Vascular risk factors and adipocyte dysfunction in metabolic syndrome

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Vascular risk factors and adipocyte dysfunction in metabolic syndrome

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in metabool syndroom
(met een samenvatting in het Nederlands)

Proefschrift

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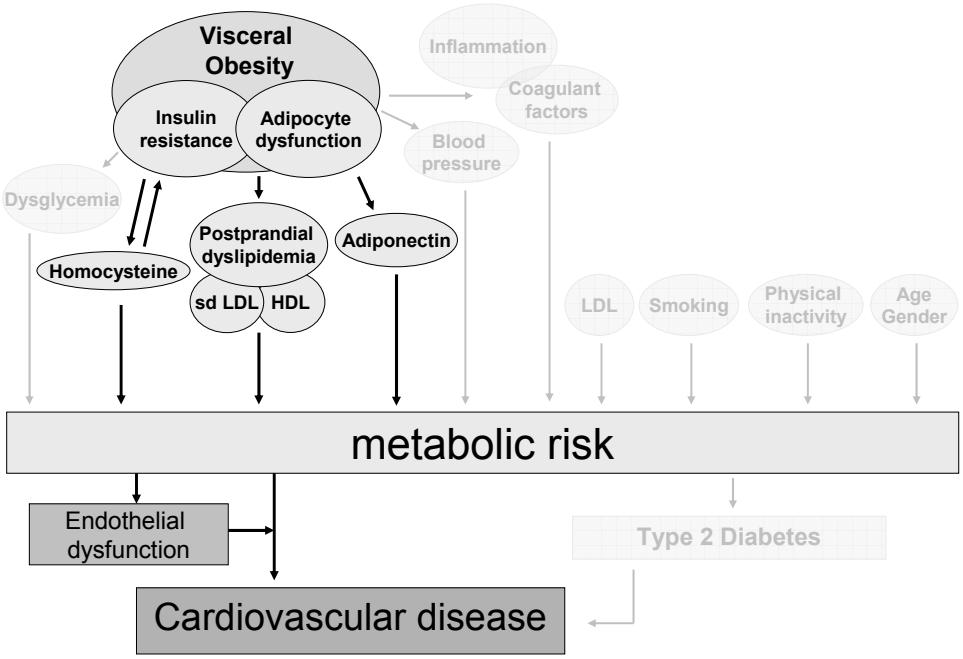
CHAPTER 1

General introduction

Obesity and cardiovascular disease

Obesity is a principal public health issue in Western countries and an increasing health problem in Africa and Asia [4-6] due to increased urbanisation, including a sedentary lifestyle, and adaptation to a high fat and high carbohydrate diet. Obesity is a major (and modifiable) risk factor for a variety of diseases including cardiovascular diseases and type 2 diabetes mellitus. Cardiovascular diseases (cerebrovascular, coronary artery and peripheral arterial diseases) are the main cause of morbidity and mortality in high-income countries [14]. Well known traditional risk factors next to obesity include smoking, hypertension, elevated low density lipoprotein (LDL)-cholesterol plasma levels, low high density lipoprotein (HDL)-cholesterol and diabetes mellitus, although many others may contribute to the risk [15] and are topic of current research (**Figure 1**).

Figure 1 *Obesity, metabolic vascular risk factors and cardiovascular disease*



Cardiovascular disease is the resultant of the presence of (non-)modifiable metabolic risk factors, including, lipid levels, lifestyle, and obesity and insulin resistant associated factors. According to this model, visceral obesity is a central feature in the development of cardiovascular disease, tightly associated with insulin resistance and adipocyte dysfunction. The highlighted potential risk factors and variables are topic of research in the current thesis.

Atherosclerosis

The development of atherosclerosis is a highly distinctive process regardless of the cause or location within the vascular tree and is characterized by (chronic) low grade inflammation, increased endothelial permeability, activated coagulation pathways and

lipid accumulation within the endothelium [16]. The endothelium regulates vascular tone and homeostasis through the release of a variety of substances like, nitric oxide (NO) and prostacyclin. Besides vasodilatory effects, NO has anti-atherogenic properties by inhibiting platelet aggregation, leukocyte adhesion, smooth muscle cell proliferation and reduces inflammatory responses [17]. Dyslipidemia, elevated levels of homocysteine, smoking, hypertension, elevated levels of tumor necrosis factor-α (TNF-α) and insulin resistance can injure the endothelium leading to reduced NO availability with impaired vasodilation, and to a pro-inflammatory and pro-thrombotic state [18,19,20]. Endothelial dysfunction plays an important role in the development of atherosclerotic vascular disease and is regarded as an early manifestation of atherosclerosis. Not only is endothelial dysfunction associated with hypertension, (postprandial) dyslipidemia, obesity [21]; presence of endothelial dysfunction is prognostic for coronary heart disease years before an event [22,23]. Endothelial function can be assed non-invasively by measuring flow-mediated vasodilation following a period of transient ischemia of the brachial artery [24] and represents the vascular function in coronary vessels [25, 26]. Preventing or reducing injury to the endothelium preserves endothelial function leading to a reduced cardiovascular risk [27, 28].

Adipose tissue and insulin resistance

Obesity is in fact an increased percentage of body fat mainly due to hypertrophic fat cells [27] and is associated with an increased risk for cardiovascular diseases, especially when obesity is defined as an increased waist circumference [11]. Adipose tissue consists of adipocytes, macrophages, neurons and vascular stroma cells [28]. For decades, adipose tissue was solely considered a storage depot for energy in form of triglycerides [29]. It is now clear that adipose tissue can be considered as a metabolic active endocrine organ producing a number of hormones and cytokines e.g. Tumor necrosis factor (TNF-α), interleukin-6 (IL-6), adiponectin, leptin, resistin, retinol binding protein-4 (RBP-4), visfatin and plasminogen activator inhibitor-1 (PAI-1) [30,31-33].

A close interaction between enlarged adipocytes and macrophages, present in adipose tissue, may be the first step in the development of insulin resistant adipocytes. As adipocytes expand they release more free fatty acids (FFAs) which can stimulate macrophages to produce TNF-α leading to a vicious cycle that aggravates inflammatory changes and insulin resistance in the adipose tissue [34, 35]. Once adipose tissue is insulin resistant the production rate of adipocytokines (except for adiponectin) increases along with the further enhanced release of free fatty acids due to impaired hormone sensitive inhibition. Adipocytokines affect adipogenesis and whole body energy balance, can induce insulin resistance, dyslipidemia, hypertension, hypercoagulability and are associated with endothelial dysfunction, diabetes type 2, increased carotid intima-media thickness and cardiovascular events [36-40].

Adiponectin is an adipocytokine being solely produced by adipocytes and has been found to improve insulin signaling and to have potentially divers anti-atherosclerotic properties *in vitro* [41, 42]. Adiponectin reduces monocyte adhesion to the endothelium, inhibit foam cell formation and suppresses proliferation of smooth muscle cells [43].

In addition, adiponectin lowers the secretion of TNF-α by macrophages, improves NO-availability and increases hepatic fatty acid oxidation [44,45]. In contrast to adiponectin, IL-6 levels are increased in obesity and IL-6 concentrations were much higher in portal vein compared to peripheral artery blood in obese subjects, illustrating the contribution of visceral fat to the IL-6 plasma concentration [46]. Due to the unique anatomical location of visceral adipose tissue in relation to the liver, the pro-inflammatory cytokine IL-6 produced by adipose tissue and released in the portal circulation, can stimulate the liver to produce fibrinogen and CRP [47]. In addition, many studies have shown that IL-6 is able to directly stimulate beta-cells to secrete insulin *in vitro* and induce insulin resistance *in vivo* [49,50].

Since these adipocytokines potentially mediate the adverse effects of obesity, it is essential to unravel the contribution of dysfunctional adipose tissue to atherogenesis and development of cardiovascular disease.

Insulin resistance is generally defined as a decline in glucose uptake in muscle cells and adipocytes, and/or defect in hepatic glucose production to a dose of insulin[51] and is the result of impaired intracellular signalling transduction pathway of the insulin receptor. Major contributors to the development of insulin resistance are elevated levels of FFAs and TNF-α which impair the phosphorylation of the insulin receptor substrate-1 by the insulin receptor after insulin binds to its membrane-bound receptor [52]. Vicious cycles can be described in which adipocyte-derived cytokines lead to insulin resistance, which contribute to increased production of adipocytokines. The initiating step of this cycle remains unclear; macrophages present in adipose tissue or an excess of nutrients may be involved [53]. Insulin resistance is strongly associated with the development of (cardio)vascular diseases and is related to a range of metabolic abnormalities including hyperinsulinemia. Whether high plasma levels of insulin are directly atherogenic independently of other metabolic disturbances seen in insulin resistance remains unclear [54].

Dyslipidemia in obesity and insulin resistance

Dyslipidemia in obese and insulin resistant subjects is characterized by elevated levels of small dense LDL particles, elevated fasting plasma levels of triglycerides and, secondary to that, low HDL-c concentrations [55] comprising an atherogenic lipid profile. Insulin resistant, dysfunctional adipocytes release large quantities of FFAs to the liver, since insulin fails to inhibit hormone sensitive lipase, resulting in increased synthesis of triglyceride-rich VLDL particles by the liver [56]. In the postprandial phase, triglyceride-rich lipoproteins (TRLs) are excessively produced by the liver (VLDL particles with apoB100 as structural protein) and by intestine (chylomicrons, with apoB48 as structural protein) [57]. Both types of TRLs compete for the same enzyme, namely lipoprotein lipase (LPL) bound to the endothelium. LPL hydrolyzes triglycerides, after which FFAs and glycerol are taken up by peri-vascular tissue. Under insulin resistant conditions, LPL activity is reduced resulting in a prolonged residence time in the circulation of TRL particles [57]. By action of cholesteryl ester transfer protein (CETP), which is also produced by adipocytes, cholesterol esters in HDL-c are exchanged for triglycerides of TRLs. Triglyceride-rich HDL particles are rapidly cleared from circulation leading to a decreased HDL-c plasma

concentration [57].
In general, LDL-c plasma levels are not elevated in an insulin resistant situation. However through similar mechanisms of CETP and hepatic lipase dependent lipid modification small dense LDL particles are formed which are therefore often present in insulin resistant subjects. Small dense LDL particles may induce endothelial dysfunction and an increased risk for cardiovascular events [58]. TRLs, their remnants and small dense LDL particles can accumulate in the sub-endothelial space, accelerating atherosclerosis by their uptake into foam cells [59]. In daily clinical practice indication for lipid-lowering treatment and monitoring of treatment largely relies on plasma levels of fasting lipids. However, before fasting dyslipidemia develops in insulin resistance, postprandial hypertriglyceridemia is present due to an excess of TRL and reduced LPL activity. It has been shown that postprandial dyslipidemia is associated with endothelial dysfunction [60, 61] and that non-fasting triglycerides are better predictors of future cardiovascular disease than fasting triglyceride concentrations [62, 63]. Apparently, fasting lipid concentrations poorly reflect postprandial plasma lipid concentrations. Since humans are in a non-fasting condition most time of day the vascular endothelium is almost constantly exposed to elevated levels of atherogenic lipoproteins. Lowering postprandiale plasma lipids may be a treatment target, in addition to reducing fasting lipid levels. It is yet unknown whether different therapeutic approaches, e.g. inhibiting the production of cholesterol in the liver and/or reducing cholesterol absorption in the intestine, lead to different postprandial lipid profiles.

