietary treatments.

Fenofibrate and fish oil

In our mouse model, we used fenofibrate as a positive control for changes in FA catabolism, as this drug represents a validated agonist of PPARα[23]. Fenofibrate indeed changed proteins involved in FA oxidation (see **Figure 2** and **Table 4a**), lowered plasma and liver TG levels, reduced plasma levels of free FA, and increased β-hydroxybutyrate levels.

The modification of plasma lipoprotein metabolism by omega-3 FA represents a major anti-atherogenic mechanism of action[33]. Dietary polyunsaturated FA (PUFA) inhibit lipogenesis by suppressing the expression of a number of hepatic enzymes involved in glucose metabolism and FA biosynthesis[34-38] through a reduced expression of sterol regulatory element binding protein-1 (SREBP-1). At the same time, PUFA induce genes encoding proteins involved in FA oxidation and ketogenesis by activation of PPARα[10,34,39]. The latter mechanism is shared by both omega-3 FA and the hypotriglyceridemic drug fenofibrate[23]. Indeed, in this study both fish oil and fenofibrate exerted similar effects at a physiological level.

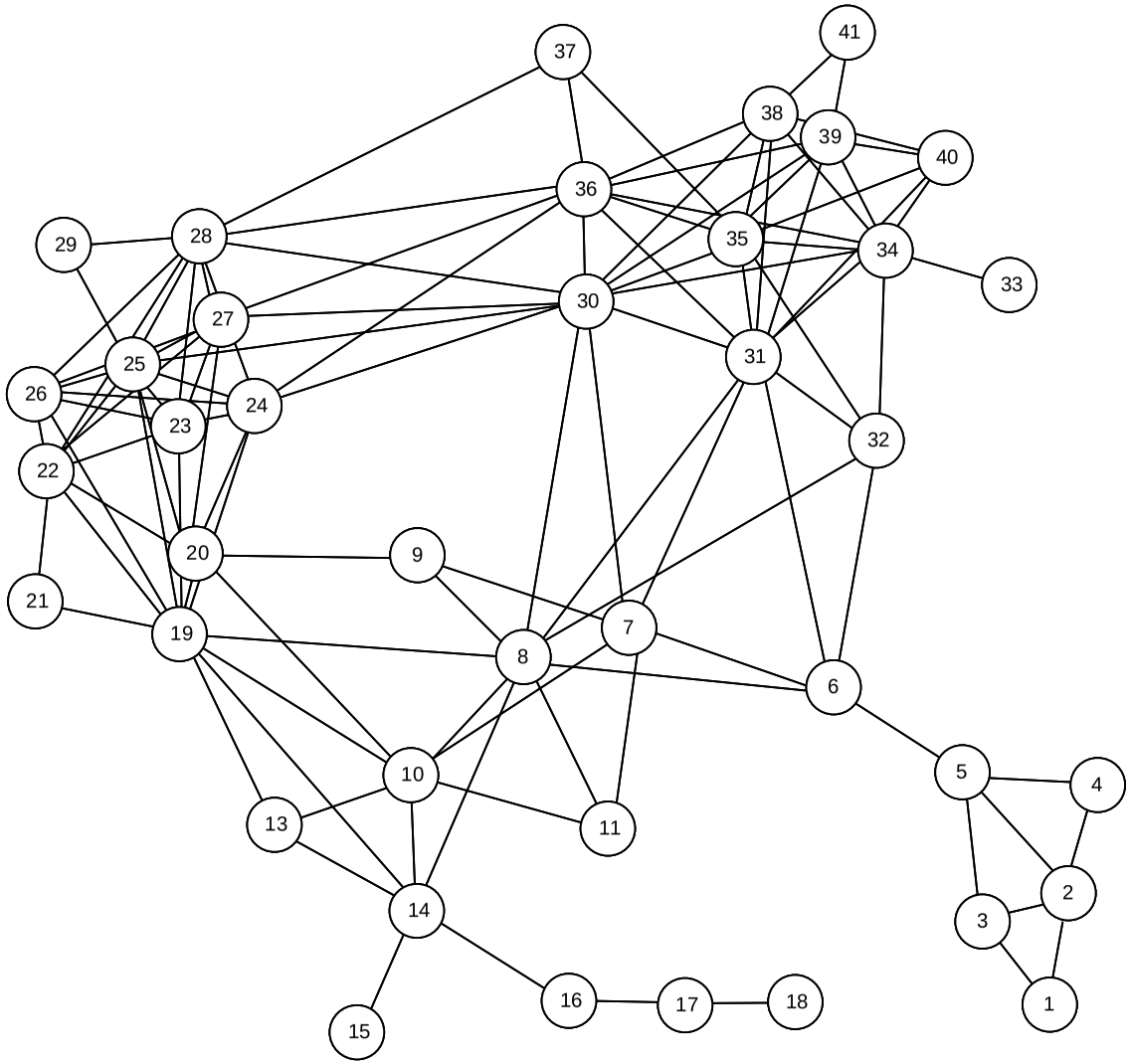


Figure 4. Network plot indicating all correlations between plasma lipid, β-hydroxybutyrate, glucose and insulin levels, liver lipid levels, and liver protein levels that had a Pearson correlation coefficient higher than 0.8, a relating P-value of 0.0053 or lower, and a relating q-value of 0.026 or lower

1 Glutathione S-transferase Mu 2
2 Long chain acyl-CoA thioester hydrolase B
3 Cysteine sulfinic acid decarboxylase
4 Long chain acyl-CoA thioester hydrolase A
5 Catalase
6 Liver Free Cholesterol
7 3-hydroxyanthranilate 3,4-dioxygenase
8 Adenosine kinase
9 Peroxyredoxin 6
10 Plasma Cholesterol
11 Senescence marker protein 30
13 Liver Cholesteryl Esters
14 Phenylanaline-4-hydroxylase
15 Glycine-N-methyltransferase
16 Ornithine aminotransferase
17 Liver weight
18 Tetranectin
19 Adenosylhomocysteinase
20 Aldehyde dehydrogenase
21 Pyrophosphatase
22 Tubulin alpha-2 chain
23 Delta-aminoevulinate dehydratase
24 Selenium binding protein 56kDa
25 Malate dehydrogenase
26 Thioether S-methyltransferase
27 Formimino cyclodeaminase
28 Nicotinate-nucleotide pyrophosphorylase
29 T-complex protein 1, beta subunit
30 Fructose biphosphatase 1
31 Plasma Free Fatty Acids
32 Glutamine synthase
33 Plasma Glucose
34 Ketohexokinase
35 Sepiapterin reductase
36 Alpha enolase
37 Phosphatidylethanolamine-binding protein
38 Plasma Triglycerides
39 Liver Triglycerides
40 Nucleoside diphosphate kinase B
41 Proteasome beta subunit

Both treatments significantly lowered plasma cholesterol and TG concentrations as compared with a saturated fat control diet, which coincided with a significant parallel reduction in levels of cholesteryl esters, free cholesterol, and TG in the liver. These effects are likely to be caused by an enhanced FA oxidation rate, as indicated by the level of β-hydroxybutyrate in plasma.

Proteome analyses of the mouse liver samples also revealed an increase in the rate of FA oxidation, as levels of catalase and long-chain acyl-CoA thioester hydrolase (both the cytosolic and the mitochondrial form) were significantly increased upon treatment with fish oil and fenofibrate. Till now, long-chain acyl-CoA thioester hydrolase has not been linked to specific dietary FA treatments. Acyl-CoA thioesterases hydrolyze CoA esters of various lengths to free FA and CoA-SH, and they are likely to play important roles in maintaining appropriate CoA-SH levels during periods of increased β-oxidation and FA overload[40]. The existence of selective acyl-CoA thioesterases could provide important control points in the oxidation of many peroxisomal substrates, and they may regulate intracellular levels of CoA esters and CoA-SH. To date, several thioesterase isoforms have been identified in peroxisomes, cytoplasm, and mitochondria, where they are thought to have distinct functions in lipid metabolism[40]. Treatment of mice with the peroxisome proliferator clofibrate also induced levels of long-chain cytosolic, mitochondrial, and peroxisomal acyl-CoA thioester activity in the liver in a previous study, although the cytosolic form was most strongly induced[41].

The lowering in liver lipids in the fish oil and fenofibrate groups matched a large reduction of the level of adipophilin in the liver on the 2D electrophoresis gels (**Figure 2**). Adipophilin is a protein associated with lipid storage droplets, which are dynamic structures that function as storage deposits for TG and cholesterol esters[42].

Although fish oil and fenofibrate are believed to share common modes of action, our PCA did show diverse treatment effects of both dietary interventions. The proteins responsible for the treatment effect of fish oil were involved in a range of metabolic functions (**Table 4a**), whereas the list of proteins responsible for the treatment effect of fenofibrate was dominated by those involved in β-oxidation of FA. This indicates that fish oil, unlike fenofibrate, triggers a more diverse range of mechanisms that could affect the physiological outcome.

*t*10,*c*12 CLA

Accumulating evidence indicates that CLA, in particular the *t*10,*c*12 isomer, may affect lipoprotein metabolism. We observed a significant increase in hepatic TG levels and a two-fold increase in liver weight upon treatment with *t*10,*c*12 CLA, and these findings were mirrored by a significant increase in hepatic levels of adipophilin. This protein also showed a high loading toward the treatment effect of *t*10,*c*12 CLA in the PCA. Increased expression of adipophilin has been associated with liver steatosis before[43], and recent attention has focused on the excessive accumulation of TG in the liver, or liver steatosis, as part of

the syndrome that involves visceral obesity and dyslipidemia, insulin resistance, and type 2 diabetes mellitus[44]. CLA-mediated liver steatosis has been observed in other studies in different strains of mice[45-53] but not in other animals. Several lines of evidence indicate that hepatic TG accumulation is also a causative factor involved in hepatic insulin resistance[44], and indeed, hyperinsulinemia in *t*10,*c*12 CLA-fed mice has been observed in several studies[50,52,54,55] as well as in our study, as evidenced by an almost three-fold increase in plasma insulin levels (**Table 2**). In a hyperinsulinemic state, a shift in fuel usage from carbohydrates to fat usually occurs, leading to an increase in the rate of β-oxidation of FA as well as an increase in ketogenesis. Indeed, we found a significant increase in levels of plasma keton bodies, as well as increased protein levels of carnitine palmitoyltransferase, catalase, and long-chain acyl-CoA thioester hydrolase upon treatment with *t*10,*c*12 CLA. Higher activity and mRNA expression of various mitochondrial and peroxisomal FA oxidation enzymes upon treatment with a CLA mixture has been described previously in C57Bl/6J mice[50].

The increased ratio of TG to cholesteryl esters in the liver upon feeding *t*10,*c*12 CLA were clearly reflected in the lipid composition of the lipoproteins. The converse effect of *t*10,*c*12 CLA on plasma TG and cholesterol suggests independent mechanisms by which CLA affects these levels. Furthermore, the effect of *t*10,*c*12 CLA on plasma TG levels in mice depends on the mouse strain used. Some studies report that CLA is effective in decreasing TG levels[48,51,56], others report no effect on plasma TG levels[50,55]. The decrease in plasma TG in previous studies has been attributed to an up-regulation of the LDL receptor. It appears therefore, that the overall effect of CLA on plasma TG is determined by two opposite actions of CLA: overproduction of VLDL and up-regulation of LDL receptors. In our APOE3*Leiden model with impaired clearance, the former action apparently dominated and resulted in hypertriglyceridemia.

Elaidic acid

Although studies investigating the mechanism of action of *trans*-FA are limited, several controlled metabolic studies have shown the unfavorable effects of *trans*-FA on lipoprotein metabolism and other biomarkers for CHD. *Trans*-fat has been shown to increase levels of LDL cholesterol and TG[1], and a high intake of *trans-fa*t has been associated with the development of insulin resistance and type 2 diabetes in humans[15,57]. However, in our mouse model, dietary elaidic acid decreased plasma levels of cholesterol and had no effect on plasma levels of TG, glucose, or insulin. However, this comparison was made against a saturated fat control diet, which might have masked the true negative effects of the elaidic acid.

Trans-FA are incorporated into membrane phospholipids and may therefore alter the packaging of the phospholipids and possibly influence the physical properties of the membrane or the activities of the membrane-associated enzymes[58]. We observed a very specific effect of elaidic acid treatment on protein levels in the liver membrane revealed by

principle component analysis. The proteins that provided the largest positive contribution to the differences between the elaidic acid treatment and the other dietary treatments were aldehyde dehydrogenase and CTP synthase. Levels of these proteins were up-regulated by more than 200% by elaidic acid. CTP synthase has been implicated in the regulation of phospholipid biosynthesis, at least in *Saccharomyces cerevisiae*[59].

Comparative analysis of all treatments

Principle component analysis was used to analyze the effects of the various treatments on the protein levels in the complete dataset. This approach visualizes the extent to which different treatments have similar or very different, effects on protein expression. Clearly, fish oil triggered a different treatment effect on cytosolic protein expression compared to all other treatments. Elaidic acid showed the strongest treatment effect on the liver membrane proteins studied.

The pair-wise correlation analysis revealed many associations, resulting in clustering of proteins that are related to each other (**Figure 4**). Some of these relations have been described before and are therefore consistent with previous studies, adding validity to the novel associations revealed by our study of the APOE*3Leiden mouse. For example, the associations within the cluster containing plasma and liver TG, plasma glucose, plasma free FA, and protein levels of hepatic fructokinase and fructose 1,6 bisphosphatase are all related to dyslipidemia and glucose intolerance, two important conditions related to the metabolic syndrome or Syndrome X. It is striking, that this cluster is observed already in a data set with relatively mild perturbations and at equal body weights but with clearly different liver weight and composition. The position of sepiapterin reductase in the middle of this cluster is unexpected. However, sepiapterin reductase is involved in the biosynthesis of tetrahydrobiopterin, an essential co-factor for eNOS activity[60], and may therefore play a role in the relationship between dyslipidemia, insulin resistance and endothelial dysfunction[61,62].

A second recognized cluster is that of the proteins catalase and two different forms of long-chain acyl CoA thioester hydrolases, which are related to the β-oxidation of FA. The addition of cysteine sulfinic acid decarboxylase to this cluster has, however, not been described before. Cysteine sulfinic acid decarboxylase is a rate-limiting enzyme for taurine biosynthesis, and taurine can be tissue-protective in many models of oxidant-induced injury[63]. Therefore, cysteine sulfinic acid decarboxylase, like catalase, might be involved in the protection of cells against oxidative stress generated by FA oxidation.