Metabolic syndrome

The clustering of metabolic risk factors (abdominal obesity, elevated plasma glucose, low HDL-c, elevated plasma triglycerides and elevated blood pressure), which is closely related to insulin resistance and visceral obesity, is often referred to as metabolic syndrome [55]. The prevalence of metabolic syndrome varies between populations; from 15% in healthy European men and woman [64] to 25% in healthy American subjects [65] up to 46% in patients with manifest cardiovascular disease [2]. Although different definitions for metabolic syndrome do exist and differ in detail, they agree on the presence of essential components as insulin resistance and abdominal obesity [55] (**Table 1**). Presence of insulin resistance as underlying pathophysiological features of metabolic syndrome [66, 67] is now generally accepted [68, 69]. In non-diabetic patients 95% of the NCEP-defined metabolic syndrome patients were insulin resistant [67]. The National Cholesterol Education program Expert Panel (NCEP) criteria are for practical reasons most used in a clinical setting, identifies patients with the worst risk factor profile [70] and patients at the highest vascular risk after PTCA [71] compared to the IDF definition. According to the NCEP definition, subjects are classified as having metabolic syndrome when at least 3 out of 5 easy to measure (metabolic) abnormalities are present (**Table 1**). Various studies have shown that presence of metabolic syndrome, defined by different criteria, is associated with an increased risk of type 2 diabetes and cardiovascular disease [72-74]. In healthy subjects, presence of metabolic syndrome was found to be associated with more vascular damage than expected from its individual components alone [75]. Furthermore, the Framingham

prediction risk-score could not completely explain the increased cardiovascular risk of in coronary heart disease patients once metabolic syndrome was present [76]. The key question remains whether the vascular risk associated with metabolic syndrome is the result of the individual criteria or that other factors associated with both obesity and insulin resistance but not part of the definition also contribute [75]. It is conceivable that adipocyte-induced low grade inflammation, impaired fibrinolysis, endothelial dysfunction, low adiponectin and high pro-inflammatory cytokines levels and/or hypercoagulability may contribute to the observed vascular risk in these metabolic syndrome patients [67, 77-79].

Table 1 *Metabolic syndrome criteria according to the ATPIII criteria*

Clinical Measure	***Any 3 of the following 5 features ATP III (2001)***	
Body Weight	♂ *Waist > 102cm* ♀ *Waist > 88cm*	
Lipid	*Triglycerides* *HDL-c* *or*	*≥1.70 mmol/l* *< 1.04mmol/l (♂),* *< 1.29mmol/l (♀)*
Blood pressure	*≥ 135/85mmHg and/or medication*	
Glucose	*Fasting glucose and or medication*	*≥ 6.1mmol/l*

ATPIII: Adult treatment Panel III

Objectives

The objectives of this thesis are 1] to determine the relationship between metabolic risk factors associated with insulin resistance and adipocyte dysfunction and the occurrence of new cardiovascular events in patients with clinical manifest vascular disease, and 2] to evaluate the effect of lipid lowering therapy on postprandial lipid metabolism and endothelial function in obese patients with metabolic syndrome.

Outline of this thesis

In **chapter 2** the concept of adipocyte dysfunction is introduced and defined while pathophysiological mechanisms are described linking abdominal obesity and adipocyte dysfunction to insulin resistance, diabetes mellitus type 2 and cardiovascular diseases.
The debate whether an elevated plasma concentration of homocysteine is a risk factor for cardiovascular disease is still ongoing, sustained by contradicting epidemiological and interventional studies. From *in vitro* studies, it is known that elevated levels of homocysteine can be both the consequence and cause of insulin resistance. In **chapter 3** we investigated

the association between metabolic syndrome and plasma levels of homocysteine and determined whether elevated homocysteine plasma levels confer an increased cardiovascular risk in the presence of metabolic syndrome.
Adiponectin is solely derived from adipocytes and has insulin-modifying and anti-atherogenic potential *in vitro*. Adiponectin plasma levels are decreased in subjects with increased adipose tissue mass and in insulin resistance *in vivo.* Aim of **chapter 4** is to test the hypothesis that low adiponectin plasma levels are a risk factor for new vascular events in patients with already clinical evident vascular disease.
High Density lipoprotein-cholesterol particles have potential anti-atherosclerotic properties. Low fasting HDL-c plasma concentrations are indeed associated with an increased cardiovascular risk in healthy subjects. In **chapter 5** we determined whether low fasting HDL-c levels are also a risk factor for recurrent vascular events in patients with clinical evident vascular disease using data from the SMART cohort. In addition, only very little is known about non-fasting HDL-c metabolism, especially in metabolic syndrome patients who have already low fasting HDL-c levels. To study postprandial HDL-c metabolism in relation to dysfunctional adipose tissue we performed oral fat loading in metabolic syndrome subjects as described in **chapter 6.**
Although in daily practice most lipid profiles are determined after an overnight fast, postprandial dyslipidemia is an early feature of insulin resistance and may even be more accurate in predicting a patient's cardiovascular risk. Much is known about the effects of lipid lowering therapy on fasting lipid levels and lipoprotein composition, less about their effects on postprandial lipid concentrations and lipoprotein composition. In **chapter 7** we examined the differences between high dose statin and low-dose statin in combination with a cholesterol absorption inhibitor on post fat load lipid levels and lipoprotein composition in obese metabolic syndrome patients.
Endothelial dysfunction is an early manifestation in the atherosclerotic process and can be enhanced by insulin resistance, low grade inflammation and postprandial dyslipidemia. In **chapter 8** we investigated whether two frequently used lipid lowering therapies affected postprandial endothelial function differently as measured with flow mediated dilation due to alterations in lipid metabolism and/or inflammation.
Finally, in **chapter 9** a summary of the results is given and the results of the thesis will be discussed.

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CHAPTER 2

Adipocyte dysfunction in obesity, diabetes and cardiovascular disease. A review

Gideon R. Hajer, Timon W. van Haeften and Frank L.J. Visseren

Introduction

The classical perception of adipose tissue as a storage place of fatty acids has been replaced over the last years by the notion that adipose tissue has a central role in lipid and glucose metabolism and produces a large number of hormones and cytokines e.g. tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), adiponectin, leptin and plasminogen activator inhibitor-1 (PAI-1) [1-3].
The increased prevalence of excessive visceral obesity and obesity related cardiovascular risk factors is closely associated with the rising incidence of cardiovascular diseases and type 2 diabetes mellitus [4,5]. This clustering of vascular risk factors in (visceral) obesity is often referred to as metabolic syndrome. The close relationship between an increased quantity of visceral fat, metabolic disturbances and cardiovascular diseases and the unique anatomical relation to the hepatic portal circulation has led to an intense endeavour to unravel the specific endocrine functions of this visceral fat depot. The objective of this paper is to describe adipocyte dysfunction, delineate the relationship between adipocyte dysfunction and obesity and to describe how adipocyte dysfunction is involved in the development of diabetes mellitus type 2 and cardiovascular disease. First, normal physiology of adipocytes and adipose tissue will be described.

Physiological role of adipocytes and adipose tissue

Adipose tissue exists of adipocytes and a vascular-stromal fraction in which macrophages, fibroblasts, endothelial cells and pre-adipocytes are present [7]. Pre-adipocytes originate from a multipotent stem cell of mesodermal origin and the potential to generate new fat cells persists during the entire human life [8].
The primary and classical roles of adipose tissue are to insulate and cushion the body, to store free fatty acids (FFAs) after food intake and to release FFAs during the fasting state to ensure sufficient energy status. During the postprandial phase FFAs are taken up from the blood in adipose tissue after hydrolysis of triglycerides from triglyceride-rich lipoproteins (VLDL-c, chylomicrons and their remnants) by lipoprotein lipase (LPL). Mobilization of this reserve occurs by hydrolysis of adipocyte triglycerides by hormone sensitive lipase (HSL). Insulin is the main regulator of adipocyte fat content, since it is both a potent inhibitor of HSL and an important activator of LPL, thereby enhancing FFA uptake and triglyceride synthesis in adipocytes.
Macrophages are more prevalent in adipose tissue of obese subjects than in adipose tissue of lean subjects and the macrophage quantity correlates with measures of insulin resistance [7]. The infiltration rate of monocytes into visceral adipose tissue is higher than into subcutaneous adipose tissue [9]. Adipose tissue harbours two types of macrophages, i.e. M1-macrophages (predominant in obesity [10]) secreting TNF-α and IL-6 thereby enhancing inflammation, and type M2 macrophages secreting anti-inflammatory cytokines such as interleukin-10, which has a function in tissue repair [11]. Both macrophages and adipocytes are capable of accumulating lipids and secreting cytokines. Interestingly, the number of macrophages in adipose tissue is reduced after weight loss [12].

Endocrine function of adipocytes: adipocytokines

Adipocytes and adipose tissue produce a wide range of hormones and cytokines involved in glucose metabolism (e.g. adiponectin, resistin), lipid metabolism (e.g. Cholesteryl Ester Transfer Protein, CETP), inflammation (e.g. TNF-α, IL-6), coagulation (PAI-1) and feeding behaviour (leptin) thus affecting metabolism and function of many organs and tissues including muscle, liver, the vasculature and brain [13-15] (**Table 1**). Plasma adipocytokine levels rise with an increase in adipose tissue and adipocyte volume, except for plasma adiponectin which is lower in obesity [16].

The essential roles of adipose tissue are exemplified by the fact that total absence of adipose tissue results in non-viability as occurs in homozygous PPAR-γ knock-out mice [17]. During evolution, fat tissue presumably obtained an intermediary role between nutritional status and essential body functions such as feeding behaviour, growth, metabolism and even fertility [18,19]. A key (co-) regulator of these functions is leptin, which is principally produced by adipocytes [20].

Leptin production is markedly augmented in large adipocytes [20], is stimulated by insulin and affected by TNF-α, estrogens, FFAs and growth hormone [21] but is not directly influenced by food uptake itself. Therefore, leptin can be considered as a signalling molecule relating the long-term nutritional and fat mass status to the brain (hypothalamus) [19,22] (**Figure 1**).

Apart from central effects, leptin increases hepatic lipid oxidation and lipolysis in skeletal muscle and adipocytes [22,23]. In subjects with decreased fat depots, e.g. in anorexia nervosa (AN), leptin levels are low [19] which may contribute to complications of AN (amenorrhea). Leptin deficient children are not only extremely obese, but remain prepubertal while exogenous leptin substitution has resulted in the onset of puberty in these children [18].

Adiponectin is exclusively produced by adipocytes [2] and circulates in plasma as trimer, hexamer and multimers and as globular form. Adiponectin synthesis is reduced in obesity, insulin resistance, metabolic syndrome and type 2 diabetes [24,25]. Men have lower plasma adiponectin levels than women [26]. Adiponectin has an array of anti-atherosclerotic effects and improves insulin sensitivity through inhibition of hepatic glucose production and enhancing glucose uptake in muscle, increasing fatty acid oxidation in both liver and muscle and augmenting energy expenditure *in vitro*, presumably by enhanced uncoupling of ATP generation in mitochondria [27]. In an insulin resistant mouse model administration of adiponectin has been shown to ameliorate hyperglycemia and hyperinsulinemia [13].

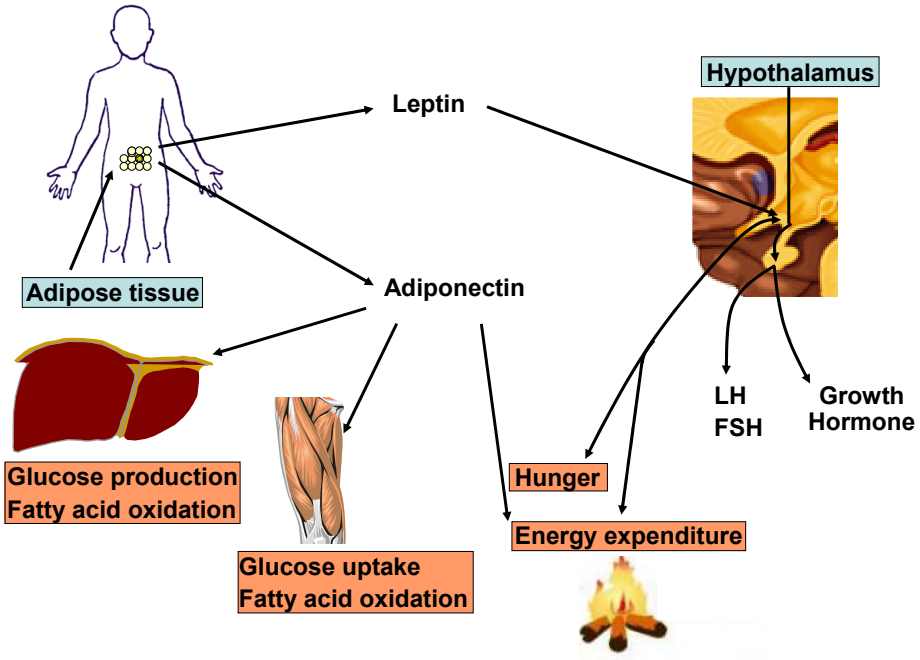
IL-6 can be produced in large quantities by abdominal adipose tissue and is a well known pro-inflammatory cytokine. Furthermore, elevated IL-6 plasma levels are associated with insulin resistance and increased risk for diabetes, independently of body weight [16]. Surprisingly, IL-6 deficient mice develop obesity with increased leptin levels [28] although conflicting results do exist. Based on these studies it is postulated that IL-6 can induce energy expenditure (including thermogenesis) and inhibit feeding behaviour at the level of the central nervous system. However, a high dose of IL-6 given peripherally results in decreased insulin sensitivity in healthy males [29]. IL-6 upregulates vascular endothelial growth factor (VEGF) production by visceral and subcutaneous adipocytes *in vitro* and in mice, thus supporting angiogenesis during adipose tissue growth [30].

Various other hormones are produced by adipocytes, such as resistin, visfatin, omentin, retinol binding protein-4 and others. The metabolic properties of these hormones are less clear yet and are the topic of current investigations.

Table 1 *Adipocytokines*

Adipocytokine	Full name	Effects on
Leptin	Leptin	Food intake, fat mass
Adiponectin	Adiponectin	Insulin resistance, inflammation
Resistin	Resistin	Insulin resistance, inflammation
Visfatin	Visfatin	Insulin resistance
Omentin	Omentin	Insulin resistance
Vaspin	Visceral adipose tissue-derived serpin	Insulin resistance
Apelin	Apelin	Vasodilatation
CETP	Cholesteryl ester transfer protein	Lipid metabolism
LPL	Lipoprotein lipase	Lipid metabolism
HSL	Hormone sensitive lipase	Lipid metabolism
A-FABP (aP2)	adipocyte fatty acid binding protein	Lipid metabolism
Perilipin	Perilipin	Lipid metabolism
RBP (4)	Renitol-binding protein (4)	Lipid metabolism
AT II	Angiotensin II	Blood pressure
ACE	angiotensin converting enzyme	Blood pressure
AGT	Angiotensinogen	Blood pressure
TNF-α	Tumor Necrosis Factor-α	Inflammation
IL-6	Interleukin-6	Inflammation
CRP	C-reactive protein	Inflammation
Adipsin	Adipocyte trypsin / Complement Factor D	Inflammation
MCP-1	Macrophage Chemo attractant Protein-1	Macrophage attraction
ICAM-1	InterCellular Adhesion Molecule-1	Macrophage activation
PAI-1	Plasminogen Activator Inhibitor-1	Fibrinolysis

Figure 1 *Leptin and adiponectin*



Leptin acts primarily via the melanocortin system (arcuate nucleus) in the hypothalamus [19] where it inhibits AMP-activated kinase [23], resulting in decreased hunger and stimulated energy expenditure [21] and, to a lesser extent, in enhancing growth (Growth Hormone) and reproduction by stimulating Gonadotrophin Releasing Hormone in both men and women [23]. In peripheral tissues, leptin increases lipid oxidation (liver) and lipolysis (skeletal muscle and adipocytes) [27]. Adiponectin inhibits hepatic glucose production while it may enhance glucose uptake in muscle, increases fatty acid oxidation in both liver and muscle, and augments energy expenditure in vitro, presumably by enhanced uncoupling of ATP generation in mitochondria [27].

Visceral versus subcutaneous adipose tissue

Subcutaneous and visceral adipose tissue show functional differences. Genes for angiotensinogen, complement factors [31], fatty acid binding protein and acylation stimulating protein (ASP) are expressed at higher levels in visceral adipose tissue than in subcutaneous fat [32]. Leptin however is mainly produced by subcutaneous adipose tissue while TNF-α is equally produced by both fat depots [32], although others report differently from *in vitro* studies [3]. In contrast to subcutaneous adipose tissue, abdominal adipose tissue drains directly onto the portal circulation. A study in extremely obese patients indicates that visceral fat is the main contributor of plasma IL-6 concentration [33]. It is therefore conceivable that viscerally produced adipocytokines directly influence liver function because IL-6 is an inducer of liver C-reactive protein (CRP) production and proteins involved in hemostasis (PAI-1, fibrinogen, tissue plasminogen activator (t-PA)). IL-6 also adds to dyslipidemia via disinhibition of Microsomal Triglycerides Transfer Protein which controls the hepatic assembly of ApoB containing lipoproteins *in vitro* [34].

Both visceral and subcutaneous adipose tissues are innervated by the autonomic nervous system, with different motor neurons separately for each depot and under control of neuro-endocrine feedback [35]. Stimulation of the parasympathetic nervous system leads to an anabolic state with decreased lipolysis, while stimulation of the sympathetic nervous system leads to a catabolic state with reduced adipogenesis and stimulated lipolysis. However, at present it is unclear whether and how these different modes of neural innervation lead to functional differences in adipose tissue.

Transcription factors in adipocytes

Two key transcription factors in the development and metabolism of adipocytes are the PPARs and Sterol Regulatory Element-Binding Proteins (SREBP) [36]. Of the various PPAR subtypes, PPAR-γ is expressed at high levels in adipose tissue. PPAR-γ activates genes involved in adipocyte differentiation and fatty acid trapping, e.g. fatty acid transport protein, LPL, fatty acid binding protein, adiponectin and Acyl CoA synthase [37]. Most of the understanding of PPAR-γ gene regulation comes from studies with thiazolidinediones (TZDs) which are PPAR-γ ligands. Whether an endogenous, high-affinity ligand for PPAR-γ exists is not yet clear but various unsaturated fatty acids and their metabolites have been shown to be able to bind PPAR-γ. Activation results in adipocyte hyperplasia with a concomitant shift of triglycerides from circulating lipoproteins and muscle tissue into adipocytes. These changes result indirectly in improved endothelial function and in decreased plasma levels of insulin, FFAs and cytokines [38].
SREBP-1c (the main SREBP-isoform) is highly expressed in most tissues, including adipose tissue. Once activated by insulin in the postprandial phase, SREBP-1c activates a cascade of genes required for endogenous lipogenesis and pre-adipocyte differentiation (fatty acid synthase, HMG-CoA synthase, LDL-receptor, adipocyte determination and differentiation factor 1) [39].

Adipocyte dysfunction and obesity

The subsequent paragraphs will mainly deal with marked changes in the secretory function of adipocytes, and to a lesser degree of macrophages and pre-adipocytes, seen in obesity, diabetes and cardiovascular disease. It has now been firmly established that obesity is associated with the appearance of a chronic, low inflammatory state [40] due to changes in function of adipocytes and macrophages. This indicates that there is not merely an increase in secretion of proteins but that a pathological state i.e. inflammation, ensues from the changes in secretory function. We use the term adipocyte dysfunction for this state of hypersecretion of pro-atherogenic, pro-inflammatory and pro-diabetic adipocytokines which is accompanied by a decreased production of adiponectin.

Obesity leads to adipocyte dysfunction

Obesity has a strong genetic predisposition, and results from an excess energy intake and/or too little energy expenditure. Obesity is in most, but not all, subjects, associated with marked changes in the secretory function of adipocytes and macrophages,

together with chronic low-grade inflammation and an increased risk to develop insulin resistance, diabetes and/or cardiovascular disease [41].