Proteomics of diet-induced changes in the liver of APOE*3Leiden mice revealed a wide array of proteins that were affected by the various dietary interventions. Our approach visualized the 500-800 most abundant proteins from a liver cell on a 2D electrophoresis gel, and this provided a detailed overview of novel and recognized alterations in lipid degradation and glycolysis pathways, reflecting changes in lipoprotein and glucose metabolism upon dietary treatment. We found, for example, that the consumption of

specific dietary FA induced a differential expression of long chain acyl-CoA thioester hydrolase protein (as an indicator of β-oxidation) and adipophilin (as an indicator of liver lipid content). Statistical analysis of our results revealed many associations, some of which are well known (like the metabolic syndrome), whereas others will be the basis of intriguing new leads for further studies.

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Chapter 5

Dietary sphingolipids lower plasma cholesterol and triacylglycerol and prevent liver steatosis in APOE*3Leiden mice

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Abstract

The prevalence of dyslipidemia and obesity resulting from excess energy intake and physical inactivity is increasing. The liver plays a pivotal role in systemic lipid homeostasis. Effective, natural dietary interventions that lower plasma lipids and promote liver health are needed. Our goal was to determine the effect of dietary sphingolipids on plasma lipids and liver steatosis. APOE*3Leiden mice were fed a Western-type diet, supplemented with different sphingolipids. Body cholesterol and triacylglycerol (TG) metabolism, as well as hepatic lipid levels and lipid-related gene expression, were determined. Dietary sphingolipids dose-dependently lowered both plasma cholesterol and TG in APOE*3Leiden mice. 1% phytosphingosine (PS) reduced plasma cholesterol and TG by 57% and 58%, respectively. PS (a) decreased the absorption of dietary cholesterol and free fatty acids by 50% and 40%, respectively, whereas intestinal TG lipolysis was not affected; (b) increased hepatic VLDL-TG production by 20%, whereas plasma lipolysis was not affected; and (c) increased the hepatic uptake of VLDL remnants by 60%. Hepatic mRNA concentrations indicated enhanced hepatic lipid synthesis and VLDL and LDL uptake. The net result of these changes was a strong decrease of plasma cholesterol and TG. The livers of 1%PS-fed mice were less pale, 22% lighter, and contained 61% less cholesteryl ester and 56% less TG than livers of control mice. Furthermore, markers of liver inflammation (SAA) and liver damage (ALAT) decreased by 74% and 79%, respectively, in PS-fed mice. We conclude that sphingolipids lower plasma cholesterol and TG and protect the liver from fat- and cholesterol-induced steatosis.

Introduction

In our modern Western society, excess energy intake and physical inactivity are the leading causes of the epidemic prevalence of obesity. Obesity in turn is associated with an increased prevalence of cardiovascular risk factors, such as dyslipidemia, insulin resistance, and hypertension (collectively called the metabolic syndrome[1]). Obesity-related dyslipidemia is characterized by mildly elevated concentrations of very low density lipoprotein (VLDL)-triacylglycerol and low density lipoprotein (LDL)-cholesterol and decreased concentrations of high density lipoprotein (HDL)-cholesterol. The liver plays a central role in the maintenance of systemic lipid homeostasis, since it synthesizes and secretes VLDL and, thus, is involved in the redistribution of lipids, primarily triacylglycerol (TG), for storage and utilization by peripheral tissues. Lipid accumulation in the liver leads to the development of steatosis, a condition closely associated with insulin resistance[2].

The metabolic syndrome is usually treated by (a combination of) life-style and dietary changes. Currently used drugs target one aspect of the metabolic syndrome. For example hypercholesterolemia is treated with HMG-CoA reductase inhibitors (statins) and cholesterol absorption inhibitors (ezetimibe, phytosterols, and stanols), hypertriglyceride-

mia is treated with PPARα agonists (fibrates), hypertension with β-blockers (atenolol, metoprolol, and propranolol), and insulin resistance with thiazolidinediones (pioglitazone and rosiglitazone) or metformin. Although recently developed compounds, such as the glitazars, target more than one aspect of the metabolic syndrome, treatment of multiple aspects of the metabolic syndrome with a single natural dietary compound could be an attractive alternative.

We recently observed in a pilot study that comsumption of a Western-type diet supplemented with sphingolipids lowered both plasma cholesterol and TG in hyperlipidemic APOE*3Leiden mice. The APOE*3Leiden mouse has a lipoprotein profile that closely resembles the human profile. In these mice, plasma cholesterol can be titrated to various concentrations by varying the amount of cholesterol in the diet[3]. Moreover, in contrast with wild-type mice, LDL-receptor-deficient mice and ApoE-deficient mice, APOE*3Leiden mice, are highly sensitive to treatment with hypolipidemic drugs, such as statins[4,5] and fibrates. We wondered, therefore, whether supplementation of the diet with sphingolipids could be used to treat the dyslipidemia characteristics of the metabolic syndrome.

Materials and methods

Sphingolipids

We used the 3 sphingolipids that represent the most abundant and simplest natural sphingolipid classes, the sphingoid bases (see **Figure 1**; I, II and III), which can be formed by enzymatic breakdown of complex sphingolipids in the intestine, and the 3 complex natural sphingolipids (**Figure 1**; IV, V and VI). Sphingomyelin (mainly N-palmitoyl-sphingosine-1-phosphocholine) from egg was obtained from Larodan Fine Chemicals (Stockholm, Sweden). Yeast-derived (semi)synthetic ceramide III (N-stearoyl-phytosphingosine), cerebroside (N- stearoyl-phytosphingosine-1-glucose) and phytosphingosine (PS) were from Cosmoferm BV (Delft, The Netherlands). Sphinganine and sphingosine were from Avanti Polar Lipids (Albaster, AL, USA).

Animals and diets

Female heterozygous APOE*3Leiden transgenic mice[3] (6 months old) were fed a Western-type diet (Hope Farms, Woerden, the Netherlands) containing 15% cocoa butter, 0.25% cholesterol, 1% corn oil, 40.5% sucrose, 20% acid casein, 10% corn starch and 5.95% cellulose (all w/w) for 5 weeks. The mice were housed in clean-conventional animal rooms (relative humidity 50-60%, temperature ~ 21°C, light cycle 6.00 A.M. to 6.00 P.M.), and supplied with food and acidified tap water *ad libitum* during the experiment. Mice were housed in macrolon cages (3 mice per cage).

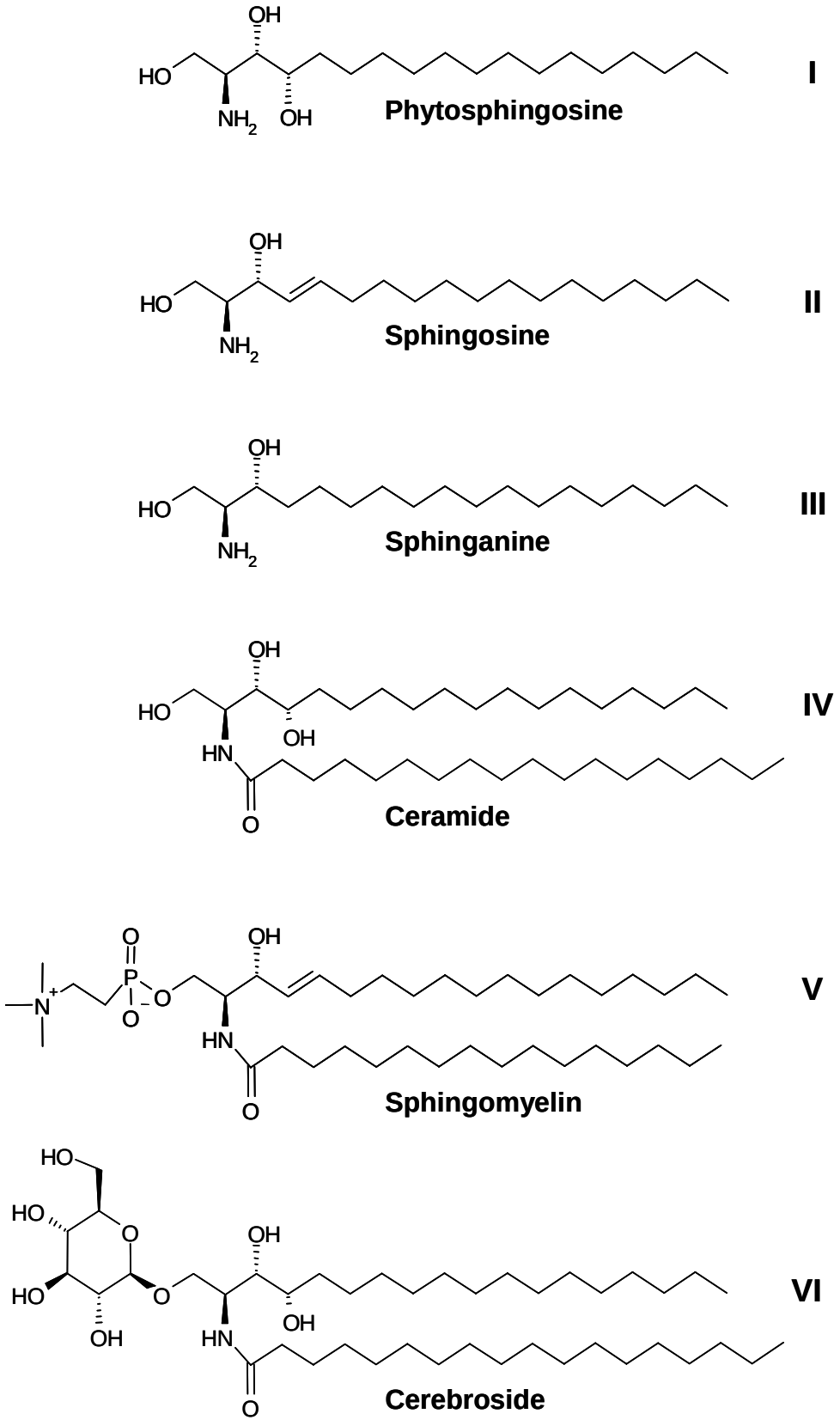

Figure 1. Structures of the sphingolipids used in this study

Body weight (20.9 ± 0.7 g) and food intake (2.5 ± 0.1 g/mouse/day) were monitored weekly. In the first experiment (**Figure 2**), the mice were randomized after these 5 weeks into 7 groups (n = 6 per group), based on plasma cholesterol, TG and body weight. Subsequently, the mice were fed the same diet with or without 0.1% (w/w) PS, sphingosine, sphinganine, cerebroside, ceramide III, or sphingomyelin for 3 weeks. Then, the sphingolipid dose was increased to 0.2% (w/w) for 3 weeks and finally to 0.4% (w/w) for 3 weeks. Tail-vein blood samples were obtained after a 4 h-fast, at randomization, and at 3, 6, and 9 weeks.

In all subsequent experiments, female APOE*3Leiden transgenic mice (6 months old) were fed the Western-type diet for 5 weeks and then randomized as described. Subsequently, the mice were fed the Western-type diet for 5 weeks or the same diet supplemented with 1.0 % (w/w) PS before being subjected to experimentation. All experiments were approved by the TNO Animal Care and Use Committee.

Plasma parameters

Tailblood samples were collected in EDTA-coated cups, or in paraoxon-coated capillaries to prevent lipolysis[6]. Plasma parameters were determined using commercial kits for total cholesterol (Roche Diagnostics, Mannheim, Germany), non-esterified free fatty acids (NEFA-C; Wako chemicals, Neuss, Germany), TG (Triglyceride GPO-Trinder; Sigma, St. Louis, MO, USA), β-hydroxybutyrate (β-HB; Sigma) and alanine aminotransferase (ALAT) (Reflotron GPT; Roche). Serum amyloid A (SAA) was measured by enzyme-linked immunoabsorbent assay (ELISA) (Biosource, Nivelles, Belgium) and fibrinogen by sandwich ELISA, as described[7]. For lipoprotein fractionation, groupwise-pooled plasma was size-fractionated by fast protein liquid chromatography (FPLC) on a Superose 6 column (Äkta; Amersham Pharmacia Biotech, Uppsala, Sweden). Fractions were assayed for total cholesterol and TG as described.

Intestinal cholesterol absorption

We assessed the cholesterol absorption using the fecal dual-isotope method described by Borgstrom[8] and Wang and Carey[9] in mice fed the diet with or without 1% PS (6 per group) for 5 weeks. The mice were housed individually. At 5.00 P.M. the mice received a dose of 200 µl of olive oil, containing [^{14}C]cholesterol (1 µCi/mouse; Amersham Biosciences, Little Chalfont, UK) and [^{3}H]sitostanol (1 µCi/mouse; ARC, St. Louis, MO, USA) by gavage. Body weight and food intake were monitored, and feces were collected for 4 days. Feces were freeze-dried, homogenized, pooled per mouse over the 4-day period, and dissolved in ethanolic potassium (3 mol/l, 60% ethanol). Radioactivity was determined in the fecal samples to assess the amount of radiolabeled cholesterol and sitostanol. Sitostanol was used as the reference compound since it is known to be poorly absorbed (< 3%) in mice[9]. The formula used to calculate cholesterol absorption was: % cholesterol absorption = ([^{14}C]/[^{3}H] dosing mixture -[^{14}C]/[^{3}H] feces) / [^{14}C]/[^{3}H] dosing mixture * 100.

Determination of neutral sterols in feces

Two mice were housed per cage, and feces were collected in 2 subsequent 3-day periods from 3 cages per group. Feces were separated, freeze-dried and weighed. 10 mg of dried feces was used to extract neutral sterols by treatment with alkaline methanol (3 parts of methanol and 1 part of 1 N NaOH) and petroleum ether with the use of 5α-cholestane as internal standard, as described previously[10]. Analysis of the sterol derivatives was performed by gas chromatography.

Intestinal TG and FFA absorption

Intestinal absorption of TG and FFA was determined in overnight-fasted mice (n = 6 per group) fed 1% PS-containing or control Western-type diet. The mice received an intragastric load of 200 µl olive oil containing [^{3}H]triolein (12 µCi/mouse) and [^{14}C]oleic acid (3.3 µCi/mouse) (Amersham Biosciences) directly after the i.v. injection of Triton WR1339 (Tyloxapol, Sigma; 500 mg/kg in 100 µl saline) to block lipoprotein lipase (LPL)-mediated TG hydrolysis[11]. For PS-fed animals, 1% PS was added to the olive oil load, since overnight-fasting might negate any direct effect that PS may have on intestinal absorption. Blood samples were collected by tail-vein incision at 1, 2, 3, and 4 h after the intragastric load into pre-cooled paraoxon-coated capillaries, and plasma radioactivity and TG were determined.