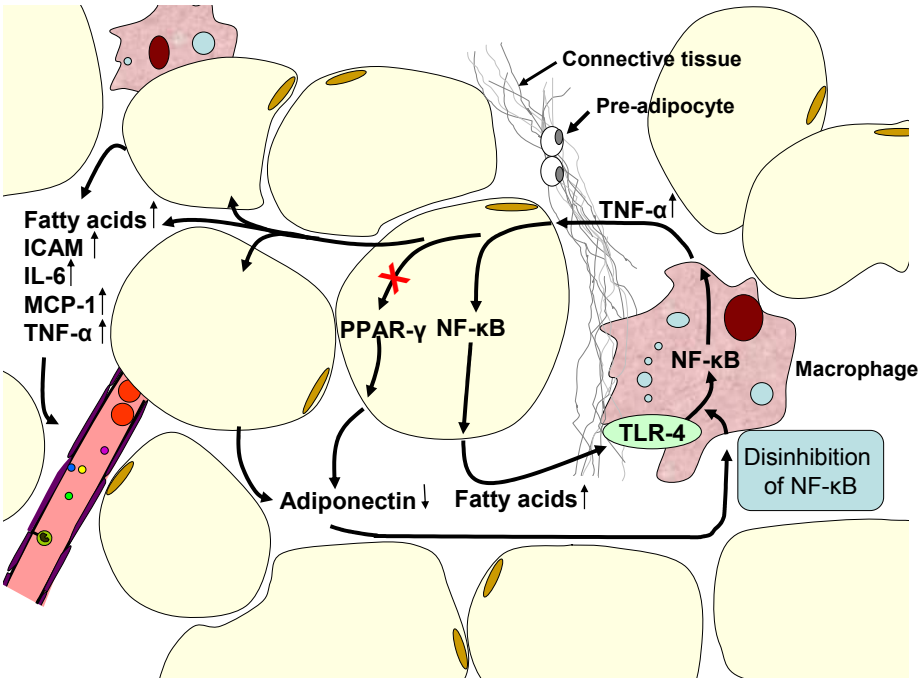
Interplay between macrophages and adipocytes by paracrine effects are presumably central in initiating and maintaining adipocyte dysfunction. Adipocytes enlarge as a consequence of hyper-alimentation. Large adipocytes release more (saturated) FFAs which can bind to macrophage Toll like receptor-4 (TLR-4) resulting in NF-κB activation ultimately leading to augmented TNF-α production [42,43]. In turn, macrophage-derived TNF-α activates human adipocytes, thereby further inducing lipolysis and enhancing the expression of various genes (Intracellular Adhesion molecule-1 (ICAM), IL-6, Macrophage Chemo attractant Protein-1 (MCP-1)) [44,45]. The diapedesis of monocytes from the blood to adipose tissue and differentiation into macrophages is further facilitated by MCP-1 and ICAM-1. This local paracrine loop involving adipocyte derived FFAs and macrophage-derived TNF-α establishes a gradual vicious cycle that presumably leads to a pro-inflammatory state of both macrophages and adipocytes. It is of note that large adipocytes produce less adiponectin. Since adiponectin normally inhibits TLR-activated NF-kB activity, it is assumed that low adiponectin levels re-enforce the above described loop (**Figure 2**). Interestingly, diet derived saturated fatty acids activate TLR-4 also directly, while poly-unsaturated fatty acids impede TLR-4 [46]. As adipocyte hypertrophy endures, local adipose tissue hypoxia occurs due to hypoperfusion. Adipocyte hypoxia elicits inhibition of adiponectin gene transcription illustrated by decreased adiponectin promoter and PPAR-γ activity, reduced adiponectin mRNA stability and finally a decline in adiponectin expression as shown in obese mice [47]. Simultaneous induction of leptin and PAI-1 gene transcription in adipose tissue suggests that the dysregulation of adipocytokine secretion is part of cellular mechanisms responding to local hypoxia and associated cellular stress.

Not only may the amount of fatty acids be relevant for mediating adipocyte function, but also the quality of the fatty acids. For example, dietary fish-oil, rich in long-chain unsaturated FFAs, increases activity and mRNA levels of FA oxidation enzymes in peroxisomes and mitochondria and is associated with increased adiponectin levels in mice presumably through PPAR-γ activation [48]. Indeed, it has been reported that poly-unsaturated fatty acids reduce mortality and morbidity in patients who suffered from a myocardial infarction [49].

Adipocyte dysfunction leads to obesity

In leptin deficient humans and in animal models, leptin administration results in reduced body mass and decreased hyperphagia. Although obese subjects have high levels of leptin, their energy expenditure and appetite are not sufficiently regulated, which has led to the concept of hypothalamic leptin resistance [50] which may be responsible for the persistent hunger and the difficulty to lose weight in obese subjects. Since insulin has similar properties as leptin in the hypothalamus, brain insulin resistance in obesity presumably adds to these effects of leptin resistance [51]. This may imply the existence of vicious circles of leptin resistance and insulin resistance which both lead to hunger and less energy expenditure thereby augmenting obesity.

Figure 2 *Adipocyte-macrophage interaction leading to dysfunction*



As adipocytes enlarge increased levels of adipocyte derived FFAs are released which can stimulate the already present macrophages to produce TNF-α. Saturated FFAs bind to the Toll like receptor-4 (TLR-4) which is expressed in macrophages resulting in NF-κB activation. In turn, macrophage-derived TNF-α activates human adipocytes in vitro, thereby enhancing the expression of various genes (Intracellular Adhesion Molecule-1 (ICAM), IL-6, Macrophage Chemo attractant Protein-1 (MCP-1). The diapedesis of monocytes from the blood to adipose tissue, and differentiation into macrophages is further stimulated by MCP-1 and ICAM-1. Low adiponectin levels re-enforce this loop by the diminished inhibition of TLR-4 activated NF-κB activity.

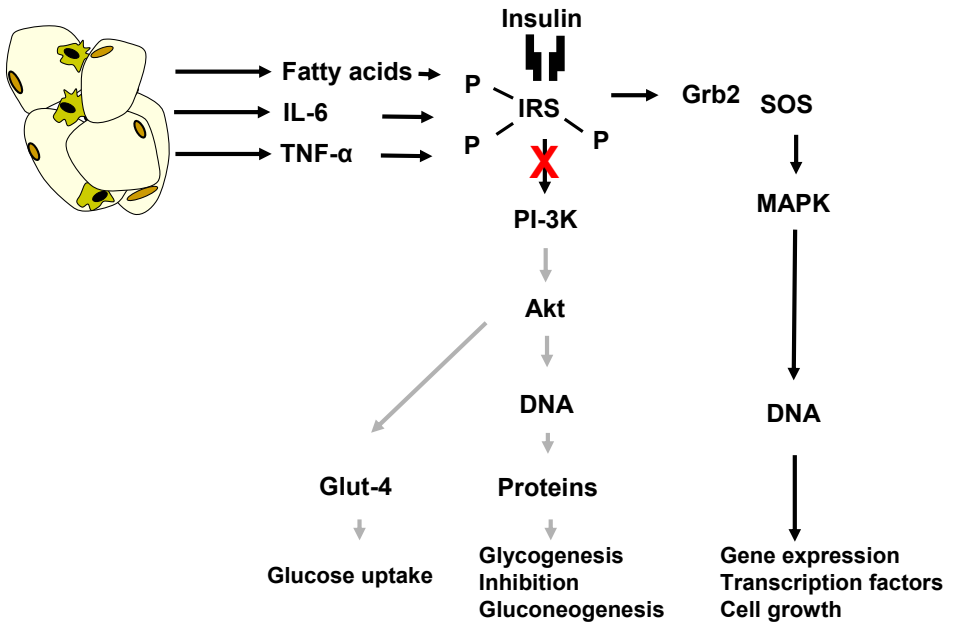
Adipocyte dysfunction, insulin resistance and type 2 diabetes mellitus

Both FFAs and TNF-α, secreted at high quantities by enlarged adipocytes play a prominent role in the development of insulin resistance [52] (**Figure 3**). Since insulin is the main regulator of HSL, the rate controlling enzyme for triglyceride hydrolysis, the inhibitory effect of FFAs on insulin sensitivity leads to enhanced lipolysis in adipocytes. This effect is augmented by the upregulation of triglyceride hydrolysis by TNF-α in adipose tissue. Besides, TNF-α also contributes to insulin resistance by inhibiting the expression of genes which are essential for insulin signalling and adipocyte differentiation (CCAAT-Enhancer-Binding Protein-α, PPAR-γ, glucose transporter type 4, insulin receptor substrate-1 protein, adiponectin and long-chain fatty acid acyl-CoA synthase) providing another molecular basis for insulin resistance [45].

Adiponectin increases insulin sensitivity by inhibiting hepatic glucose production and increasing fatty acid oxidation in both liver and muscle as a result of improved AMP-activated Kinase (AMPK) activity [53]. Single-nucleotide polymorphisms (SNPs) of the promoter region of the adiponectin gene may relate to the development of insulin resistance, obesity and type 2 diabetes [54,55]. In a study in morbidly obese subjects, SNPs in the adiponectin promoter gene were associated with a doubling of the risk for type 2 diabetes [56].

The high prevalence of non-alcoholic fatty liver disease in obese, insulin resistant and diabetic subjects may, at least in part, be due to adipocyte dysfunction. The increased flux of fatty acids and IL-6 through the portal circulation results in increased hepatic lipid accumulation. Leptin is considered to be a mediator of liver fibrosis after chronic liver injury in mouse models. However, this action of leptin may be reduced in leptin resistance [57]. Substitution of adiponectin ameliorates hepatomegaly and steatosis in mouse models of fatty liver disease in part due to antagonistic effects against TNF-α [58].

Figure 3 *Insulin resistance*



TNF-α, IL-6 and FFAs induce serine phosphorylation of IRS-1 and IRS-2, which reduces the capacity of IRS proteins to be phosphorylated by the insulin receptor in vitro and may even inhibit insulin receptor autophosphorylation (tyrosine kinase) activity, thereby further attenuating the insulin signalling cascade [98-100]. FFAs presumably act via activation of Protein Kinase-C isoforms (PKC) after formation of diacylglycerol, while TNF-α acts via activation of c-Jun N-terminal kinase-1 [60]. In muscle, FFA related generation of acyl-coA derivatives (e.g. ceramide) can diminish Akt1 activity and hence insulin action. In liver, IRS-2 is involved in inhibition of gluconeogenesis, which is often augmented in an insulin resistant state, possibly via activation of both PKC and c-Jun N-terminal kinase-1 by FFA and TNF-α.

Type 2 diabetes mellitus

In a prospective cohort study of women, 61% of the acquired cases of type 2 diabetes could be attributed to overweight and obesity. Already a mild increase in BMI increases the risk for type 2 diabetes: for example, women with a BMI between 23 to 25 kg/m^2 have an almost three-fold increased risk for developing diabetes compared to women with a BMI below 23 kg/m^2. This relative risk increases to 20 for women with BMIs of 35 or higher [59].

Type 2 diabetes is now generally accepted to be due to a combination of insulin resistance and relatively diminished insulin secretory function of pancreatic β-cells [60]. β-cell dysfunction is the most important risk factor for type 2 diabetes as shown in normoglycemic subjects [60]. Expansion of β-cell mass has been reported from pancreata of obese subjects and is related to increased intake of nutrients (glucose and FFAs). When insulin resistance increases, insulin production by pancreatic β-cells also increases but if this adaption fails diabetes will ensue. In most studies low adiponectin and elevated levels of other adipocytokines are associated with an increased risk for diabetes. This presumably relates not only to their effects on insulin sensitivity but also to their effects in the pancreas leading to β-cell failure [60] (**Figure 4**).

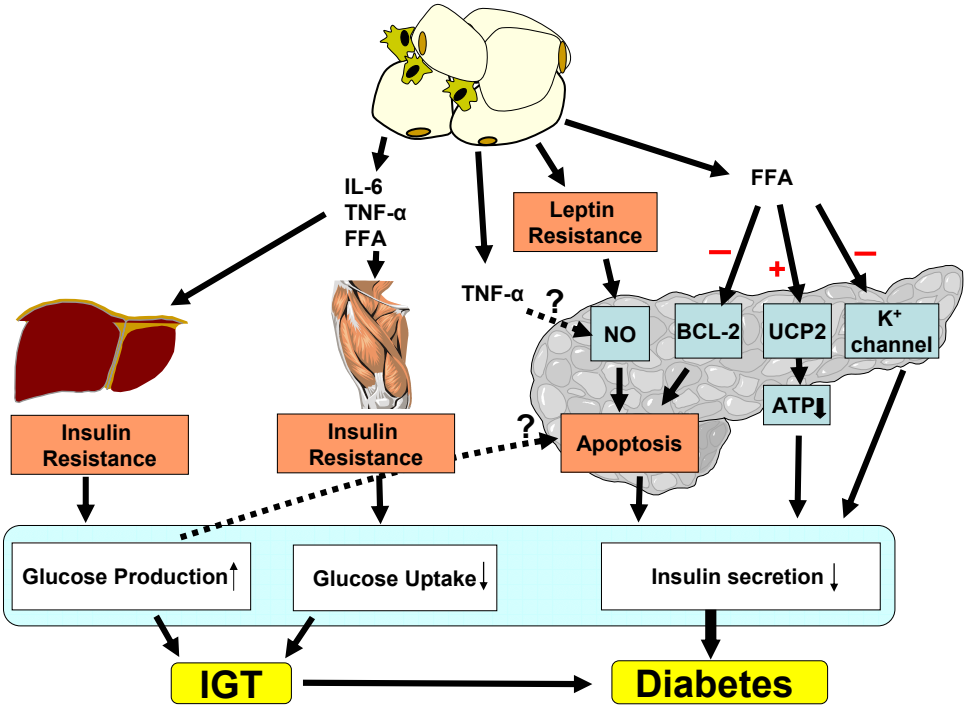
While FFAs acutely raise insulin secretion, chronically elevated plasma FFA levels as seen in obesity inhibit secretion [61]. Various proposed mechanisms have been put forward. In the presence of hyperglycemia oxidation of FFA is inhibited, resulting in accumulation of long-chain fatty-acyl-coA [61,62]. Long-chain fatty-acyl-CoA and FFA can open β-cell potassium channels which diminishes insulin secretion [60,61]. FFAs also enhance expression of uncoupling protein-2 (UCP-2), which would diminish ATP production necessary for insulin secretion. In addition, FFA can induce β-cell apoptosis via an endoplasmic stress response [61] and by inhibiting expression of the anti-apoptotic factor Bcl-2.

Since leptin has a restraining effect on normal insulin secretion by the pancreas, it has been proposed that in obesity leptin resistance might occur in β-cells, thus adding to hyperinsulinemia observed in obese subjects. Moreover, leptins anti-apoptotic effects in β-cells could be diminished in the leptin resistant state. Anti-apoptotic effects of leptin may include inhibition of nitric oxide (NO) production via reduction of triglyceride content. NO has been proposed to induce apoptosis via depletion of calcium stores in the endoplasmic reticulum (ER) leading to the ER stress response with induction of C/EBP homologue protein (CHOP) expression [61].

TNF-α inhibits glucose induced insulin secretion *in vitro* possibly via NO synthesis, which may cause damage to the insulin DNA strand [63], and may enhance apoptosis in β-cells via Bcl-2 [64]. Insulin signalling in the β-cell via β-cell insulin receptors is in itself of great importance for normal insulin secretory function, and TNF-α is capable of inhibiting insulin signalling. However, whether these *in vitro* phenomena are of real importance for obesity related mechanisms in type 2 diabetes is unclear since plasma TNF-α levels are lower than levels necessary to obtain the above effects.

Finally, adiponectin has no effect on normal insulin secretion, but diminishes the pro-apoptotic effects of cytokines and FFA on β-cells [61]. In the presence of insulin resistance in mice (due to high fat diet) adiponectin augments insulin secretion in response to high glucose while inhibiting insulin secretion at low glucose plasma concentrations.

Figure 4 *Adipocyte dysfunction leads to type 2 diabetes*



Chronically elevated FFA levels inhibit insulin secretion [61]. In vitro, long-chain fatty-acyl-CoA and FFA can open beta cell potassium channels which diminishes insulin secretion [60,61]. FFAs enhance expression of uncoupling protein-2 (UCP-2), which diminishes ATP production necessary for insulin secretion. FFAs can also induce β-cell apoptosis via an endoplasmic stress response [61] and by inhibiting expression of the anti-apoptotic factor Bcl-2. Leptin has been shown to have anti-apoptotic effects in β-cells, which may also be diminished in the (obese) leptin resistant state. Anti-apoptotic effects of leptin include inhibition of nitric oxide (NO) production via reduction of triglyceride content. NO has been proposed to induce apoptosis via depletion of calcium stores in the endoplasmic reticulum (ER) leading to the ER stress response [61]. By inhibiting insulin signalling in the β-cell and by induction of NO synthesis TNF-α may reduce insulin secretion in vitro. At the same time NO may cause DNA damage enhancing β-cell apoptosis [64].

Adipocyte dysfunction and cardiovascular disease

Atherosclerotic vascular disease may also be an important clinical consequence of adipocyte dysfunction. Dysfunctional adipocytes contribute directly and indirectly (through insulin resistance) to the development of cardiovascular risk factors and cardiovascular disease.

Adipocyte dysfunction and common vascular risk factors

Elevated blood pressure, low plasma HDL-c and elevated triglycerides, all independent cardiovascular risk factors, are closely associated with abdominal obesity and can often be controlled by dietary changes and weight reduction.

A growing body of evidence suggests that an activated renin-angiotensin-aldosterone system (RAS) and leptin are involved in obesity-associated hypertension by influencing the salt-fluid homeostasis and vascular tone. In obese subjects, plasma angiotensinogen (AGT) and renin concentrations are elevated and angiotensin converting enzyme (ACE) activity is increased [65]. Dysfunctional adipocytes of obese subjects produce AGT and angiotensin II [1], contributing to systemic blood pressure levels [66]. Weight loss of only 5% and especially a decrease in waist circumference is associated with reduced activity of all RAS-components and is accompanied with a 7 mm Hg decrease in blood pressure [65]. Remarkably, treatment with RAS-inhibitors prevents or delays the development of type 2 diabetes [67]. Angiotensin II may impair intracellular insulin signalling similarly to TNF-α and FFAs leading to reduced glucose uptake and diminished adipocyte differentiation [14,68]. Secondly, adiponectin gene expression may be directly increased by RAS-blockade, independently of body mass, as shown in essential hypertensive patients in whom ACE-inhibition and angiotensin II receptor blockers lead to improvements in insulin sensitivity without affecting adiposity [68]. Indeed treatment with RAS-inhibitors increases plasma adiponectin levels, improves whole body insulin sensitivity and decreases adipocyte size.

Leptin deficient subjects are normotensive despite the presence of considerable obesity [69]. Indeed, weight loss (resulting in decreased leptin levels) in obese subjects with hypertension by a calorie restricted diet resulted in lower blood pressure [65]. The concept of leptin-provoked hypertension is based on the findings that leptin up regulates Na^+/K^+-ATPase in the renal cortex and medulla [70]. In the brain leptin leads to an increased sympathetic nerve activity directed to the kidneys and peripheral vasculature which leads to increased heart rate and elevated blood pressure levels in mice, a response that is preserved in leptin resistance [71]. In addition to leptin, renal sodium reabsorption is enhanced under insulin resistant conditions and associated hyperinsulinemia [72].

A combination of elevated plasma triglycerides levels and decreased plasma HDL-cholesterol due to release of large quantities of FFAs and CETP by adipocytes is the typical dyslipidemia seen in obesity and insulin resistance. The increased fasting and postprandial FFA flux into the portal circulation contributes directly to the development of insulin resistance, endothelial dysfunction and increased VLDL-c synthesis in the liver. Elevated levels of FFAs are independently associated with an increased cardiovascular risk [73] although other studies show conflicting results. CETP facilitates the cholesteryl ester transfer from HDL-c to ApoB containing lipoproteins and the counter flux of triglycerides (TG) [74] resulting in elevated plasma levels of TG-rich HDL-c particles. These TG-rich HDL-c particles are rapidly hydrolyzed and cleared from the circulation resulting in low HDL-c levels [74]. CETP-deficient subjects have high HDL-c levels and are at low cardiovascular risk [74]. However, pharmacological inhibition of CETP-activity increases HDL-c plasma levels with 63% but fails to reduce progression of atherosclerosis as measured with carotid IMT in patients with mixed dyslipidemia [75]. Insulin resistance contributes to chronic hypertriglyceridemia due to less suppression by insulin of HSL and a reduction in insulin-activated LPL-activity, both leading to an enhanced flux of TGs from adipocytes to the liver [76].

Inhibition of the endocannabinoid system is a new option to improve cardiovascular risk factors besides essential lifestyle modifications. The receptors of the endogenous cannabinoid system are located at both the central nervous system and peripheral cells

(including adipocytes) and are proposed to modulate physiological functions such as drug dependency and feeding behaviour. Twelve months treatment of overweight patients with dyslipidemia resulted in a reduction in waist circumference (-5.8 cm), improvement of dyslipidemia (HDL-c +10% and TGs -13%) and increased plasma adiponectin levels [77].

Adipocyte dysfunction in visceral fat and vascular disease

Waist-to-hip ratio (WHR) and waist circumference, good indicators of abdominal obesity, are more closely associated with the risk for myocardial infarction than BMI [5]. After controlling for cardiac risk factors, including BMI, women with a WHR of at least 0.76 were more than twice as likely to develop coronary heart disease compared to women with a WHR beneath the 0.72. Women with a WHR higher than 0.88 were even more than 3 times as likely to develop coronary heart disease [78].

Presence of the metabolic syndrome is associated with lower adiponectin plasma levels, an indicator of adipocyte dysfunction [79] and with a 2- to 4- times increased risk for both the development of type 2 diabetes and cardiovascular disease [80]. Weight loss results in a decrease in the size of existing adipocytes (not to a decreased number of cells) and is associated with improvements in blood pressure, insulin sensitivity, dyslipidemia, low grade inflammation and adiponectin plasma levels [81].