Hepatic VLDL-TG production

The rate of hepatic VLDL-TG production, *de novo* apoB secretion, and VLDL composition were determined in overnight-fasted mice. Mice were anaesthetized with fluanisone/fentanyl/midazolam i.p., and injected i.v. with 0.1 ml PBS containing 100 µCi Tran^{35}S-label^{TM} (ICN Biomedicals, Irvine, CA, USA) to measure *de novo* apoB synthesis. After 30 min, the animals received a Triton WR1339 injection (500 mg/kg body weight) to prevent systemic lipolysis of newly secreted hepatic VLDL-TG[12]. Blood samples were drawn at 0, 15, 30, 60, and 90 min after Triton WR1339 injection, and plasma TG concentrations were determined. After 90 min, the animals were sacrificed and blood was collected by retro-orbital bleeding for isolation of VLDL.

VLDL composition

VLDL particles (d < 1.019) were separated from other lipoproteins in the plasma by density gradient ultra-centrifugation, as described previously[13]. The protein content of the VLDL fraction was determined by Lowry's assay[14], and TG and total cholesterol were determined as described above. Phospholipids and free cholesterol were determined using standard commercial kits (Wako Chemicals, Neuss, Germany). The [^{35}S]-apoB content of VLDL was measured after selective precipitation of apoB with isopropanol[15,16].

In vivo clearance of VLDL-like TG-rich particles

To determine whether 1% PS accelerates the clearance of TG-rich lipoproteins from plasma, we used radiolabeled emulsion particles as a tool. VLDL-like emulsion particles containing 200 µCi [^{3}H]triolein and 20 µCi [^{14}C]cholesteryl oleate were prepared and characterized as described[15,17]. Fed mice (n = 6) were anaesthetized as described above and laparotomy was performed. Emulsion particles were injected into the vena cava inferior, at a dose of 300 µg TG per mouse. At 2, 5, 10, 20, and 30 min, blood samples (50 µl) were taken from the vena cava inferior and liver samples were tied off, excised, and weighed. [^{3}H] and [^{14}C]-activities were counted in 10 µl of serum and corrected for total serum volume (ml) calculated as 0.04706 * body weight (g)[18]. After the last liver and blood samples were taken, the remainder of the liver, heart, spleen, hind limb muscle, and gonadal, perirenal and intestinal white adipose tissues were harvested. Lipids were extracted overnight at 60°C in 500 µl of Solvable™ (Perkin Elmer, Wellesley, MA, USA), and radioactivity was counted[17].

RNA isolation and RT-PCR

Livers from 4 h-fasted mice fed 1% PS-containing diet or control Western-type diet for 5 weeks were removed immediately after sacrifice, flushed with cold 0.9% NaCl and snap-frozen in liquid nitrogen. Total RNA was isolated as described by Chomczynski and Sacchi[19] by use of RNA-Bee™ (Campro Scientific, Berlin, Germany). cDNA synthesis was performed according to Bloks et al.[20] Real-time quantitative PCR[21] was performed using an Applied Biosystems (Nieuwerkerk aan den IJssel, The Netherlands) 7700 Sequence detector. Primers were obtained from Invitrogen (Paisley, UK) and fluorogenic probes, labeled with 6-carboxyfluorescein (6-FAM), and 6-carboxytetramethylrhodamine (6-TAMRA), were made by Eurogentec (Seraing, Belgium). Primers and probes used were described earlier[22-24]. All expression data were subsequently standardized for hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA concentrations.

Liver lipid concentration

Liver samples taken from 4 h-fasted mice fed 1% PS-containing or control Western-type diet for 5 weeks were homogenized in phosphate-buffered saline (PBS) (10% wet wt/vol) and the protein content was measured by Lowry's assay[14]. The lipid content was determined by lipid extraction using the Bligh and Dyer method[25], followed by lipid separation using high performance thin layer chromatography (HPTLC) on silica gel plates as described[26] and analysis by TINA2.09 software[27] (Raytest Isotopen Meβgeräte, Straubenhardt, Germany).

Liver histology

Livers from 4 h-fasted mice fed 1% PS-containing or control Western-type diet for 5 weeks were fixed in 10% formalin and paraffin-embedded. Liver sections were stained with hematoxylin-phloxine-saffron (HPS) for morphological analysis.

Statistics

Differences in responses during the intervention period between the control group and the treatment groups were analyzed by two-way ANOVA. In case of a significant overall effect, this analysis was followed by comparison of all treatment groups with the control group (Dunnett's test). When only 2 groups (control and treatment) were compared, Student's t-test, Welch's t-test or the Mann-Whitney U test was used. Time-course experiments were analyzed by two-way ANOVA. In all statistical tests performed, the null hypothesis was rejected at the 0.05 level of probability. All data are presented as mean ± SD. Statistical analyses were performed using SPSS11.0 (SPSS, Chicago, IL).

Results

Sphingolipids lower plasma cholesterol and TG in APOE*3Leiden mice

For our initial experiment, to evaluate the effect of sphingolipids on plasma cholesterol and TG concentrations in APOE*3Leiden mice, we used 3 simple and 3 complex sphingolipids (**Figure 1**). The data were analyzed by two-way ANOVA. After demonstrating that the interaction between dose and treatment was significant ($P < 0.001$), we analyzed the data for each dose separately by ANOVA, followed by Dunnett's test. With 0.1% of these sphingolipids in the diet, no significant effect on plasma cholesterol ($P = 0.978$) and TG ($P = 0.398$) was seen (**Figure 2**). At a dose of 0.2% (w/w), the sphingolipids (ceramide and cerebroside excepted) significantly decreased plasma cholesterol concentration by 20-40% ($P = 0.0096$; **Figure 2A**). At a dose of 0.4% sphingolipid, plasma cholesterol was decreased even more, and ceramide also had a significant cholesterol lowering effect ($P = 0.0009$; **Figure 2A**). The decrease in TG concentration was ~ 40% for all sphingolipids at dietary sphingolipid concentrations of 0.2% and 0.4%, a significant decrease for all compounds both at week 6 and at week 9; **Figure 2B**). No differences in food intake or body weight were observed throughout the experiment between the mice fed sphingolipids and the control animals (data not shown). Remarkably, the simplest sphingolipids, the sphingoid bases (**Figure 1**), had the same potent cholesterol- and TG-lowering effect as their complex sphingolipid derivatives.

To study the mechanisms underlying the cholesterol- and TG-lowering effects, we performed studies in mice fed the Western-type diet with or without 1% (w/w) PS for 5 weeks. This sphingolipid was chosen for all subsequent studies because it is one of the simplest in the sphingolipid class; it is the central structural element of ubiquitous sphingolipids of plants and yeasts that are part of our diet; and this lipid may be formed *in situ* in the intestine by enzymatic degradation of complex sphingolipids. No effects on body weight or food intake were ever observed (data not shown).

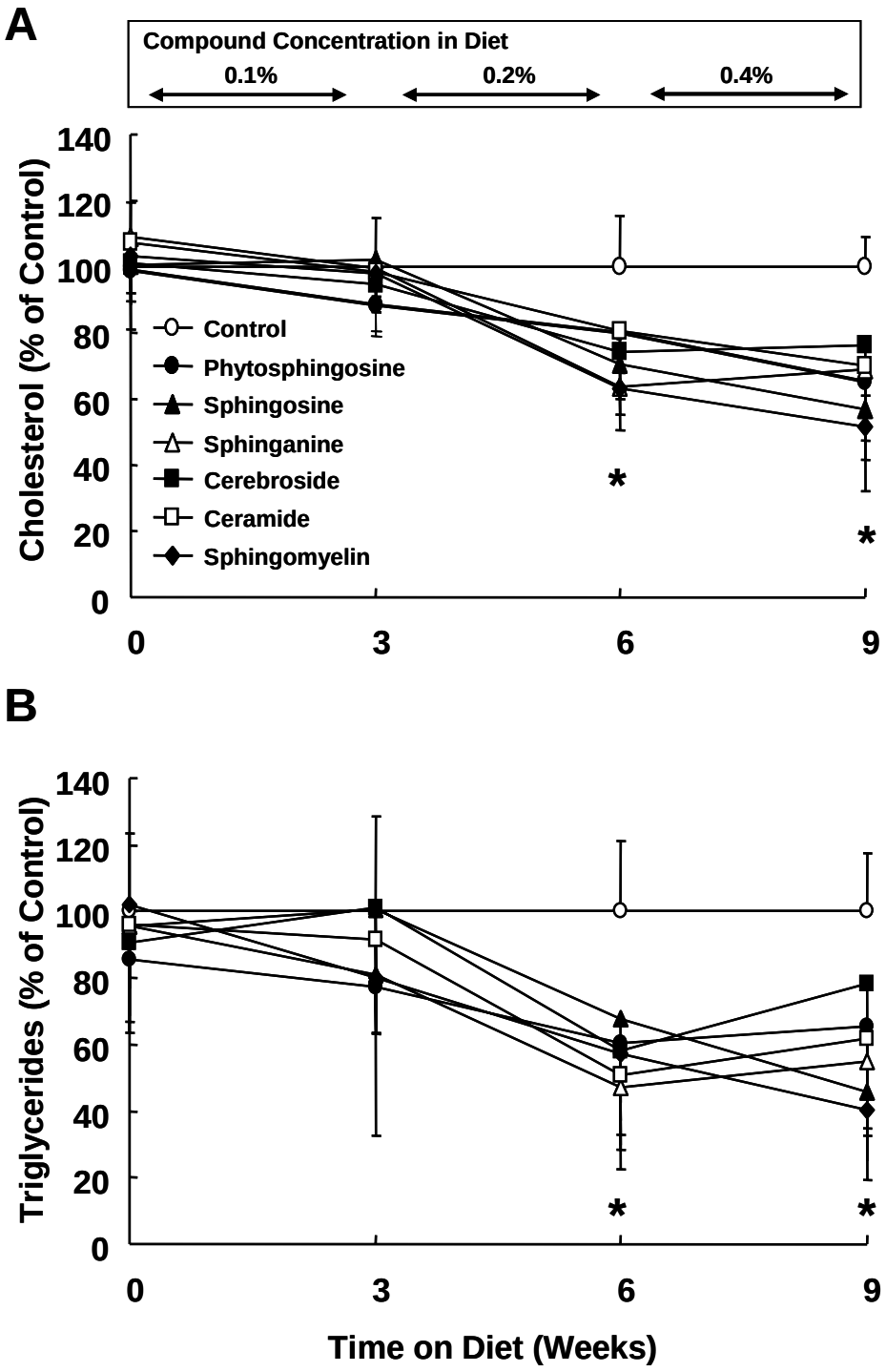

Figure 2. Plasma cholesterol (A) and triacylglycerol (B) concentrations in APOE*3Leiden mice after a 4h-fast

After a run-in period of 5 weeks with Western-type diet, female APOE*3Leiden mice were fed control Western-type diet, or this diet supplemented with increasing doses (0.1, 0.2 or 0.4% (w/w)) of the various sphingolipids (see also **Figure 1**) for periods of three weeks each (n = 6 per group). Baseline values for cholesterol and triacylglycerol were 15.0 ± 1.9 and 2.3 ± 0.5 mmol/l, respectively. Data shown are mean ± SD of percentages relative to the control group. *P < 0.05 vs. control using ANOVA, followed by Dunnett's test

Table 1. Plasma parameters in 4 h-fasted APOE*3Leiden mice fed a control Western-type diet or a 1% PS-containing Western-type diet for 5 weeks

	Control	1% PS	%Δ
Total cholesterol (mmol/l)	14.0 ± 1.2	6.1 ± 1.1***	-57
Triacylglycerol (mmol/l)	2.80 ± 0.52	1.19 ± 0.39***	-58
Free fatty acids (mmol/l)	1.67 ± 0.31	1.06 ± 0.14***	-36
β-hydroxybutyrate (mmol/l)	0.40 ± 0.25	0.49 ± 0.39	NS

Values shown are mean ± SD of 6 mice per group. ***P=0.001 vs. control, using the Student t-test. NS not significant, PS phytosphingosine

As can be seen in **Table 1**, mice fed a 1% PS-containing diet showed, as expected, a strong and significant decrease in plasma cholesterol and TG. Plasma FFA concentration also significantly decreased, whereas plasma β-hydroxybutyrate (a liver-derived keton body) did not change significantly (**Table 1**). Groupwise-pooled plasma was used to determine lipoprotein profiles of these mice. The lipoprotein profiles showed that the decrease in cholesterol and TG was confined to the VLDL and IDL/LDL fractions, while HDL cholesterol did not change (**Figure 3**).

Phytosphingosine reduces intestinal cholesterol, TG, and FFA absorption

We assessed the effects of dietary sphingolipids on intestinal cholesterol absorption by measuring absorption with the fecal dual-isotope method, using [^{14}C]cholesterol and [^{3}H]sitostanol, in mice fed a 1% PS-containing or control Western-type diet for 5 weeks. No differences in body weight, food intake, and fecal output were observed between the two groups (data not shown). We observed that the intestinal cholesterol absorption in PS-fed mice was only one-half of that in the control diet-fed mice (**Figure 4A**). This reduction was also reflected by the fecal neutral sterol excretion, which was twice as high in the PS-fed mice as in control mice (**Figure 4B**). We next determined whether the observed decrease in plasma TG and FFA was also due to decreased intestinal absorption. Mice fed a control or 1% PS-containing Western-type diet for 5 weeks were used. After an overnight-fast, the mice were i.v. injected with Triton WR1339 to inhibit lipoprotein lipolysis, followed by an intragastric gavage of [^{3}H]triolein and [^{14}C]oleic acid in olive oil. In the group fed 1% PS, the olive oil contained, in addition to [^{3}H]triolein and [^{14}C]oleic acid, 1% PS. Serum TG, [^{3}H]-activity, and [^{14}C]-activity were assayed over a 4h-period. The time-dependent appearance of plasma [^{3}H]-activity (a measure of intestinal TG uptake) is depicted for the two groups in **Figure 5A**. PS reduced the intestinal [^{3}H]TG uptake by 33% after 4 h. The plasma appearance of [^{14}C]oleate (a measure of intestinal FFA uptake) was 43% lower in PS-fed mice than in the control mice at 4 h after administration (**Figure 5B**).

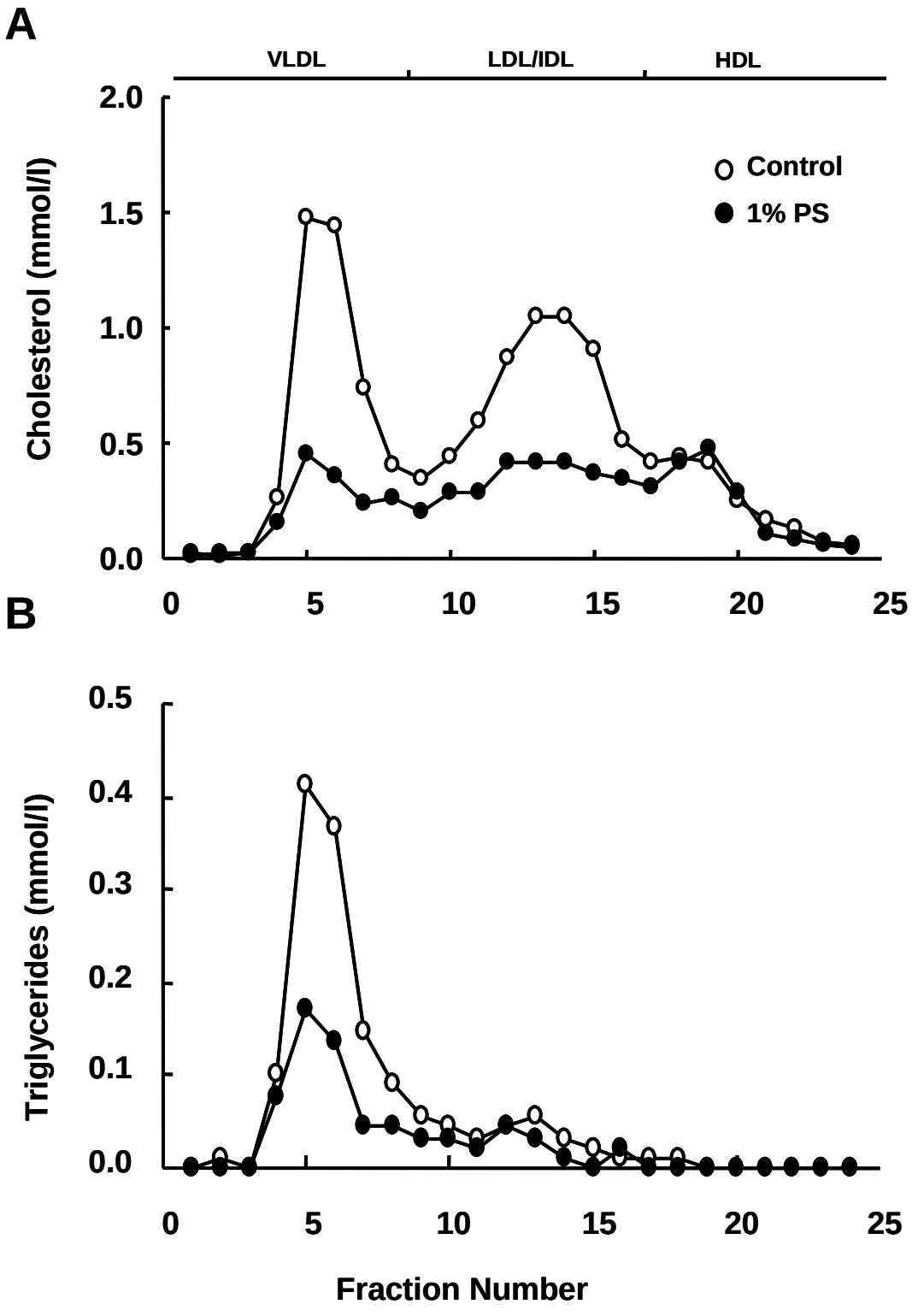

Figure 3. Cholesterol (A) and triacylglycerol (B) profiles after FPLC separation of plasma lipoproteins
Pooled plasma of six 4 h-fasted APOE*3Leiden mice fed a control Western-type diet (open circles) or this diet supplemented with 1% (w/w) PS (closed circles) for 5 weeks was used. Cholesterol and triacylglycerol concentrations were determined in the individual fractions after separation by fast-liquid chromatography (FPLC). IDL intermediate density lipoprotein, HDL high density lipoprotein, LDL low density lipoprotein, PS phytosphingosine, VLDL very low density lipoprotein

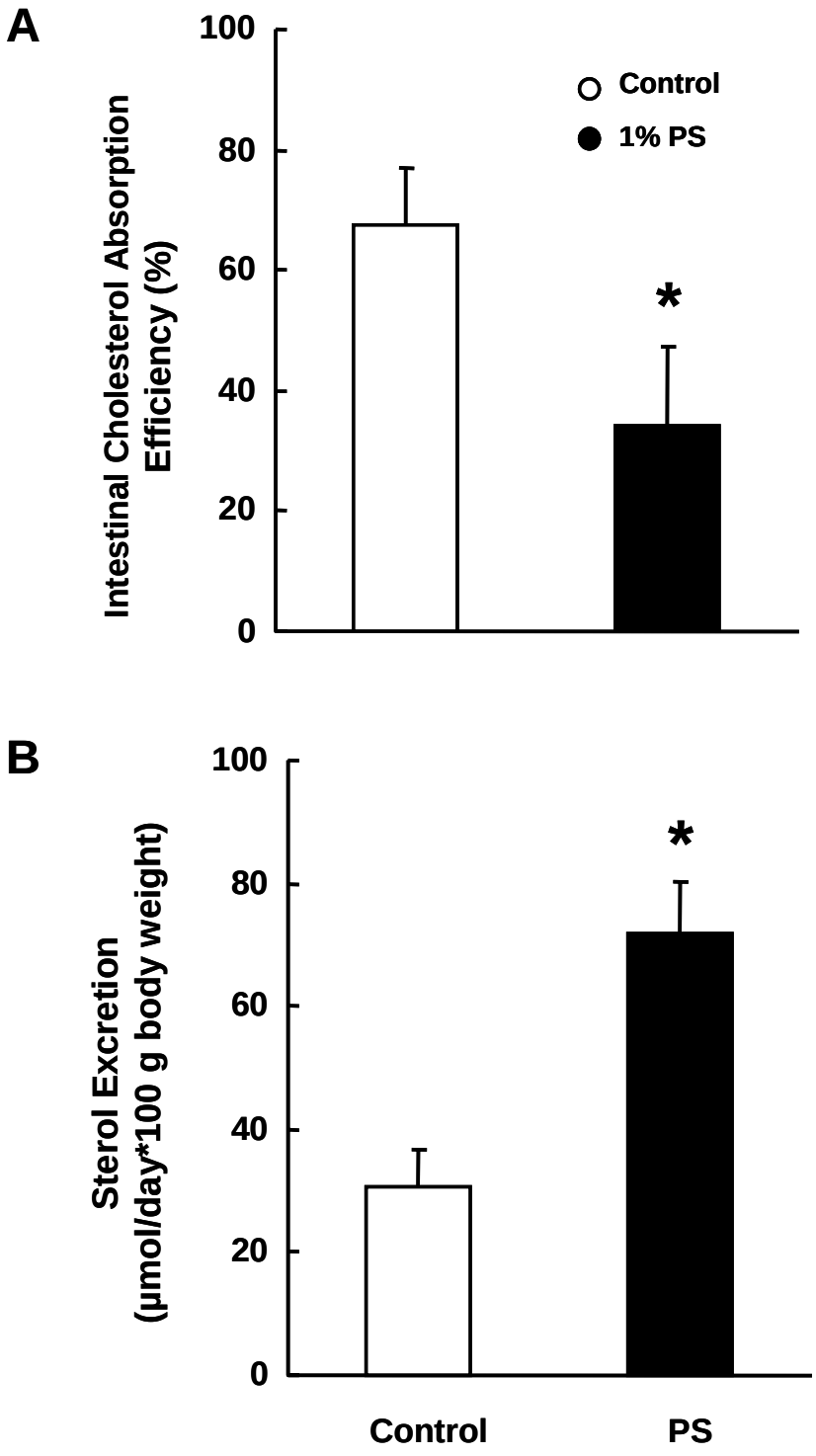

Figure 4. Intestinal cholesterol absorption (A) and neutral sterol excretion (B)
Mice (n = 6 per group) were fed 1% PS containing (closed bars) or control (open bars) Western-type diet for 5 weeks. The mice received a dose of 200 µl of olive oil, containing [^{14}C]cholesterol (1 µCi/mouse) and [^{3}H]sitostanol (1 µCi/mouse) by gavage. Feces were collected for 4 days. Radioactivity was determined in the fecal samples to assess the amount of radiolabeled cholesterol and sitostanol. Sitostanol was used as the reference compound (A). For neural sterol excretion analysis two mice were housed per cage, and feces were collected in 2 subsequent 3-day periods from 3 cages per group. Feces were analyzed as described in the materials and methods section (B). Data shown are mean ± SD. *P < 0.05 vs. control (Student's t-test)

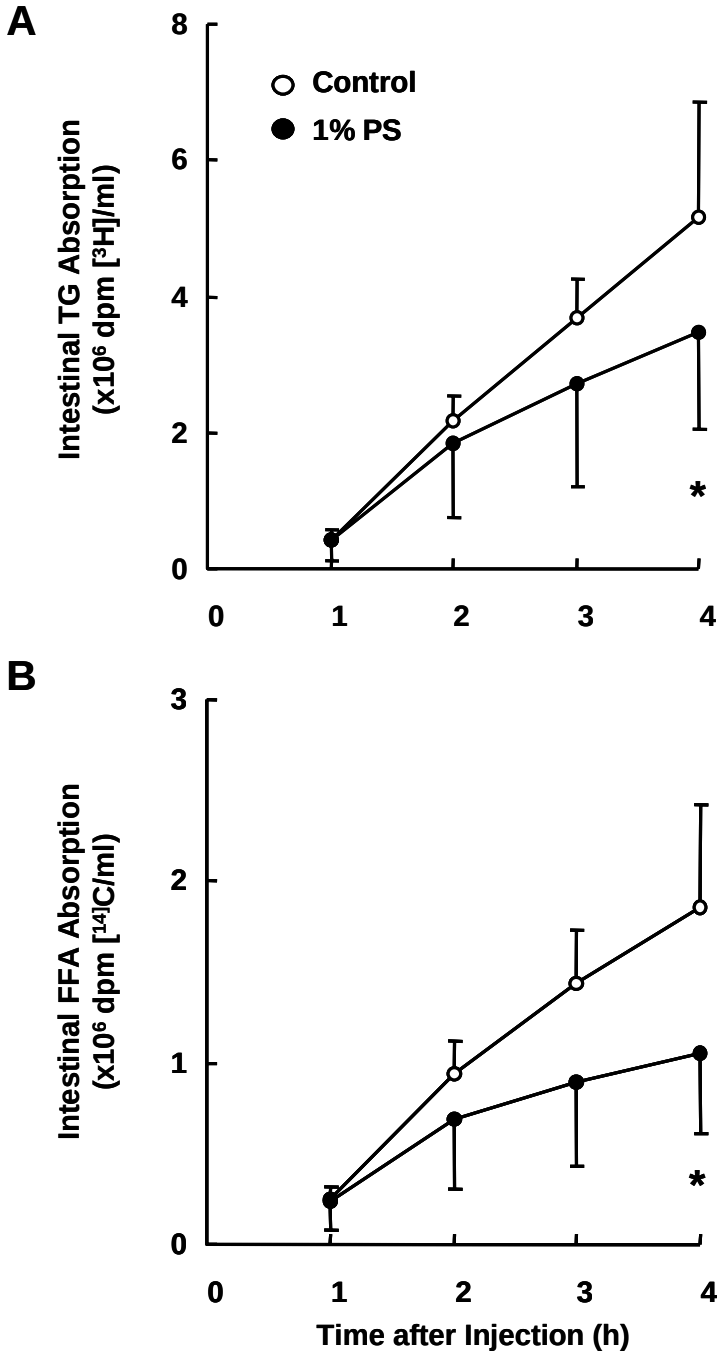

Figure 5. Intestinal TG (A) and FFA (B) absorption

After an overnight fast, mice (n = 6 per group) fed 1% PS-containing (closed circles) or control Western-type diet (open circles) for 5 weeks were injected with Triton WR1339 to block LPL-mediated TG hydrolysis and were given an intragastric load of 200 µl olive oil containing [^{3}H]triolein (12 µCi/mouse) and [^{14}C]oleic acid (3.3 µCi/mouse). For PS-fed animals, 1% PS was added to the olive oil load, since overnight fasting might annihilate any direct effect that PS may have on intestinal absorption. Plasma samples were obtained to determine the appearance of TG derived [^{3}H]-FA (A) or [^{14}C]-FFA (B) in the blood. Data shown are mean ± SD. After establishing significant interaction between time and treatment by two-way ANOVA (Figure A and B both $P < 0.05$), differences between control and PS-treated mice were calculated (*$P < 0.05$ vs. control). FFA free fatty acids, PS phytosphingosine, TG triglycerides

Phytosphingosine increases hepatic VLDL-TG production

Hepatic VLDL-TG production was studied in overnight-fasted mice by use of the Triton WR1339 method and by measuring plasma TG accumulation. In these experiments, plasma TG concentration increased faster in the mice fed 1% PS-containing diet than in mice fed the control Western-type diet. The VLDL-TG production rate, as determined from the slope of the curves, was 20% higher in PS-fed mice than in control mice (**Table 2**). Analysis of the composition of the VLDL particles (isolated by ultracentrifugation) revealed that the TG content as well as the phospholipid content increased by 66% and 17%, respectively, in VLDL particles derived from PS-fed mice (**Table 2**). Total cholesterol, in contrast, decreased by 51% in the VLDL particles from the PS-fed mice. The *de novo* total apoB production rate in newly synthesized VLDL particles did not differ between mice fed PS-containing diet and the control mice (**Table 2**). The data indicate that the number of VLDL particles secreted by the liver was not affected; however the VLDL particles from PS-fed mice contained less cholesterol, but more TG.