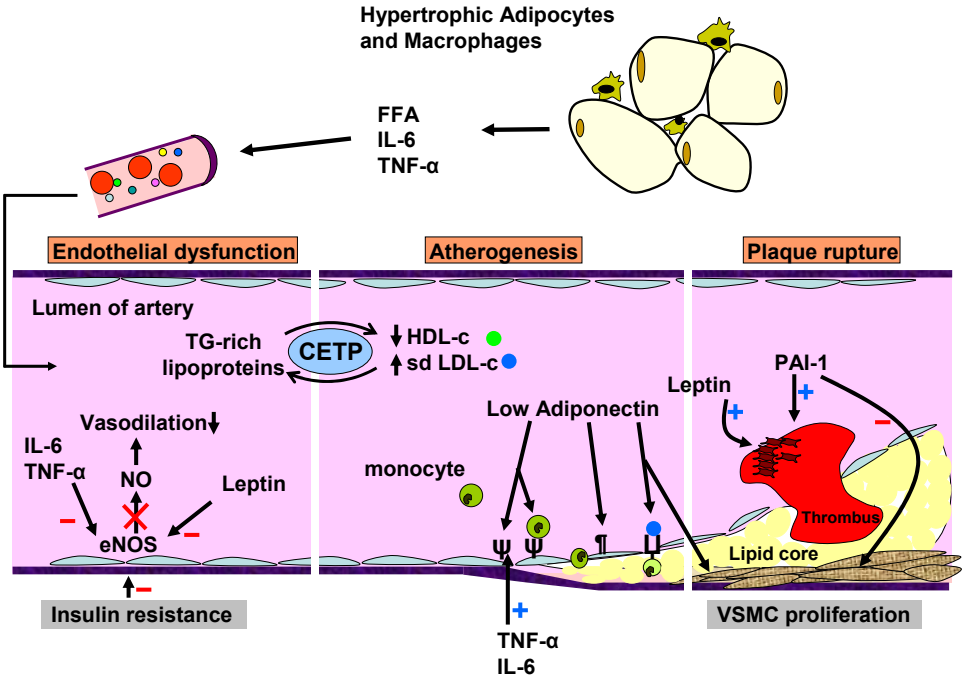
Adipocytokines and vascular disease

Independently of body weight, plasma adiponectin levels can be increased through inhibition of the endocannabinoid receptor system [77]. Being solely produced by adipocytes, a low plasma adiponectin concentration is a good representative of adipocyte dysfunction. Based on the anti-atherosclerotic properties *in vitro* adiponectin may be an important causal link between dysfunctional adipocytes and the development of cardiovascular diseases [53,82] (**Figure 5**). In various populations of healthy subjects and high risk patients, low plasma levels of adiponectin are independently predictive for future cardiovascular disease [26,83-85]. However, other studies among women in the primary care setting and in American Indians show discrepant results [86]. The discrepancy may be explained by functional differences between adiponectin isoforms and their ratios in plasma [87,88]. The High Molecular Weight (HMW)-form has better correlations with insulin sensitivity in subjects with and without type 2 diabetes compared to total or LMW-adiponectin levels, suggesting that HMW-adiponectin is the most active form [87]. Indeed, HMW-adiponectin levels were selectively suppressed in obese patients with ischemic heart disease and were restored after weight loss [89].

Results from prospective and case-control studies have pointed towards a possible contribution of coagulation factors and proteins of the fibrinolytic system in the development of cardiovascular events [90]. PAI-1 is a primarily regulator of fibrinolysis and is largely produced by visceral adipocytes [91] under influence of TNF-α, insulin, FFAs and glucocorticoids *in vitro* [92]. Elevated plasma PAI-1 levels (due to genetic polymorphisms or to obesity) are associated with an increase in cardiovascular risk [93,94].

This is due to a shift in the balance between fibrinolysis and thrombosis towards thrombosis facilitating the formation of micro-thrombi [15] and by PAI-1's ability to inhibit plasminogen-induced migrating of VSMCs resulting in plaques prone to rupture with thin fibrous caps, necrotic cores and rich in macrophages.
Elevated plasma levels of leptin are associated with adipocyte dysfunction, the presence of risk factors (increased BMI, CRP, LDL-c and TG) [95,96] and with increased cardiovascular risk, although prospective studies on the relationship between leptin and cardiovascular disease show inconsistent results. In older adults leptin was indeed associated with the extent of coronary artery calcifications, however this relationship was dependent of other risk factors (blood pressure, lipid levels and insulin resistance) [97].

Figure 5 *Adipocyte dysfunction leads to atherosclerosis*



Ψ ICAM-1, ¶ VCAM-1, Ц Scavenger receptor class A-1, Platelets
Elevated levels of IL-6, TNF-α and presence of insulin resistance lead to a decrease in production and availability of eNOS resulting in endothelial dysfunction. Increased adipocyte derived CETP plasma concentrations lead to lower levels of HDL-c and an increased number of small dense LDL-c particles.
Adiponectin has inhibitory effects on the development of atherosclerosis by inhibiting the expression of adhesion molecules (ICAM-1, VCAM-1)(induced by IL-6 and TNF-α) on endothelial cells by activating AMPK (in vitro) [82], by inhibiting NF-κB [82] and by the inhibition of scavenger receptor class A-1. The latter leads to reduction of cholesterol uptake in macrophages and to transformation of macrophages into foam cells [82]. Furthermore, adiponectin reduces vascular smooth muscle cell proliferation (VSMCs), migration and apoptosis by attenuating DNA synthesis inducing effects of growth factors including platelet-derived growth factor and fibroblast growth factor [53]. Increased levels of PAI-1 can inhibit plasminogen-induced migration of VSMCs leading to plaques prone to rupture with thin fibrous caps, necrotic cores and rich in macrophages. Leptin is capable to induce ADP-dependent platelet activity and aggregation in healthy subjects.

Conclusions

The classical perception of adipose tissue as a storage depot for free fatty acids has now been replaced by the notion that adipose tissue has a central role in lipid and glucose metabolism and produces a large number of hormones and cytokines involved in the development of metabolic syndrome, diabetes mellitus and cardiovascular diseases. The concept of adipocyte dysfunction contributes to the present understanding of the relationship between obesity and cardiovascular diseases, metabolic syndrome and type 2 diabetes mellitus. By understanding the roles of adipocytes and adipocytokines in normal physiology and in diseases, new strategies for preventing cardiovascular diseases and development of type 2 diabetes mellitus may be developed.

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CHAPTER 3

Levels of homocysteine are increased in metabolic syndrome patients but are not associated with an increased cardiovascular risk, in contrast to patients without the metabolic syndrome

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Abstract

Aim

The metabolic syndrome is associated with increased cardiovascular risk. Elevated plasma homocysteine may cause or result from insulin resistance, and may indicate vascular risk or be actively involved in atherogenesis. The aim of the study was to investigate the relationship between homocysteine, the metabolic syndrome and the incidence of cardiovascular events in patients with manifest vascular disease.

Methods

A cohort of 2169 patients with manifest vascular disease was followed for a mean period of 2.8 years. Plasma homocysteine was measured at baseline. Metabolic syndrome was defined by NCEP criteria.

Results

Homocysteine levels were higher in metabolic syndrome patients compared to patients without the metabolic syndrome (14.9 µmol/l; 95%CI: 14.5 to 15.3 µmol/l v 14.1 µmol/l; 95% CI: 13.8 to 14.5 µmol/l; p=0.002) and increased with the presence of its components (from 0 to 5) (12.7 to 15.9 µmol/l; p<0.001). During follow-up, 52 strokes, 67 myocardial infarctions, 5 fatal ruptures of aortic aneurysms and 53 vascular deaths occurred. Patients without the metabolic syndrome and homocysteine levels in the highest tertile had increased risk for events (HR 1.9; 95% CI 1.0 to 3.5) compared to patients without the metabolic syndrome and homocysteine levels in the lowest tertile. The presence of the metabolic syndrome increased the risk (HR 2.2; 95% CI 1.2 to 4.2), but elevated homocysteine levels further increased the risk only marginally (2.5; 95% CI 1.4 to 4.6).

Conclusions

Metabolic syndrome patients have elevated homocysteine levels, but these higher levels are not associated with an increased risk for new cardiovascular events. In contrast, elevated homocysteine levels confer increased risk in patients without the metabolic syndrome.

Introduction

The clustering of risk factors associated with central obesity (elevated glucose, dyslipidaemia and elevated blood pressure), often referred to as the metabolic syndrome, is associated with a two- to fivefold increased risk for the development of type 2 diabetes and a three- to fourfold increased incidence of cardiovascular diseases [1-5]. The prevalence of the metabolic syndrome varies from 9% to 27% depending on geographical location and age of the study population [4-6]. In patients with manifest vascular disease, the prevalence of the metabolic syndrome is even higher (46%) and associated with advanced vascular damage [7,8]. In patients who have experienced a myocardial infarction, the presence of the metabolic syndrome worsens the prognosis of survival and increases the incidence of future cardiovascular events [9].

Insulin resistance is considered to be the major underlying pathophysiological feature of the metabolic syndrome, as it interferes in many metabolic pathways [10]. It is not yet known whether the increased cardiovascular risk associated with the metabolic syndrome can be explained by the individual components only, or whether other risk factors associated with both atherosclerosis and insulin resistance are involved.

Homocysteine, a thiol-containing amino acid which is produced during the metabolism of methionine, is considered to be a risk factor or an indicator of risk for the development of cardiovascular disease [11-13]. In an insulin resistant state, elevated homocysteine plasma levels may be the result of hyperinsulinaemia, as observed in animal models [14,15]. At the same time, homocysteine may lead to insulin resistance through inhibition of insulin-receptor kinase activity in vitro [16,17]. Therefore, homocysteine may be a cause and/or a consequence of insulin resistance.

Studies investigating the association between the metabolic syndrome and homocysteine levels have shown conflicting results [18-25]. The aim of the present study is to determine whether the presence of the metabolic syndrome is associated with elevated levels of homocysteine and to determine the relationship between homocysteine and the incidence of new cardiovascular events in patients with manifest vascular disease with and without the metabolic syndrome.

Methods

Study design and patients

Data from patients enrolled in the SMART study (Second Manifestations of ARTerial disease), an ongoing single centre prospective cohort study carried out at the University Medical Center Utrecht, were used. Patients newly referred to our institution with clinically manifest atherosclerotic vascular disease (coronary heart disease, cerebrovascular disease, peripheral arterial disease or abdominal aortic aneurysm) or with cardiovascular risk factors (hyperlipidaemia, diabetes or hypertension) are included. The aims of the SMART study are to determine (i) the risk factors for atherosclerosis, (ii) the prevalence of additional vascular disease and (iii) the incidence of future cardiovascular events. The Medical Ethics Committee has approved the study and all patients gave written informed consent. Patients were asked to complete a health questionnaire covering

medical history, risk factors, smoking habits and medical treatment. A standardised diagnostic protocol was followed consisting of physical examination and laboratory testing in a fasting state. A more detailed description of the design of the study has been published previously [26].
The present study was based on the data of 2169 consecutive patients included in the SMART study between September 1996 and November 2004. Only data from patients with manifest vascular disease were used for analyses with the reservation that participants did not use folate therapy; 72 patients (3%) on folic acid therapy were therefore excluded from analyses.

Definitions

The metabolic syndrome was defined according to the Adult Treatment Panel III (ATPIII) criteria of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults [27]. Subjects were diagnosed with the metabolic syndrome if three or more of the following abnormalities were present:

- Abdominal obesity: waist circumference >102 cm in men or >88 cm in women.
- High blood pressure: ≥130 mm Hg systolic or ≥85 mm Hg diastolic or use of blood pressure lowering agents.
- Hypertriglyceridaemia: serum triglycerides ≥1.70 mmol/l (150 mg/dl).
- Low HDL-cholesterol: serum HDL-cholesterol <1.04 mmol/l (40 mg/dl) in men or <1.29 mmol/l (50 mg/dl) in women.
- High fasting glucose: fasting serum glucose ≥6.1 mmol/l (110 mg/dl) or use of glucose lowering agents.

Diabetes mellitus was defined as the use of glucose lowering agents and/or a fasting serum glucose concentration ≥7.0 mmol/l [28]. Smoking and alcohol consumption were defined as smoking or use of alcohol within the last 12 months. Creatinine clearance (ml/min) was estimated by the Cockroft-Gault formula.