Table 2. Production rates of VLDL-TG and VLDL-apoB, and VLDL particle composition in APOE*3Leiden mice fed a control Western-type diet or the same diet supplemented with 1% PS for 5 weeks

	Control	1% PS	%Δ
Production rates of VLDL			
VLDL-TG production rate (µmol TG/h)	3.01 ± 0.44	3.63 ± 0.52***	+20
apoB production rate ($x10^4$ dpm/ml/h)	12.80 ± 2.57	13.47 ± 2.45	NS
Composition of VLDL relative to protein			
Total cholesterol (µmol/mg VLDL protein)	24.39 ± 3.73	11.94 ± 2.16**	-51
Triacylglycerol (µmol/mg VLDL protein)	22.28 ± 2.37	36.93 ± 7.17**	+66
Phospholipid (µmol/mg VLDL protein)	6.47 ± 0.92	7.57± 1.13*	+17

Mice were fasted overnight and injected with ^{35}S label and Triton WR1339. Timed blood samples were taken to determine TG concentration. At 2 h, VLDL (d < 1.006g/ml) was isolated by ultracentrifugation, and TG, total cholesterol, phospholipid and protein content were determined. Values represent mean ± SD for 10 mice per group. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control using Student's or Welch's t-test; NS, not significant. Apo apolipoprotein, PS phytosphingosine, TG triglycerides, VLDL very low density lipoprotein

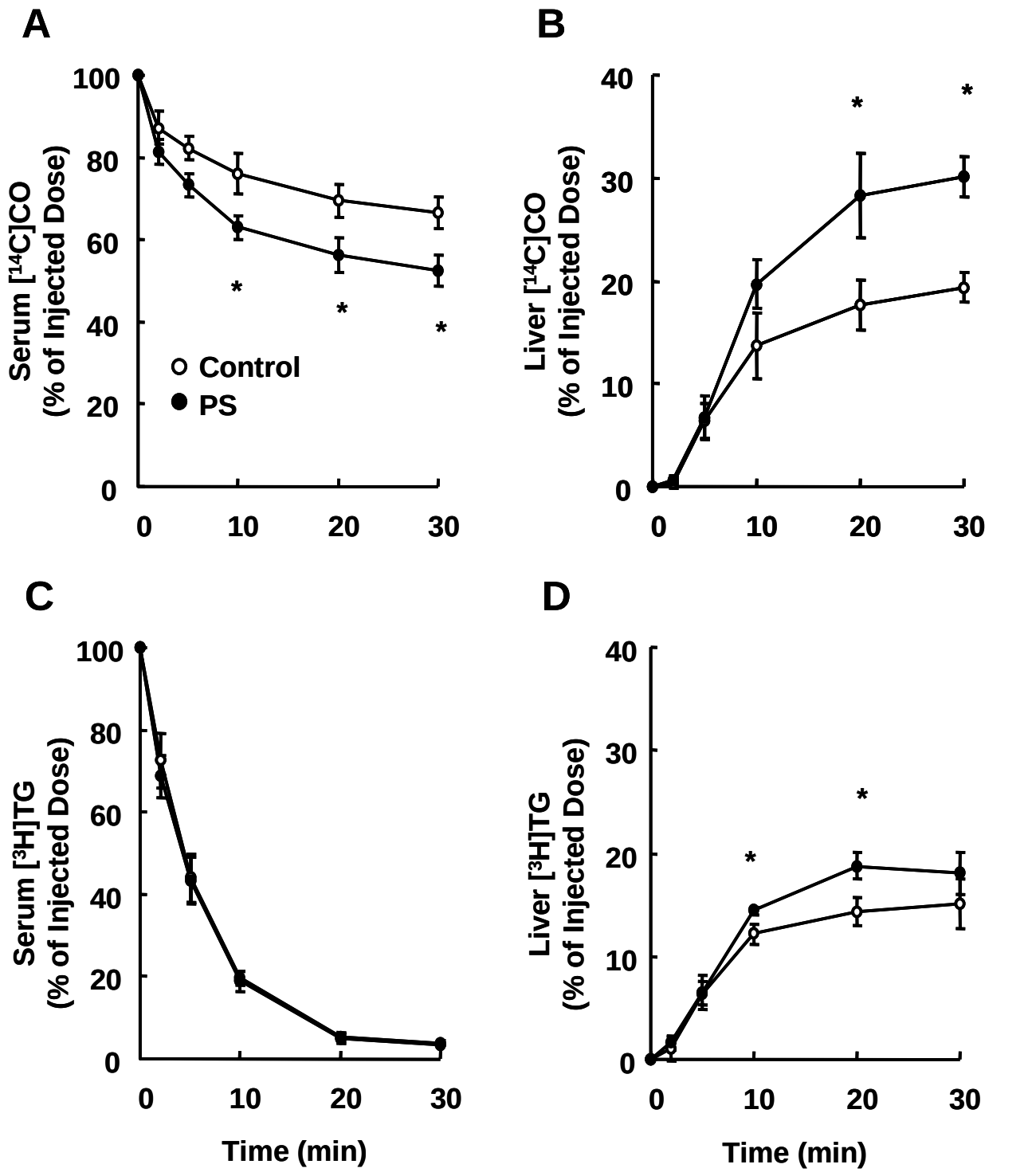

Figure 6. *In vivo* clearance of VLDL-like emulsion particles
After 5 weeks of 1% PS-containing (closed circles) or control (open circles) Western-type diet, fed mice (n = 6 per group) were injected into the vena cava inferior with VLDL-like emulsion particles, containing [^{3}H]triolein (TG) and [^{14}C]cholesteryl oleate (CO), at a dose of 300 µg TG per mouse. Over time, serum (A,C) and liver (B,D) samples were taken to determine radioactivity. Data shown are mean ± SD. After establishing significant interaction between time and treatment by two-way ANOVA (Figure A, B and D all $P < 0.05$), differences between control and PS-treated mice were calculated (*$P < 0.05$ vs. control). In Figure 6C, differences were not significant. PS phytosphingosine

Phytosphingosine increases liver-mediated clearance of plasma cholesterol but not plasma TG

Plasma cholesterol and TG concentrations are not only determined by their production rate but also by their clearance, *i.e.*, by their uptake, lipolysis, or both. VLDL-like particles containing [^{3}H]triolein and [^{14}C]cholesteryl oleate, which were previously shown to mimic the metabolic behavior of TG-rich lipoproteins[17,28], were used to determine the effects of an 1% PS-containing Western-type diet on plasma clearance. PS accelerated the plasma clearance of [^{14}C]cholesteryl oleate ($t_{½}$ 39.5 ± 5.3 vs. 74.5 ± 9.9 min in PS-treated vs. control mice, respectively; $P < 0.05$; **Figure 6A**). The enhanced removal of cholesterol from the blood is corroborated by the increased liver uptake of [^{14}C]cholesteryl oleate (+60% at 20 min; **Figure 6B**). Although lipoprotein lipase (LPL)-dependent serum clearance of [^{3}H]TG was not affected ($t_{½}$ 4.7 ± 0.3 vs. 4.9 ± 0.3 min in PS-treated vs. control mice; **Figure 6C**), [^{3}H]TG uptake in the liver 10 min and 20 min after injection increased significantly in the PS-fed mice (**Figure 6D**). No effects were observed on the uptake of [^{14}C]cholesteryl oleate or [^{3}H]TG-derived radioactivity by various peripheral muscle and adipose tissues (data not shown). Taken together, the results of this experiment indicate an increase in VLDL particle remnant uptake in the liver.

Hepatic mRNA concentrations indicate increased lipid synthesis

The hepatic expression of several genes was studied by using RT-PCR on liver samples of 4 h-fasted mice given the 1% PS-containing or the control Western-type diet for 5 weeks (**Table 3**). The mRNA concentrations of genes involved in FA and TG synthesis and secretion (*srebp1c, fas, mttp* and *dgat2*) increased. *Acc1, dgat1, aco and apob* transcription concentrations were unaltered. Furthermore, mRNA concentrations of cholesterol homeostasis genes, such as *srebp2*, *ldlr*, and *hmgcoAred,* also increased. In strong contrast, the two genes involved in bile salt formation studied decreased (*cyp7a1* and *lxrβ*), while *lxrα* and *fxr* increased. *Pparα*, *pparγ* and *abca1* expression did not change (**Table 3**). Overall, these changes suggest increased hepatic lipid and cholesterol synthesis and decreased bile formation, which indicates a shift in hepatic TG and cholesterol homeostasis compared with the control situation.

Phytosphingosine protects livers from steatosis

Increased remnant uptake and increased mRNA expression of genes involved in FA and TG synthesis suggested that intrahepatic TG and cholesterol concentrations increased in our setting. However, at autopsy of these mice, it was noted that the livers of the mice fed the 1% PS-containing Western-type diet were of normal size and had a dark-red appearance, whereas the livers of the control mice fed the Western-type diet without PS were enlarged and yellowish (**Figure 7A** and **B**). This indicates that the hepatic lipid content was lower in the PS-fed mice than in the control mice. Microscopical examination of HPS-

stained sections showed that, compared with the controls, the PS-fed mice had less lipid-filled vacuoles in the liver cells (**Figure 7C** and **D**). The livers of PS-fed mice weighed significantly less (22%) than those of the control mice (**Table 4**). Lipid analysis revealed that livers of PS-fed mice contained less TG (56%) than livers of control mice. Furthermore, liver cholesteryl esters were decreased by 61% as was free cholesterol by 11% in PS-fed mice. All these differences were significant (**Table 4**).

Table 3. Hepatic mRNA expression of genes involved in FA and TG and cholesterol homeostasis of APOE*3Leiden mice fed a control or a 1% PS-containing Western-type diet for 5 weeks, as determined by RT-PCR

mRNA of	Control	1% PS	Change
FA/TG homeostasis			
srebp1c	100 ± 7 %	194 ± 3 %**	↑
fas	100 ± 6 %	237 ± 33 %*	↑
mttp	100 ± 4 %	167 ± 5 %**	↑
dgat2	100 ± 17%	185 ± 23 %*	↑
acc1	100 ± 6 %	138 ± 29 %	NS
dgat1	100 ± 25 %	112 ± 27 %	NS
aco	100 ± 12 %	56 ± 9 %	NS
apob	100 ± 15 %	105 ± 25 %	NS
Cholesterol homeostasis			
srebp2	100 ± 9 %	317 ± 33 %*	↑
ldlr	100 ± 7 %	209 ± 14 %**	↑
hmgcoAred	100 ± 7 %	444 ± 71 %**	↑
cyp7a1	100 ± 10 %	7 ± 2 %*	↓
lxrβ	100 ± 18 %	16 ± 8 %*	↓
lxrα	100 ± 4 %	162 ± 13 %*	↑
fxr	100 ± 15 %	164 ± 58 %*	↑
pparα	100 ± 1 %	125 ± 10 %	NS
pparγ	100 ± 7 %	119 ± 10 %	NS
abca1	100 ± 12 %	79 ± 6 %	NS

Values represent mean ± SD of 5 mice per group. mRNA expression was normalized to HPRT. *P < 0.05, **P < 0.01 vs. control using the Mann-Whitney U test. abca1 ATP-binding cassette transporter, acc acetyl-CoA carboxylase, aco acyl-coenzyme A oxidase, apo apolipoprotein, cyp7a1 Cholesterol 7-alpha-hydroxylase, dgat acyl:diacylglycerol transferase, fas fatty acid synthase, fxr farnesoid X receptor, hmgcoAred 3-hydroxy-3-methyl glutaryl coenzyme A reductase, HPRT hypoxanthine guanine phosphoribosyl transferase, ldlr LDL receptor, lxr liver X receptor; mttp microsomal TG transfer protein, NS not significant, ppar peroxisome proliferator-activated receptor, PS phytosphingosine, srebp, sterol regulatory binding protein

Phytosphingosine lowers plasma inflammatory markers

Hepatic steatosis is often associated with liver inflammation. We determined plasma ALAT concentration, as a measure for liver damage. The ALAT concentration decreased by 79% (P < 0.05) after PS-feeding. A 74% (P < 0.05) decrease in the concentration of the acute phase marker SAA was found in PS-fed mice. The fibrinogen concentration was 42% higher (P < 0.05) in PS-treated mice compared with controls (**Table 4**).

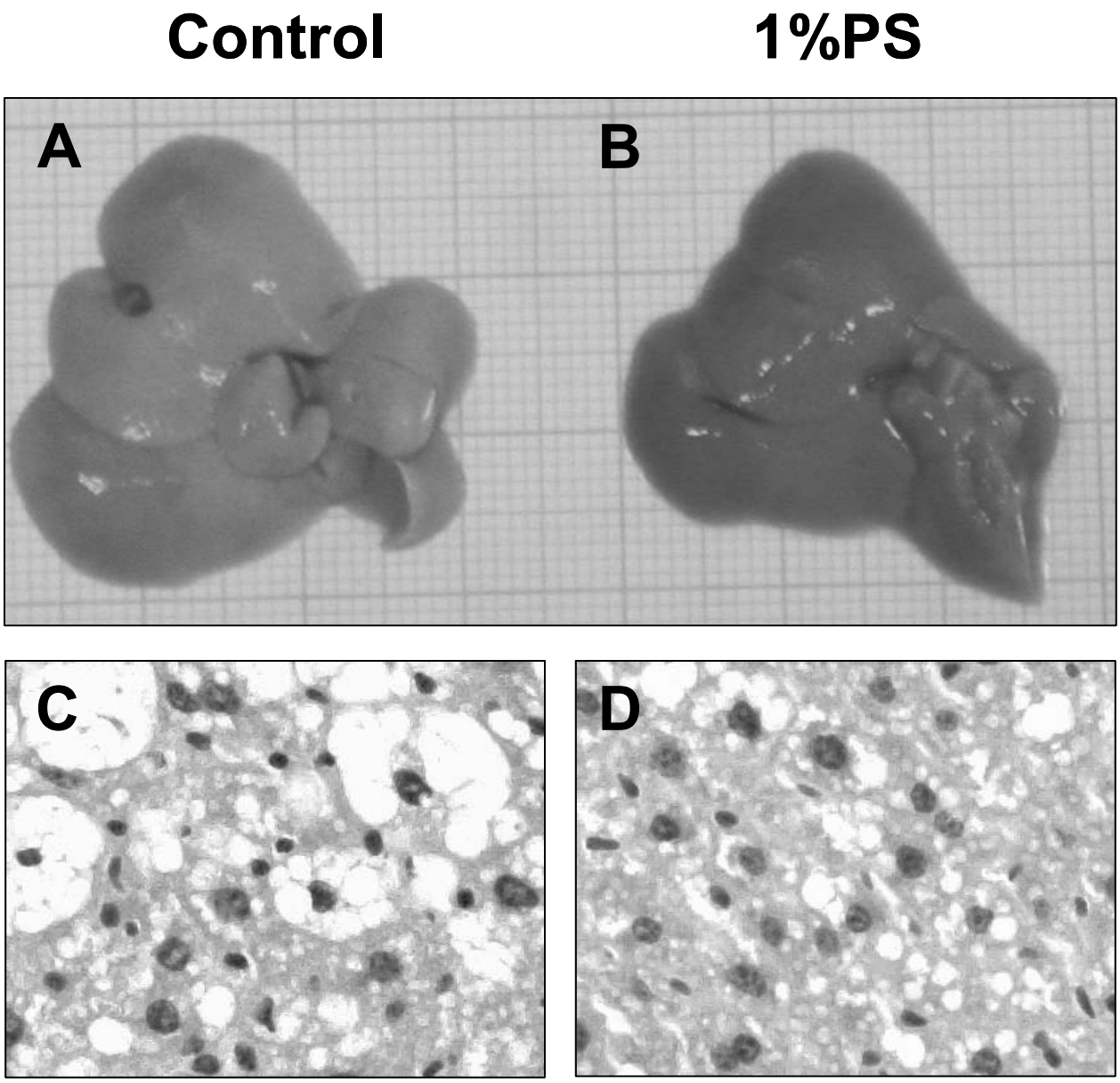

Figure 7. Liver analysis
Macroscopic appearance of a liver from a mouse fed the control Western-type diet for 5 weeks (A) and from a mouse fed the same diet with 1% PS (B). Haematoxylin-phloxine-saffron stained histological micrographs of paraffin-embedded livers from a mouse fed the Western-type diet for 5 weeks (C) and a mouse fed the same diet containing 1% PS (D). PS phytosphingosine.