Homocysteine measurement and methionine loading test

High performance liquid chromatography with fluorescence detection was used to determine plasma homocysteine levels. From September 1996 until May 1998, as part of a sub-study within the SMART cohort aiming at identifying risk factors in young patients with a recent clinical manifestation of atherosclerosis, methionine loading tests were performed in all consecutive patients <50 years of age. Plasma homocysteine was measured when fasting and 6 h after standardised methionine loading (100 mg/kg bodyweight). From May 1998 on, only fasting homocysteine levels were measured in all patients. As there are no international reference values for abnormal methionine loading tests, hyperhomocysteinaemia was defined as levels of homocysteine >55 µmol/l or an increase of 30 µmol/l above fasting levels as recommended by the Netherlands Heart Foundation [29].

Vascular endpoints

During follow-up, patients were asked twice a year to fill in a questionnaire on hospitalisations and outpatient clinic visits. When a possible event was reported by a participant, correspondence and relevant data were collected. Vascular death was reported by relatives of the participant, the general practitioner or by the vascular specialist to members of the SMART study group. Based on the information from the questionnaire and/or the family, all events were audited by three members of the SMART study Endpoint Committee, comprising physicians from different departments. In cases of disagreement, the opinion of other members of the Endpoint Committee was sought and final adjudication was based on the majority of the classifications obtained. The endpoints of interest for the present study were vascular death, ischemic stroke, coronary ischemic disease and these vascular events combined. If a patient had multiple events, the first recorded event was used in the analysis. In the present study of 2169 patients, 105 patients were excluded from follow-up analysis because of limited follow-up time (<6 months), and four subjects left the study directly after inclusion.

Statistical analyses

Data are presented as percentages with number of patient in parenthesis for categorical variables, as mean ± standard deviation (SD) for normally distributed variables and as median with the interquartile range in parenthesis for non-normally distributed variables.

The mean levels of homocysteine and 95% confidence intervals (95% CI) were calculated firstly for patients with and without the metabolic syndrome and secondly according to the number of metabolic syndrome components. Adjustment for age, gender and creatinine clearance was carried out with the general linear model (SPSS) since these factors were considered to be confounders. p-Values <0.05 were considered significant. Binary logistic regression was used to analyse the association between the methionine loading test and the metabolic syndrome. Cox proportional hazard analysis was performed to estimate adjusted hazard ratios with 95% CI for the association between tertiles of homocysteine levels and the occurrence of cardiovascular events. To investigate whether relationships between homocysteine and vascular events were modified by the presence of the metabolic syndrome, we compared the model with the interaction term metabolic syndrome and homocysteine tertiles with a model without that interaction term and compared the -2log likelihood of the two models. Patients without the metabolic syndrome and levels of homocysteine in the lowest tertile were the reference group. All statistical analyses were performed with the Statistical Package for Social Sciences version 12.0 (SPSS) for Windows.

Results

Study population

Patient characteristics of the 2169 study subjects with manifest vascular disease are presented according to tertiles of homocysteine plasma levels in **Table 1**. In the study population 1021 patients were recently diagnosed with coronary heart disease, 506 with

cerebrovascular disease, 462 with peripheral arterial disease and 180 with an abdominal aortic aneurysm.
In the study population, 78% of patients were male (from 69% within the lowest homocysteine tertile to 86% in the highest) and the mean age increased from tertile 1 (57 ± 10 years) to tertile 3 (63 ± 10 years). The prevalence of the metabolic syndrome within the total study population was 43%. Patients within the highest homocysteine tertile had lower creatinine clearance compared to patients within the lowest tertile (67 v 86 ml/min). Of the entire study population, 55% had a creatinine clearance between 60 and 90 ml/min and 19% had a creatinine clearance <60 ml/min. Diabetes mellitus was almost equally distributed between the tertiles, although the use of glucose lowering agents was more prevalent in the lowest tertile (13% v 10%). No difference was observed in the proportion of patients using blood pressure lowering agents in all tertiles.

Table 1 *Patient characteristics according to tertiles of homocysteine at baseline.*

	T1	**T2**	**T3**
	(n=724)	**(n=734)**	**(n=711)**
Homocysteine (µmol/l)*	9.8 ± 1.3	13.3 ± 1.0	20.4 ± 8.5
Male gender (% (n))	69 (503)	78 (576)	86 (610)
Age (years)*	56.8 ± 9.8	59.3 ± 9.8	62.8 ± 10.4
Body mass index (kg/m^2)*	26.7 ± 3.8	26.8 ± 3.8	26.4 ± 3.6
Smoking (% (n)) †	30.9 (224)	32.3 (237)	34.0 (242)
Alcohol use (% (n)) †	73.1 (529)	65.7 (482)	68.5 (487)
Total cholesterol (mmol/l)*	5.3 ± 1.1	5.3 ± 1.2	5.3 ± 1.0
Diabetes mellitus (% (n)) ‡	22 (161)	19 (139)	20 (139)
Creatinine clearance (ml/min) (Cockroft)	86.0 ± 17.8	79.3 ± 18.4	67.2 ± 20.8
Glucose-lowering agents (% (n))	13 (96)	10 (74)	10 (70)
Blood pressure-lowering agents (% (n))	27 (193)	31 (229)	41 (290)
Lipid-lowering agents (% (n))	15 (110)	15 (111)	17 (118)
Metabolic syndrome Yes (%(n))	43.5 (315)	40.5 (289)	44.8 (328)
Components of the metabolic syndrome			
Waist circumference (cm)*	94.7 ± 10.7	97.0 ± 10.5	96.9 ± 10.6
Blood pressure systolic (mmHg)*	138 ± 22	139 ± 20	144 ± 23
Blood pressure diastolic (mmHg)*	80 ± 11	80 ± 11	81 ± 12
HDL-cholesterol (mmol/l) #	1.16 (0.96 - 1.40)	1.14 (0.94 - 1.39)	1.16 (0.93 - 1.38)
Triglycerides (mmol/l) #	1.6 (1.1 - 2.2)	1.6 (1.1 - 2.2)	1.6 (1.2 - 2.4)
Fasting glucose (mmol/l)*	6.4 ± 2.3	6.3 ± 1.8	6.2 ± 1.6

** Mean ± standard deviation, # median with interquartile range.*
HDL: High-Density Lipoprotein;
† Still smoking, drinking or recently stopped smoking, drinking respectively;
‡ Fasting serum glucose ≥ 7.0 mmol/L or self-reported diabetes.

Homocysteine concentrations and the metabolic syndrome

In **Table 2** we present the homocysteine plasma concentrations (mean (95%CI)) in patients with and without the metabolic syndrome and by the number of metabolic syndrome components. Patients with the metabolic syndrome had significantly increased homocysteine fasting plasma levels compared to subjects without the metabolic syndrome after adjustment for age, creatinine clearance and gender (14.9 µmol/l; 95% CI: 14.5 to 15.3 µmol/l v 14.1 µmol/l; 95% CI: 13.8 to 14.5 µmol/l; p=0.002). The last part of **Table 2** shows a gradual and significant increase in plasma homocysteine concentrations with increase in the number of metabolic syndrome components after adjustment for age, gender and creatinine clearance (from 12.7 µmol/l; 95% CI: 11.7 to 13.7 µmol/l to 15.9 µmol/l; 95% CI: 14.8 to 17.0 µmol/l; p<0.001).

Table 2 *Homocysteine plasma concentration in patients with and patients without the metabolic syndrome, and by the number of components of the metabolic syndrome.*

Metabolic Syndrome	**Homocysteine, µmol/l**	
	mean (95% CI)	**p-Value**
Crude		
No	14.4 (14.0 to 14.8)	
Yes	14.5 (14.1 to 14.9)	0.6
Adjusted for age, gender and creatinine clearance		
No	14.1 (13.8 to 14.5)	
Yes	14.9 (14.5 to 15.3)	0.002
Number of metabolic syndrome components		
Adjusted for age, gender and creatinine clearance		
0	12.7 (11.7 to 13.7)	
1	13.6 (13.1 to 14.2)	
2	14.8 (14.3 to 15.3)	
3	14.6 (14.0 to 15.1)	
4	15.2 (14.5 to 15.9)	
5	15.9 (14.8 to 17.0)	<0.001

Methionine loading test

Methionine loading tests were performed in a group of 114 patients (mean age 44 ± 5 years, 62% male). At the time of inclusion, 27 of these patients were diagnosed with cerebrovascular disease, 42 with peripheral arterial disease and 45 with coronary arterial disease. Forty four patients (38%) fulfilled the criteria of the metabolic syndrome. The proportion of patients with abnormal levels of plasma homocysteine after the

methionine loading test was similar in patients with (22.7%) and without the metabolic syndrome (22.8%, odds ratio 1.1, 95% CI 0.4 to 2.8). The same analysis was carried out with a different cut-off level (50 µmol/l) for the methionine loading test. The odds ratio remained unchanged [30].

Homocysteine and vascular events

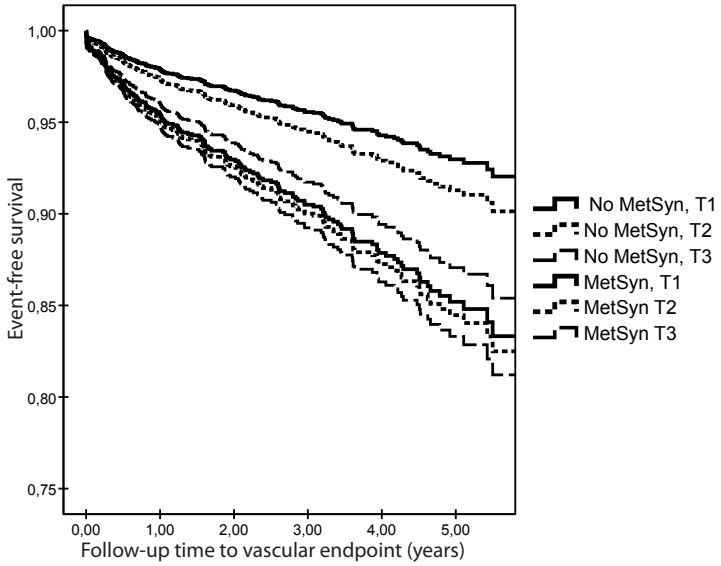
In the 2060 patients who were followed up, 177 vascular events occurred in a mean period of 2.8 years (range 0.1 - 7.5 years). During follow-up, 52 strokes, 67 myocardial infarctions, 5 fatal ruptures of abdominal aorta aneurysms, 29 acute vascular deaths and 24 other vascular deaths occurred. For the study population in total, patients with homocysteine levels in the highest tertile had a 40% increased risk for new cardiovascular events compared to patients with homocysteine levels in the lowest tertile (hazards ratio (HR) 1.4, 95% CI 0.9 to 2.2). For patients in the middle tertile, this risk was increased by 10% (HR 1.1, 95% CI 0.7 to 1.7).
The adjusted hazard ratios for new vascular events in patients with and without the metabolic syndrome for different homocysteine levels are shown in **Table 3**. In patients without the metabolic syndrome, increasing levels of homocysteine were associated with an increased risk of future cardiovascular events. Patients within the highest homocysteine tertile had an increased risk, HR 1.9 (95% CI 1.0 to 3.5), of experiencing a new vascular event compared to the reference group, who were patients without the metabolic syndrome and levels of homocysteine in the lowest tertile. However, in patients with the metabolic syndrome there was no relationship between homocysteine level and future cardiovascular events (p value for interaction= 0.049). The HR in the highest homocysteine tertile for developing cardiovascular events was 2.5 (95% CI 1.4 to 4.6), while patients with the metabolic syndrome and low levels of homocysteine (tertile 1) had a 2.2-fold (95% CI 1.2 to 4.2) increased risk compared to patients without the metabolic syndrome and within the lowest homocysteine tertile. **Figure 1** displays a Cox proportional hazard survival curve according to the homocysteine tertiles and the presence of the metabolic syndrome and adjusted for age, gender and creatinine clearance.