Table 4. Liver lipid parameters and plasma inflammation markers determined in 4 h-fasted APOE*3Leiden mice after feeding a control or a 1% PS-containing Western-type diet for 5 weeks

	Control	1% PS	%Δ
Liver parameters			
Liver weight (g)	1.34 ± 0.12	1.05 ± 0.08***	-22
Triacylglycerol (µg/mg protein)	34.62 ± 2.23	15.28 ± 3.84***	-56
Free cholesterol (µg/mg protein)	7.81 ± 0.51	6.95 ± 0.78*	-11
Cholesteryl ester (µg/mg protein)	13.44 ± 0.79	5.20 ± 0.79***	-61
Plasma inflammation markers			
ALAT (U/l)	189 ± 49	41 ± 21***	-79
SAA (µg/ml)	11.2 ± 3.6	3.0 ± 0.3**	-74
Fibrinogen (mg/ml)	1.6 ± 0.5	2.3 ± 0.7***	+42

Values represent mean ± SD of 6 mice per group. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control using Student's or Welch's t-test. ALAT alanine aminotransaminase, PS phytosphingosine, SAA serum amyloid A

Discussion

From this study we conclude that sphingolipids dose-dependently decrease plasma cholesterol and TG concentrations in APOE*3Leiden mice fed a Western-type diet. This cholesterol- and TG-lowering effect is mediated through inhibition of intestinal absorption of both cholesterol and TG and, eventually, leads to protection of the liver from fat and cholesterol-induced steatosis.

Since the intestinal absorption of FA and TG were inhibited to the same extent, we conclude that intestinal TG lipolysis *per se* is not inhibited by sphingolipids but that the absorption of FA is impaired. We do not know the exact mechanism underlying the PS-mediated inhibition of FA absorption. However, the FA-PS complex formation via an ionic interaction between the negatively charged carboxylic acid group of FA and the positively charged primary amine of PS might be an explanation. Although complex sphingolipids do not have such a primary amine group, in the intestine they can be lipolyzed to some extent into sphingoid bases and, thus, the same mechanism will, eventually, also hold for these complex molecules. However, it has been observed that some dietary sphingolipids are not fully digested and are partly excreted via the feces[29].

The intestinal FA absorption experiments (**Figure 5**) were performed applying an intragastric olive oil load. As compared with conditions of normal *ad libitum* food intake, intragastric olive oil loads represent extreme conditions of intestinal FA absorption. Thus, quantitative extrapolation of the observed inhibitory effect of PS on the FA absorption during a intragastric fat load to FA absorption under normal feeding conditions is hazardous. Under normal feeding conditions the effect of PS on the FA absorption is assumed to be more modest, and the mice may compensate with increased glucose utilization. The observation that a PS-containing diet does not significantly affect food intake or body weight in the long term is in line with this reasoning.

Intestinal absorption of cholesterol depends on bile salts, and is favored by the presence in the intestine of TG-derived FA that form mixed micelles with bile salts in which cholesterol is solubilized[30]. Since we observed no effect of PS on intestinal TG hydrolysis (**Figure 5**), disturbance of the composition of these mixed micelles by sphingolipids, leading to hampered solubilization of cholesterol, seems unlikely. The formation of stable cholesterol and sphingomyelin (or sphingosine) complexes has been described[29,30] and could, as such, be the cause of a reduced intestinal absorption of cholesterol. However, whether the formation of cholesterol-sphingolipid complexes also occurs in the intestine has been questioned, since the high affinity of cholesterol for sphingomyelin is lost in the presence of bile salts[31,32]. We found that both simple and complex sphingolipids (**Figures 1** and **2**) decrease plasma cholesterol and TG concentrations in APOE*3Leiden mice. Because of the diversity in chemical structure between the various sphingolipid species, a wide range in physical and chemical properties may be expected and, thus, the inhibition of intestinal absorption of cholesterol and TG by PS is not likely to be explained by specific complex formation with bile salts or disturbance of bile salt micelles in the intestinal lumen. However, other yet unknown biochemical processes may be influenced by dietary sphingolipids or their metabolites. For example, the effect of the sphingolipid diet on the bile salt profile was not investigated, but changes in that profile (and in bile salt production) potentially may affect the intestinal lipid absorption and may thus influence plasma lipid concentrations. This hypothesis is sustained by the observation that genes involved in the bile salt synthesis are indeed affected by the sphingolipid diet (see **Table 3**).

The lipid-lowering effect of both cholesterol and TG of PS-feeding in APOE*3Leiden mice clearly differs from the effects of feeding stanol esters, which results in cholesterol-lowering only, while plasma TG remains unaffected[5]. Importantly, in contrast with the present dietary sphingolipid study, the expression of hepatic key genes involved in lipid metabolism (*i.e.*, *ldlr* and *cyp7a1*), were not affected in APOE3*Leiden mice fed with plant stanol esters[33]. In addition, PS proved to be about twice as effective at decreasing plasma cholesterol in APOE*3Leiden mice as stanol esters. At a dose of 1% of plant stanol ester for 9 weeks, a 33% decrease in total plasma cholesterol was observed[5], whereas PS-feeding at the same concentration reduced plasma cholesterol by 57%. Reciprocally, as did PS, dietary stanol esters decreased hepatic cholesteryl esters, free cholesterol, and TG concentrations in APOE*3Leiden mice[33].

Since blood samples were taken from animals that were fasted for 4 hours and are thus not expected to have intestine-derived chylomicrons in their plasma, the decreased concentration of plasma cholesterol and TG could not be directly ascribed to the PS-mediated inhibition of intestinal cholesterol and TG absorption. We reasoned that reduction in the absorption of dietary plus biliary cholesterol leads to a reduction in the liver cholesterol pool as presented in **Table 4**. A reduction in the cholesterol pool in the liver leads to a reduction in bile acid synthesis as reflected by a reduced expression in the liver of bile

acid synthesis genes such as *lxrβ* and *cyp7a1*, concomitant with an increased expression of genes involved in hepatic cholesterol synthesis (*hmgcoA* reductase) and hepatic cholesterol uptake from plasma (*ldlr*) (**Table 3**). It can also be concluded from the results presented in **Table 3** that the expression of genes involved in lipid synthesis (*srebp1c*, *fas* and *dgat2*) and VLDL production (*mttp*) increased. Indeed, as presented in **Table 2**, measurement of VLDL-TG production *in vivo* revealed a 20% enhanced secretion of VLDL-TG by the liver, whereas the number of VLDL particles secreted into the circulation (apoB synthesis) remained constant.

The observations that these newly synthesized VLDL particles exhibit a 50% reduction in cholesterol content (**Table 2**) and a strongly increased hepatic uptake of their remnants (**Figure 6B**), the latter is in line with the increased expression of the hepatic *ldlr* (**Table 3**), offers an explanation for the cholesterol-lowering effect of dietary PS. In contrast, the increased hepatic secretion of VLDL-TG does not sustain the TG-lowering effect of PS. From **Figures 6B** and **D** it can be concluded that, after LPL-mediated lipolysis more than 80% of VLDL-derived TG is taken up by peripheral tissues for within 20 minutes; the remainder is taken up by the liver (**Figure 6D**). A difference in plasma clearance of VLDL-TG between PS- and control-fed mice could not be observed under the experimental conditions used (**Figure 6C**). However, as depicted in **Figure 6D** the hepatic uptake of VLDL-TG increased significantly in the PS-fed group, thereby sustaining the TG-lowering effect of PS-feeding. In PS-fed mice, the newly synthesized VLDL particles were larger and relatively enriched in TG compared with control fed mice (**Table 2**). Since the affinity of TG-rich emulsion particles[17] and lipoproteins[34] for LPL is higher with increasing particle size, this change in lipid composition could also (partly) explain the TG-lowering effect of PS-feeding.

Although sphingolipids have been suggested to be PPARα agonists[35], we found no changes in expression of PPARα or in genes that are under PPARα control, such as *aco* (**Table 3**). Furthermore, we did not observe enlarged livers in PS-treated mice, as would be expected with PPARα agonists in rodents[36,37]. In contrast, the compared with control mice, the livers of PS-fed mice weighed even significantly less, than those of the control mice, contained less lipid (cholesteryl esters and TG, **Table 4**), and contained smaller lipid-filled vacuoles in the parenchymal cells (**Figure 7D**). To maintain its lipid homeostasis, the liver compensates for the lower PS-mediated dietary and biliary cholesterol and TG supply from the intestine by increasing its endogenous cholesterol and FA synthesis, as is reflected in the increased hepatic mRNA concentrations of *fas* and *hmgcoAred*. However, hepatic lipid synthesis is well regulated by a feed-back mechanism. Consequently, on PS-feeding, hepatic cholesterol or TG synthesis increased but lipids do not accumulate in the liver (see **Table 4**).

In steatotic livers, lipid accumulation is accompanied by an increase in plasma ALAT and SAA, which are markers of liver damage and liver inflammation, respectively. In

PS-fed mice both markers decreased dramatically, pointing to a true hepato-protective effect of dietary PS under conditions of Western-type diet feeding. Liver steatosis, and elevated ALAT and SAA concentrations are associated with insulin resistance and the metabolic syndrome in mice and humans[38,39]. Furthermore, inflammatory reactions are involved in the atherosclerotic process[40]. Therefore, dietary sphingolipids should be considered as compounds for treating or ameliorating not only the lipid component of cardiovascular disease, but also the inflammatory processes involved in atherosclerosis and insulin resistance. We suggest that dietary sphingolipids hold great potential to treat multiple aspects of the metabolic syndrome, such as dyslipidemia, insulin resistance and cardiovascular diseases.

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Chapter 6

Discussion

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Future Perspectives

The metabolic syndrome is an increasing problem in our Western society. Many of the features of the metabolic syndrome like obesity, hepatic steatosis, insulin resistance and dyslipidemia are established risk factors for cardiovascular diseases. Growing evidence shows that handling of free fatty acids (FA) and/or body distribution of triglycerides and free FA plays a central role in the pathogenesis of the problems associated with the metabolic syndrome. In this thesis we present different studies aimed at unraveling the pathophysiological mechanisms underlying the development of obesity, dyslipidemia, insulin resistance and hepatic steatosis.

Several mouse studies indicate that decreased lipoprotein lipase (LPL) activity in adipose tissue decreases the propensity to develop obesity[1-5]. However, it was unclear whether the opposite was true as well, *i.e.*, whether activation of LPL leads to an enhanced susceptibility to diet-induced obesity and associated insulin resistance. We showed that, during high-fat feeding, the absence of apolipoprotein (apo) C3, a strong inhibitor of LPL, indeed leads to a higher adipose tissue mass, concomitant with insulin resistance and mild hepatic steatosis compared with wild-type littermates (**chapter 2**). It would be of interest, to investigate whether apoC3 overexpression leads to less adipose tissue mass.

Obesity does not necessarily lead to insulin resistance in the liver. As long as the FA flux toward the adipose tissue does not lead to increased FA flux to the liver, hepatic insulin resistance is not expected to occur. For instance, peroxisome proliferator-activated receptor (PPAR) γ agonist treatment leads to increased adipose tissue LPL expression[6], concomitant with more adipocyte differentiation[7] and increased whole body insulin sensitivity. It may be concluded that growing obese *per se* is not detrimental to the development of diabetes type 2 and cardiovascular health. Diminishing FA fluxes from liver and plasma to adipose tissue, as well as increasing adipose tissue lipolysis are two possibilities to prevent obesity, but may be detrimental, rather than favorable to reducing the incidence of the metabolic syndrome. It is obvious, that not the adipose tissue mass *per se* but rather the FA fluxes determining the adipose tissue mass are fundamental to the metabolic relationship between obesity and insulin resistance.

However, it is not only FA fluxes. Recent studies clearly showed that various endocrine factors are secreted by adipose tissue, like leptin, resistin and adiponectin. These hormones are known to affect insulin sensitivity and are correlated with adipose tissue mass[8-11]. In this thesis we described that apoC3-deficient mice indeed had increased plasma leptin levels, in accordance with an increase in adipose tissue mass. Although it is likely that the hyperleptinemia observed in these mice is the consequence rather than the cause of insulin resistance, as has been observed earlier in humans[8,12-15].

Our results show that the liver can modulate plasma lipid levels and at the same time fat mass and insulin sensitivity through production of just one protein, apoC3. It warrants further investigation, whether in man apoC3 is a potential therapeutic target for

treatment of obesity and/or obesity-related insulin resistance and at the same time for prevention of cardiovascular risk.

Next to adipose tissue mass, lipogenesis, chylomicron- and very low density lipoprotein (VLDL)-remnant uptake, VLDL production and secretion, as well as β-oxidation of FA, are fundamental to hepatic steatosis and, eventually, hepatic insulin resistance. We wondered whether inhibition of hepatic β-oxidation increases hepatic steatosis, VLDL production and/or secretion, or both (**chapter 3**). Therefore, we used methyl palmoxirate (MP), an inhibitor of carnitine palmitoyl transferase I (CPTI), to acutely inhibit hepatic FA oxidation. Indeed, within 2 hours after oral dosing of MP, plasma keton bodies dropped and remained less than 10% for up to 8 hours after gavage. Since plasma keton bodies are solely derived from hepatic β-oxidation, we concluded that hepatic β-oxidation of long-chain FA was almost completely inhibited by the applied dose of MP.

As expected, inhibition of hepatic β-oxidation led to significant accumulation of TG in the liver. This increased hepatic TG accumulation was not associated with increased hepatic VLDL-TG production and/or changes in VLDL-composition. However, we did observe an increase in mRNA expression of microsomal triglyceride transfer protein (*mttp*), involved in hepatic VLDL assembly and secretion, in the livers of MP-treated mice. Therefore, we cannot exclude that chronic, long-term inhibition of hepatic β-oxidation does induce hepatic VLDL-TG production.

Several studies have demonstrated that β-oxidation inhibitors (like etomoxir and MP) are effective at lowering both keton body and glucose levels in rodents, dogs and humans[16-20]. In our overnight-fasted mice plasma glucose levels were similar between MP-treated and control mice. Also, we observed that acute inhibition of β-oxidation was associated with strongly decreased plasma insulin levels. This is in line with Boden et al.[21] who show that in humans there is a positive correlation between plasma keton body concentrations and insulin secretion capacity.

Our study clearly showed that, in contrast to adipose tissue-mediated hepatic steatosis, hepatic steatosis as a consequence of inhibition of β-oxidation does not lead to hepatic insulin resistance. In addition, β-oxidation related hepatic steatosis does not result in increased VLDL production. Thus, the metabolic relationship between hepatic steatosis on one hand, and insulin resistance and VLDL production on the other hand, seems to be dependent on the pathway, via which the TG have been accumulated. These findings argue against a common assumption that the production of VLDL in the liver is substrate-driven. In that respect it would be interesting to know whether stimulation of β-oxidation, by for instance tetradecylthioacetic acid administration[22], would result in decreased hepatic steatosis, concomitant with increased hepatic insulin sensitivity and decreased VLDL production.

Whole-body FA metabolism is driven by the FA homeostasis in adipose tissue and liver, and is a strong determinant of plasma lipid levels and cardiovascular risk. In this respect, much attention has been paid to the effect of specific dietary FA: saturated, (poly)unsaturated, *trans*- and *cis*-unsaturated FA and conjugated linoleic acids (CLA). The mechanisms underlying the various effects of these FA on plasma lipid levels and/or cardiovascular risk has to date not become clear, and the results obtained from dietary studies are often inconsistent due to differences in study design and different animal models used. Therefore, we decided to study the effect of various specific FA on plasma and hepatic lipid levels using a single animal model, with a human-like lipoprotein metabolism, that has been proven to be sensitive to relatively mild perturbations in the diet, the APOE*3Leiden mouse (**chapter 4**). Indeed, our results showed that the various FA differ clearly in their effects on plasma and liver lipid levels in this animal model. To obtain more insight in the underlying mechanisms, we focused on the liver as a central organ in lipid/lipoprotein metabolism by applying a proteomics approach. The results showed that the different specific dietary FA have different effects on protein composition of the liver. Although the combination of proteomics with physiology gave us more insight in the mechanisms by which these FA (may) regulate lipid metabolism and related pathways, the current study is an example of the very beginning of the application of the “omics” approach in finding new relevant molecular pathways.

The statistical analyses of our results revealed many associations, some of which are well known, including the associations with aspects of the metabolic syndrome, whereas many others will be the basis of intriguing new leads for further studies. In the future, these studies should be repeated and extended with other “omics” approaches, like metabolomics and transcriptomics. By doing so, many new promising and less promising molecular pathways underlying the metabolic syndrome and cardiovascular diseases are expected to be found. Subsequently, additional (classical) biochemical/physiological studies have to be executed to evaluate the relevance of the respective pathways. Since the number of possible pathways will be quite extensive, it will be a great challenge to choose the most promising pathway right from the start of this inevitable and necessary “post-omics” era.

It is obvious, that the liver plays a pivotal role in both lipid and glucose homeostasis. Glucose and lipid metabolism are tightly interrelated and a steatotic liver is often the culprit for disturbances in both glucose and lipid metabolism. Interventions to improve liver TG content, and as a consequence, insulin sensitivity and plasma lipid levels is highly needed, especially in Western society where the obesity-related metabolic syndrome is highly prevalent and responsible for the high risk of cardiovascular diseases.

At present, several drugs are available to treat one or maximally two aspects of the metabolic syndrome at a time. Treatment of multiple aspects of the metabolic syndrome with a single natural dietary compound would be an attractive alternative.

Although far from clearly conclusive, various animal studies have been published in the past claiming health benefits for dietary sphingolipids regarding lowering plasma lipid levels. Sphingolipids are membrane constituents in plants, yeasts and animals and are present in our daily diet. In **chapter 5** we first questioned whether sphingolipids supplemented to the Western-type diet indeed decrease plasma cholesterol and/or triglycerides in our “humanized” hyperlipidemic APOE*3Leiden mouse model. We found that both simple and complex sphingolipids decrease plasma lipid levels in this mouse model, the primary underlying mechanism being the inhibition of the intestinal absorption of both cholesterol and TG.

More importantly, we clearly observed that the livers of phytosphingosine (PS)-fed mice weighed significantly less than livers of control mice, and contained less cholesteryl esters and TG, and less lipid-filled vacuoles in the parenchymal cells. In addition, plasma levels of ALAT and SAA, markers for liver damage and liver inflammation, respectively, were strongly decreased. These results point to a true hepatoprotective effect of dietary PS under conditions of Western-type diet feeding. Since inflammatory parameters are involved in both atherosclerotic and diabetes/insulin resistance related processes, dietary sphingolipids may therefore be considered as compounds useful in treating or ameliorating not only the lipid component of cardiovascular disease, but also the insulin-resistance components of the metabolic syndrome.

In studies not presented in this thesis we indeed showed that PS added to the diet improves obesity-related insulin resistance in mice. In a pilot study with human volunteers we showed that daily supplementation of one gram of PS also resulted in a reduction of total plasma cholesterol. A more extended clinical study with metabolic syndrome patients is currently being designed. In that study, next to evaluation of the effect of PS on plasma lipid, glucose and insulin levels, strong focus will be on hepatic steatosis as measured by non-invasive magnetic resonance imaging (MRI) analyses. We expect to conclude from such a study, that sphingolipids hold great potential to treat or prevent metabolic syndrome and, eventually cardiovascular disease.

Besides obesity, administration of some drugs and also alcohol consumption often lead to steatotic livers. Although these forms of hepatic steatosis may be metabolically different from obesity-related steatosis, it is tempting to investigate whether the addition of sphingolipids to the diet can also prevent or cure these forms of hepatic triglyceride accumulations. Chronic liver diseases as caused by hepatitis B, hepatitis C and heavy alcohol consumption have previously been shown to be major risk factors for developing liver cancer[23,24]. Until recently, diabetes alone was also seen as a risk factor for liver cancer, most probably via the high prevalence of obesity-related fatty liver. In this respect, it is important to note that in the developed Western world the incidence of liver cancer is rising parallel to the prevalence of obesity. Whether dietary sphingolipids help in preventing liver cancer might be worth studying in the near future.

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Summary

The metabolic syndrome is an increasing problem in our Western society. Many of the features of the metabolic syndrome, like obesity, insulin resistance, dyslipidemia, and hepatic steatosis are established risk factors for cardiovascular disease. Growing evidence supports the important role of body free fatty acid handling and/or body distribution of triglycerides in the pathogenesis of the metabolic syndrome-associated problems. Since many of the features of the metabolic syndrome are major threats to human health, prevention of the development and/or treatment of the metabolic syndrome is a desirable goal. We used several different approaches to study the development of obesity, insulin resistance, dyslipidemia, and liver steatosis.

In **chapter 2** our aim was to study whether the absence of apolipoprotein (apo) C3, a strong inhibitor of lipoprotein lipase (LPL), accelerates the development of obesity, and consequently insulin resistance. After 20 weeks of high-fat feeding *apoc3*$^{-/-}$ mice showed decreased plasma triglyceride levels and increased body weight compared with wild-type littermates. The observed increase in body weight was entirely explained by increased body lipid mass. We showed that the underlying mechanism for the increased fat mass was increased LPL-dependent triglyceride-derived fatty acid (FA) uptake by adipose tissue in *apoc3*$^{-/-}$ mice, while LPL-independent albumin-bound FA uptake did not differ. As expected, the increased body weight and fat mass led to decreased insulin sensitivity, both peripheral and liver-specific, as measured by hyperinsulinemic-euglycemic clamps. We concluded that the absence of apoC3, a natural LPL inhibitor, enhances FA uptake from plasma triglycerides in adipose tissue. This leads to increased susceptibility to diet-induced obesity, followed by more severe development of insulin resistance. Therefore, we have shown that regulation of body distribution of triglycerides, in a LPL-dependent process, plays an important role in obesity development. Down-regulation of adipose tissue LPL activity might contribute to treatment and/or reduction of obesity development. ApoC3 may be a potential target in this strategy. Nevertheless, because of the risk of cardiovascular diseases the effects of reduced LPL activity on plasma lipoprotein levels need to be carefully monitored.

In another set of experiments, we used methyl palmoxirate (MP), an inhibitor of carnitine palmitoyl transferase I (CPTI), to acutely inhibit hepatic FA β-oxidation in hyperlipidemic APOE*3Leiden mice. We investigated whether FA in the liver are rerouted into very low density lipoprotein (VLDL) production and secretion, and if so, whether this rerouting affects hepatic insulin sensitivity regarding glucose production in **chapter 3**. Administration of MP to the mice led to a strong inhibition of the hepatic β-oxidation (as measured by a reduction in plasma β-hydroxybutyrate [= keton body] levels) compared with vehicle-treated mice. Plasma free FA and cholesterol levels were increased, while insulin levels were decreased in MP-treated mice compared with controls. Although MP treatment led to

an increase in liver triglyceride content, no effect on hepatic VLDL-triglyceride production was observed between both groups. In addition, the capacity of insulin to suppress endogenous glucose production was unaffected in MP-treated mice compared with controls. We concluded from these studies, that acute inhibition of the β-oxidation of FA indeed increases hepatic lipid content, but neither stimulates hepatic VLDL secretion nor reduces insulin sensitivity. Apparently, accumulation of FA-metabolites induced by impaired β-oxidation *per se* does not affect VLDL-production or insulin sensitivity in the liver. It seems likely, that the FA-metabolites trigger a chronic (inflammatory) signal that eventually leads to VLDL overproduction and insulin resistance.

FA are known to have different effects on health. In **chapter 4** we studied the effects of a saturated-fat diet supplemented with fish oil, *trans*10,*cis*12 conjugated linoleic acid (CLA), elaidic acid, or fenofibrate on lipid and glucose metabolism and on liver protein levels in hyperlipidemic APOE*3Leiden mice. We found that all treatments significantly lowered serum cholesterol. Fish oil and fenofibrate significantly lowered triglyceride levels, while CLA significantly increased triglyceride levels in plasma. These changes in plasma cholesterol and triglyceride levels were mirrored by the changes in liver lipid composition: fish oil and fenofibrate decreased, and CLA increased liver triglyceride levels. Serum glucose was decreased by fish oil and fenofibrate, and CLA and fish oil significantly increased serum insulin. Proteomics analyses identified significant changes in the levels of several liver cytosolic and membrane proteins. Principal component analysis revealed a major treatment effect of fish oil on cytosolic protein levels and of elaidic acid on membrane protein levels. Proteins that provided the largest contribution to the treatment effects were involved in glycolysis/gluconeogenesis, lipid metabolism and oxidative stress. This study shows that the combination of proteomics with relevant physiological parameters in a sensitive animal model, is a powerful tool, which will aid in identifying workingmechanisms of various dietary FA. Understanding these workingmechanisms is of interest because some FA are known to have beneficial effects on plasma lipids/lipoproteins, and therefore on human health. It seems that, although effects of different FA on plasma lipid/lipoprotein levels measured are equal, the underlying mechanisms are completely different. Further research in defined metabolic and physiological parameters, such as hepatic VLDL-triglyceride production, absorption, LPL-dependent triglyceride clearance, will enable us to specifically define what changes are induced by specific FA.

In **chapter 5** we questioned whether dietary sphingolipids decrease plasma cholesterol and/or triglycerides in hyperlipidemic APOE3*Leiden mice. The six sphingolipids tested dose-dependently decreased both plasma cholesterol and triglyceride levels. The hypolipidemic effect of specifically phytosphingosine (PS) was the net result of: (a) decreased absorption of dietary cholesterol and free FA; (b) increased hepatic VLDL-triglyceride

production; and (c) increased hepatic uptake of VLDL-remnants. These changes also resulted in less pale livers, which weighed less and contained fewer cholesteryl esters and triglycerides compared with livers of control mice. Furthermore, in PS-fed mice markers for liver inflammation (SAA) and liver damage (ALAT) were decreased. We concluded that sphingolipids protect the liver from fat and cholesterol-induced steatosis. Since sphingolipids are nutritional compounds present in several daily foods, such as milk and meat, addition of sphingolipids to the diet may decrease traditional cardiovascular risk factors, such as plasma cholesterol and triglycerides. Furthermore, the ability of sphingolipids to lower liver inflammation markers and liver-specific steatosis may contribute in targeting the metabolic syndrome. It seems that, sphingolipids may prove to be very beneficial for both cardiovascular and diabetes risk factors. Further research on beneficial effects of sphingolipids on hepatic steatosis and insulin sensitivity might reveal an interesting potential.

Nederlandse

Samenvatting

Het metabole syndroom is tegenwoordig een veel voorkomend ziektebeeld dat getypeerd wordt door een combinatie van verschillende aandoeningen. Voornamelijk in de westerse landen wordt het probleem steeds groter. Dit hangt samen met de levensstijl in deze landen: veel suiker- en vetrijk eten gecombineerd met weinig beweging. Het metabole syndroom is een verzameling aandoeningen, waaronder obesitas (zwaarlijvigheid), insuline resistentie (gebrek aan reactie van organen op de werking van insuline), dyslipidemie (onbalans tussen bloedvet gehalten namelijk een te hoog LDL-cholesterol en/of triglyceriden en/of een laag HDL-cholesterol gehalte) en leversteatose (leververvetting). Deze specifieke aandoeningen zijn ook bekende risico factoren voor hart- en vaatziekten. Naarmate onderzoek vordert, wordt er meer bewijs gevonden dat aangeeft dat de manier waarop het lichaam omgaat met vetten, en de manier waarop deze vetten verdeeld worden over het lichaam, een zeer grote rol speelt in de pathogenese van de problemen die met het metabole syndroom gepaard gaan. Omdat veel van de aandoeningen die vallen onder het metabole syndroom een ernstige bedreiging zijn voor onze gezondheid, is voorkoming en/of behandeling van het metabole syndroom, of onderdelen hiervan, een doel in de gezondheidszorg en wetenschap. In dit proefschrift hebben we verschillende benaderingen gebruikt om de ontwikkeling van obesitas, dyslipidemie en leversteatose te bestuderen en om eventuele aangrijpingspunten voor preventie/behandeling aan te wijzen.

In **hoofdstuk 2** hebben we bestudeerd of verhoging van de activiteit van het enzym lipoproteïne lipase (LPL), een enzym dat zorgt voor opname van bloedvetten door weefsels voor opslag en energie, de ontwikkeling van obesitas en uiteindelijk insuline resistentie versnelt. LPL speelt een grote rol bij de verdeling van de bloedvetten naar verschillende weefsels en het is bekend dat overmatige opslag van vetten kan leiden tot insuline resistentie. Verschillen in activiteit van LPL bij specifieke weefsels leidt dan ook tot verschillende bloedvet en orgaanvetverdelingen in de weefsels. Om in dit onderzoek de LPL activiteit te verhogen hebben we gezorgd voor afwezigheid van het eiwit apolipoproteïne (apo) C3. ApoC3 is een sterke remmer van LPL. Muizen die geen apoC3 kunnen maken (*apoc3*$^{-/-}$ muizen), hebben minder remming van LPL, waardoor er meer bloedvetten beschikbaar kunnen komen voor opname in weefsels. Deze *apoc3*$^{-/-}$ muizen hebben we 20 weken een dieet met een hoog vetgehalte gevoerd. Na deze periode was in de *apoc3*$^{-/-}$ muizen de bloedtriglyceride waarde verlaagd. Ook waren de *apoc3*$^{-/-}$ muizen zwaarder vergeleken met controle muizen (die wel apoC3 maken) uit hetzelfde nest (wild-type muizen). De toename in lichaamsgewicht werd verklaard door een toename in lichaamsvet in de *apoc3*$^{-/-}$ muizen. Het mechanisme achter het verhoogde lichaamsvetgehalte bleek, na een experiment naar orgaanspecifieke opname van door LPL vrijgemaakte vetzuren, een toename in vetzuuropname in het vetweefsel in *apoc3*$^{-/-}$ muizen. Zoals verwacht leidde het toegenomen lichaamsgewicht en de toegenomen vetmassa tot verlaagde insuline gevoeligheid in de lever en de andere weefsels (insuline resistentie). Uit deze gegevens

concluderen wij dat de afwezigheid van apoC3, een remmer van LPL, vetzuuropname vanuit het bloed naar vetweefsel verhoogt. Dit leidt tot verhoogde gevoeligheid voor obesitas door dieet en heeft uiteindelijk een ernstigere vorm van insuline resistentie tot gevolg. Met dit onderzoek hebben we laten zien dat regulatie van lichaamsverdeling van triglyceriden een belangrijke rol speelt in de ontwikkeling van obesitas. Verlaging van LPL activiteit in het vetweefsel kan mogelijk bijdragen aan de behandeling en/of voorkoming van obesitas. ApoC3 zou een mogelijk aangrijpingspunt zijn in deze strategie. Echter, wegens het risico op hart- en vaatziekten moeten de effecten van verlaagde LPL activiteit op bloedvetgehaltes goed gecontroleerd worden.

In **hoofdstuk 3** hebben we de manier waarop de lever met vetzuren omgaat bestudeerd. Over het algemeen wordt aangenomen dat de aanmaak van VLDL (een lipoproteïne dat zorgt voor transport van triglyceriden door het bloed) afhankelijk is van de hoeveelheid vet dat beschikbaar is in de lever. Om deze theorie te toetsen hebben we de vetzuurverbranding in de lever acuut geremd met een eenmalige dosis methyl palmoxirate (MP), een chemische stof die een belangrijk enzym in de vetverbanding en ketogenese in de lever, namelijk CPTI, remt. Doordat de lever geen vetzuren meer kan verbranden, zouden er meer vetzuren beschikbaar komen voor de aanmaak van VLDL en/of opslag van vetten. Deze mogelijke veranderingen en een eventuele verandering van de glucoseproductie in de lever hebben we bestudeerd in muizen. Het toedienen van MP aan muizen leidde tot een sterke verlaging van bloedketonlichamen ten opzichte van controle muizen, waaruit we kunnen afleiden dat de vetzuurverbranding inderdaad geremd is in de lever. In MP behandelde muizen namen we meer vetstapeling in de lever (leversteatose) waar, terwijl de VLDL aanmaak onveranderd was. Ook de glucoseproductie in de lever was onveranderd, wat betekent dat er geen insuline resistentie opgetreden was. We concluderen uit deze experimenten dat door acute remming van vetzuurverbranding de beschikbare vetzuren in de lever wel opgeslagen worden, maar dat de hepatische VLDL secretie of de insuline gevoeligheid niet verandert.

Deze resultaten suggereren dat stapeling van vetzuren (en vetzuurmetabolieten) in de lever niet *per se* invloed heeft op VLDL productie of insuline resistentie. Het is waarschijnlijk dat de stapeling van vetzuren in de lever wel een chronisch (ontstekings) signaal veroorzaakt wat op de lange termijn kan zorgen voor VLDL overproductie en insuline resistentie.

Van verschillende vetzuren zijn effecten op de gezondheid bekend, zo is visolie ‘goed’ en zijn verzadigde vetzuren ‘slecht’ voor de gezondheid. Hoe deze verschillende effecten bereikt worden door de verschillende vetzuren is grotendeels onbekend. We hebben de effecten van een dieet met een hoog verzadigd vet gehalte, waaraan visolie, *trans*10,*cis*12-geconjugeerd linolzuur (CLA), of *trans*vetzuur toegevoegd is, op het vet- en

glucose metabolisme en op levereiwit gehaltes in muizen bestudeerd in **hoofdstuk 4**. Aangezien van visolie en CLA wordt gedacht dat ze via een bepaalde receptor werken, hebben we, om dit te bestuderen, een extra groep muizen ingezet op een dieet waaraan fenofibraat, een bekend vetverlagend medicijn dat op die receptor werkt, toegevoegd is. Nadat de muizen 3 weken met de diëten gevoerd waren, bleken alle diëten de bloedcholesterol waarden te verlagen ten opzichte van het controle dieet. De muizen die visolie of fenofibraat dieet kregen, hadden een verlaagd bloedtriglyceride gehalte, terwijl CLA bloedtriglyceriden juist verhoogde. Deze veranderingen in bloedvetten waren ook in de levervetgehalten terug te zien. Visolie en fenofibraat verlaagden en CLA verhoogde het levertriglyceride gehalte. Bloedglucose waarden werden verlaagd in de muizen die visolie of fenofibraat dieet hadden gehad. CLA en visolie verhoogden bloedinsuline waarden. Analyse van de levereiwitten (proteomics) gaf aan dat zowel cytoplasma- als membraaneiwitten verschillend tot expressie komen onder invloed van de verschillende diëten. De grootste verschillen tussen de dieet-effecten werden gezien bij eiwitten betrokken bij glucoseaanmaak, vetmetabolisme en oxidatieve stress. Deze studie laat zien dat proteomics, gecombineerd met relevante fysiologische parameters in een passend muizenmodel, een krachtig hulpmiddel kan zijn dat kan helpen bij de identificatie van de werkingsmechanismen van verschillende vetzuren uit ons dieet. Het begrijpen van het werkingsmechanisme van vetzuren is interessant, omdat van verschillende vetzuren een gunstig effect op bloed lipiden en/of lipoproteinen, en dus op gezondheid, bekend is. Hoewel de gemeten effecten, op bijvoorbeeld bloed cholesterol, door verschillende vetzuren vergelijkbaar kunnen zijn, hoeven deze effecten niet via hetzelfde mechanisme bewerkstelligd te worden. Onderzoek naar gedefinieerde metabole en fysiologische parameters, zoals lever-VLDL aanmaak, vetabsorptie, LPL-afhankelijke triglyceriden-klaring, zal het onderzoekers mogelijk maken specifiek aan te geven welke veranderingen geïnduceerd worden door bepaalde vetzuren.

In **hoofdstuk 5** onderzochten we eigenschappen van sphingolipiden. Deze vetzuren komen voor in ons voedsel, bijvoorbeeld in melk, ei of vlees. Van één van de vetzuren uit deze vetzuurgroep, sphingomyeline, is aangetoond dat het cholesterolverlagende eigenschappen heeft. Onze onderzoeksvraag was: verlagen verschillende sphingolipiden bloed cholesterol en/of triglyceriden in een passend muizenmodel? Zes verschillende sphingolipiden bleken zowel bloedcholesterol als -triglyceriden op een dosisafhankelijke manier te verlagen in de muizen waarbij de sphingolipiden waren toegevoegd aan het dieet. De manier waarop sphingolipiden bloedvetten verlagen hebben we bestudeerd door de muizen phytosphingosine (PS) te voeren. Muizen op een PS-dieet hadden een verlaagde opname van dieet cholesterol en vrije vetzuren in de darmen. Hierdoor komen minder vetten in het bloed terecht na een maaltijd. De VLDL aanmaak in de lever, welke bijdraagt aan bloedtriglyceride waarden, was tegen verwachtingen in verhoogd. Tenslotte werd er

een verhoogde opname van VLDL door de lever gezien in muizen op het PS-dieet. Het netto resultaat van deze veranderingen is, naast verlaagd bloed cholesterol en triglyceriden, kleinere, minder lichtgekleurde levers, die bovendien minder cholesteryl ester en triglyceriden bevatten. De levers van de controle muizen daarentegen waren groter en lichter van kleur en vertoonden vetstapeling (leversteatose). De markers voor leverontsteking (SAA) en leverschade (ALAT) waren sterk verlaagd in muizen die PS-dieet hadden. We concluderen uit deze gegevens dat sphingolipiden, de bloedvet waarden verlagen en de lever beschermen tegen vet- en cholesterol geïnduceerde leversteatose.

Omdat sphingolipiden natuurlijke vetzuren in ons dagelijkse voedsel zijn kan toevoeging van sphingolipiden aan het dieet mogelijk de risico factoren op hart- en vaatziekten, namelijk bloedcholesterol en -triglyceriden, verlagen. Het vermogen van sphingolipiden om leverspecifieke vetstapeling en bijbehorende bloedontstekingsmarkers te verlagen kan mogelijk bijdragen aan het bestrijden van het metabole syndroom. Daarom lijkt het erop dat sphingolipiden erg gunstig kunnen zijn voor zowel hart- en vaatziekten en diabetes-gerelateerde risico factoren. Verder onderzoek naar gunstige effecten van sphingolipiden op leververvetting en insuline-gevoeligheid kan veel informatie opleveren.

Abbreviations

2D	2-dimensional
6-FAM	6-carboxyfluorescein
6-TAMRA	6-carboxytetramethylrhodamine
ABCA1 / *abca1*	ATP-binding cassette transporter A1
ACC / *acc*	Acetyl-CoA carboxylase
ACO / *aco*	Acyl-CoA oxidase
ALAT	Alanine aminotransferase
Apo / *apo*	Apolipoprotein
ATGL	Adipose tissue triglyceride lipase
ATP	Adenosine triphosphate
β-HB	β-hydroxybutyrate
BMI	Body mass index
BSA	Bovine serum albumin
cDNA	Complement DNA
CHD	Coronary heart disease
CLA	Conjugated linoleic acid
CM	Chylomicron
CO	Cholesterol oleate
CoA	Coenzyme A
CPCA	Cyclopropane carboxylic acid
CPT	Carnitine palmitoyl transferase
CT	Carnitine/acylcarnitine translocase
Cyp7A1 / cyp7a1	Cholesterol 7-α-hydroxylase
DGAT / *dgat*	Acyl:diacylglycerol transferase
DHA	Docosahexaenoic acid
DTT	DL-dithiothreitol
ELISA	Enzyme-linked immunoabsorbent assay
EPA	Eicosapentaenoic acid
FA	Fatty acid(s)
$FABP_c$	Cytosolic fatty acid binding protein
$FABP_{pm}$	Plasma membrane fatty acid binding protein
FACS	Fatty acid CoA synthase
FAS / *fas*	Fatty acid synthase
FAT	Fatty acid translocase
FATP	Fatty acid transport protein
FFA	Free fatty acid(s)
FPLC	Fast protein liquid chromatography
FXR / *fxr*	Farnesoid X receptor
G-6-P	Glucose-6-phosphate
(n-)HDL	(nascent-)high density lipoprotein
HFC	High-fat/cholesterol
HL	Hepatic lipase
HMG-CoA	3-hydroxy-3-methylglutaryl CoA
hmgs	HMG-CoA synthase
HPRT	hypoxanthine guanine phosphoribosyl transferase

HPS	Hematoxylin-phloxine-saffron
HPTLC	High pressure thin layer chromotography
HSL	Hormone sensitive lipase
HSPG	Heparin sulphate proteoglycans
IDL	Intermediate density lipoprotein
IL	Interleukine
IPG	Immobilized pH gradient
LBM	Lean body mass
LC-FA	Long chain fatty acid
LDL	Low density lipoprotein
LDLR / *ldlr*	LDL receptor
LPL / *lpl*	Lipoprotein lipase
LRP	LDLR-related protein
LXR / *lxr*	Liver X receptor
MALDI-TOF	Matrix-assisted laser desorption/ionisation –time of flight
MCAD / *mcad*	Medium chain acyl-CoA dehydrogenase
MP	Methyl palmoxirate
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MTP / *mttp*	Microsomal triglyceride transfer protein
NS	Not significant
PBS	Phosphate buffered saline
PC	Principle component
PCA	Principle component analysis
(RT)-PCR	(Reverse transcriptase)-Polymerase chain reaction
PEPCK / *pepck*	Phosphoenol pyruvate carboxykinase
PK / *pk*	Pyruvate kinase
PPAR / *ppar*	Peroxisome proliferator-activated receptor
PS	Phytosphingosine
PUFA	Polyunsaturated fatty acids
RIA	Radioimmuno assay
RXR / *rxr*	Retinoic X receptor
SAA	Serum amyloid A
SD	Standard deviation
SR-B1	Scavenger receptor B1
SREBP / *srebp*	Sterol regulatory element binding protein
*t*10,*c*12	*Trans*10,*cis*12
TG	Triglyceride(s)
TNF	Tumor necrosis factor
VLDL	Very low density lipoprotein
WAT	White adipose tissue
WHO	World health organization
WT	Wild-type

List

of

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Curriculum

Vitae

Ilse Duivenvoorden werd geboren op 9 september 1977 in Heemstede. In 1996 behaalde zij haar VWO diploma aan het R.K. Lyceum Sancta Maria te Haarlem. In september van datzelfde jaar begon zij aan de studie Medische Biologie aan de Vrije Universiteit te Amsterdam. Het propedeutisch examen werd in september 1997 behaald, gevolgd door het doctoraal examen medische biologie in september 2000. In het kader van het doctoraal examen werden twee onderzoeksstages uitgevoerd. De eerste stage werd verricht bij de afdeling Nucleaire Geneeskunde aan de Vrije Universiteit Medisch Centrum (VUMC) onder begeleiding van Prof. Dr. G.J.J. Teule. De tweede stage werd verricht bij de onderzoeksgroep Lipiden, afdeling Vaat en Bindweefsel Onderzoek bij TNO Preventie en Gezondheid te Leiden onder begeleiding van Dr. H.M.G. Princen. Van november 2000 tot en met februari 2005 was zij aangesteld als assistent in opleiding (AIO) aan het Leids Universitair Medisch Centrum (LUMC) op de afdeling Algemene Inwendige Geneeskunde op een door NWO/ZonMw gesubsidieerd project (903-39-179). Tijdens deze periode was zij gedetacheerd bij TNO Preventie en Gezondheid (nu TNO Kwaliteit van Leven) en werkzaam in het Gaubius laboratorium te Leiden onder begeleiding van Prof. Dr. Ir. L.M. Havekes. De resultaten van dit promotieonderzoek staan beschreven in dit proefschrift. Sindsdien is zij werkzaam in de farmaceutische industrie.