Table 3 *Cardiovascular risk in patients with and without the metabolic syndrome according to homocysteine plasma concentrations.*

Metabolic Syndrome	Tertile	Number of patients	Number of CV events	Hazard ratio* (95% CI)
No	1	391	37	Reference
	2	399	48	1.3 (0.7 to 2.4)
	3	384	87	1.9 (1.0 to 3.5)
Yes	1	302	49	2.2 (1.2 to 4.2)
	2	275	61	2.3 (1.2 to 4.4)
	3	309	85	2.5 (1.4 to 4.6)

** Adjusted for age, gender and creatinine clearance. CV, cardiovascular*

Figure 1 *Survival curve according to the presence of the metabolic syndrome and tertiles of homocysteine levels.*



No MetSyn/MetSyn: patients without and with the metabolic syndrome.
T1–T3: tertiles of plasma homocysteine concentrations, from low to high levels.
Adjusted for age, gender and creatinine clearance.

Discussion

In the present study we show that clustering of risk factors related to central obesity, often referred to as the metabolic syndrome, in patients with manifest vascular disease is associated with elevated fasting plasma levels of homocysteine compared to patients without the metabolic syndrome and show that homocysteine increases with the number of metabolic syndrome components. Elevated levels of plasma homocysteine are associated with an increased incidence of new cardiovascular events in patients without the metabolic syndrome but not in patients with the metabolic syndrome. The relationship between insulin resistance and levels of homocysteine has been investigated previously in different populations but with conflicting results [18-25]. Studies in non-diabetic healthy men and women showed that plasma homocysteine concentrations are not influenced by insulin resistance [18,22], while in similar populations, others found an inverse relationship between homocysteine and insulin resistance [19-24]. In yet other study populations (healthy non-obese, obese non-diabetic subjects and patients with the metabolic syndrome), a positive relationship was found between homocysteine and insulin resistance [20-23, 25].

Several mechanisms may explain the association between elevated homocysteine levels and insulin resistance or the metabolic syndrome. Increased homocysteine plasma concentrations may be the cause and/or the consequence of insulin resistance.

Decreased insulin receptor activity was observed in rat hepatoma cells overexpressing the human insulin receptor after incubation with homocysteine thiolactone [16,17], a metabolite of homocysteine which is present in human vascular endothelial cells [31]. This resulted in reduction of glycogen synthesis and reduced insulin-stimulated DNA and protein synthesis. Folate therapy given to patients with the metabolic syndrome (NCEP criteria) resulted not only in lower homocysteine concentrations but, more interestingly, also in reduced levels of insulin and improved insulin sensitivity [32]. Conversely, induction of insulin resistance in rats resulted in elevated plasma homocysteine levels [14,15].
Cystathionine-β-synthase, the key enzyme of the transsulfuration pathway in homocysteine metabolism, is downregulated in an insulin resistant state [14]. During methionine loading tests this pathway is more challenged than the remethylation pathway [33], and so these loading tests may therefore identify more precisely disturbances in homocysteine metabolism. The results of the present study do not indicate any differences in homocysteine metabolism after methionine loading in patients with or without the metabolic syndrome.
Different studies have investigated the impact of elevated levels of homocysteine on the development of cardiovascular events but lack indisputable proof of causality [34,35]. Elevated plasma homocysteine levels may be a cause of insulin resistance and be actively involved in atherogenesis, and therefore may be a risk factor. On the other hand, elevated levels of homocysteine could be considered an indicator of vascular risk and only be used for risk estimation. In the present study elevated homocysteine levels were associated with an increased risk of future cardiovascular events in patients with manifest vascular disease but without the metabolic syndrome. This risk was similar to that observed in patients with a MTHFR gene mutation, a mutation which leads to increased homocysteine levels [13]. In contrast, elevated levels of homocysteine did not substantially increase the already increased cardiovascular risk in patients with the metabolic syndrome. Apparently, in metabolic syndrome patients the cardiovascular risk is mainly determined by the individual metabolic syndrome components and other vascular risk factors associated with insulin resistance (for example, inflammation, hyperinsulinaemia, hypoadiponectinaemia). Another argument suggesting that elevated homocysteine levels are an indicator of risk instead of a risk factor is suggested by the results of two recent homocysteine lowering trials showing no positive effect on the cardiovascular risk in patients with manifest vascular disease [36,37]. These results are supported by previous findings [38,39], although other studies show conflicting results [40,41]. In the present study the relatively high homocysteine plasma concentrations in patients with and without the metabolic syndrome may be explained by the high prevalence of decreased renal function [24,42].
We acknowledge study limitations. In the present study we used the Adult Treatment Panel III definition for the metabolic syndrome [27]. Although this definition is most often used, other definitions for the metabolic syndrome do exist [43]. Secondly, the cut-off values for hyperhomocysteinaemia after methionine loading are arbitrary because no stringent normal ranges have been defined. However, levels above 55 µmol/l or an increase of 30 µmol/l after methionine loading are considered as elevated according to the Netherlands Heart Foundation [29]. It is likely that cardiovascular risk increases gradually and there is no particular threshold. Finally, it could be argued that plasma concentrations of vitamins

B6, B12 and folate may provide additional information on the relationship between the metabolic syndrome and cardiovascular disease. Information on these vitamins were not available in the present study. However, it is not likely that vitamins are confounders or effect modifiers in the analyses reported.
In conclusion, patients with the metabolic syndrome have elevated plasma homocysteine levels, but these elevated homocysteine levels are not associated with an increased risk for new cardiovascular events. These data indicate that elevated plasma homocysteine levels are not a risk factor for cardiovascular events in metabolic syndrome patients in contrast to patients without the metabolic syndrome.

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CHAPTER 4

Low plasma levels of adiponectin are associated with a low risk for future cardiovascular events in patients with clinical evident vascular disease

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Abstract

Background

Adiponectin is considered to have anti-inflammatory, insulin-sensitizing and anti-atherosclerotic properties. In the present prospective study, the relationship between metabolic syndrome (Adult Treatment Panel III) and adiponectin plasma levels and the relationship between plasma adiponectin levels and future cardiovascular events were investigated.

Methods

A case-cohort study of 431 patients with clinical evident vascular disease from the Second Manifestations of ARTerial Disease study. The relationship between adiponectin plasma levels and new vascular events was investigated with Cox regression, adjusted for potential confounders and effect modifiers (age, sex, renal function (modification of diet in renal disease), body mass index, high sensitive C-reactive protein, use of angiotensin converting enzyme-inhibition and/or AII antagonists and presence of metabolic syndrome or impaired renal function).

Results

Plasma adiponectin levels were lower in patients with metabolic syndrome as compared with patients without (7.9 ± 0.3 vs 5.2 ± 0.3 μg/ml) and decreased with the number of components. During a mean follow-up of 2.3 years, 216 patients had a new cardiovascular event. Lower adiponectin plasma levels were associated with a lower risk for future cardiovascular events (hazard ratio 0.50, 95% confidence interval 0.25–0.99). This relationship was not influenced by renal function, body mass index, and renin-angiotensin sytem-blocking agents or modified by metabolic syndrome and impaired renal function.

Conclusion

In patients with clinical evident vascular disease, lower adiponectin levels were associated with a lower cardiovascular risk. Therefore, it may be hypothesized that the potential antiatherosclerotic properties of adiponectin do not apply for patients with already established vascular disease.

Introduction

Adipose tissue, previously solely regarded as an energy storage depot, is currently considered an active endocrine organ producing a large number of adipokines and cytokines, which are involved in glucose and lipid metabolism and influence inflammation and hemostasis [1,2]. The clustering of vascular risk factors associated with excessive visceral adipose tissue (elevated plasma glucose levels, dyslipidemia, and elevated blood pressure) is often referred to as metabolic syndrome and is associated with an increased risk for diabetes type 2 and cardiovascular diseases [3]. Although the precise causes of metabolic syndrome are still unknown and the question remains whether the cardiovascular risk associated with metabolic syndrome is more than could be expected from the separate risk factors alone [4], insulin resistance, disturbed hemostasis and low-grade inflammation are considered to be essential pathophysiological features [5,6]. It has been hypothesized that a changed secretion rate of adipocytokines by dysfunctional adipocytes in obesity and metabolic syndrome contribute to the already increased cardiovascular risk in these patients [1,7]. Adiponectin, an adipokine solely derived from adipocytes, is considered to have anti-inflammatory, insulin sensitizing and antiatherosclerotic properties and may therefore be important in the development of obesity-induced cardiovascular disease [2,8]. Adiponectin plasma levels are decreased in obesity and related features; insulin resistance, metabolic syndrome, type 2 diabetes and inflammation [7,9] can be increased by renin-angiotensin sytem (RAS)-blocking agents [10]. In addition, adiponectin levels are lower in male compared with female subjects [11]. High levels of adiponectin in healthy subjects and in patients with severe chronic renal failure [11] have been associated with a lower risk for future ischemic heart disease [12,13], cerebrovascular disease and peripheral arterial disease [14,15]. A similar result was found in patients with familial hypercholesterolemia [16]. However, studies in healthy females and American Indians did not show a protective vascular effect of adiponectin [17,18]. The aim of the present study was 1) to determine the relationship between metabolic syndrome and adiponectin plasma levels and 2) to investigate the relationship between adiponectin plasma levels and new vascular events in patients with already clinical evident vascular disease and how this association is influenced by potential confounders (age, sex, renal function, body mass index, high sensitive C-reactive protein (hsCRP) and RAS-blocking agents) and effect modifiers (metabolic syndrome, renal function).

Methods

Study Population

Between 1996 and 2003, 2398 subjects with (a history of) clinical evident vascular disease were enrolled in the Second Manifestations of ARTerial disease (SMART) study, an ongoing prospective cohort study at the University Medical Center Utrecht, designed to establish the prevalence of concomitant arterial diseases and risk factors for cardiovascular disease in a high-risk population [19]. The Medical Ethics Committee approved the study, and all subjects gave their written informed consent. Clinical evident vascular disease includes coronary artery disease (angina pectoris and

myocardial infarction), cerebrovascular disease (transient ischemic attack, cerebral infarction, amaurosis fugax, and retinal infarction), an aneurysm of the abdominal aorta and peripheral artery disease (symptomatic and documented obstruction of distal arteries of the leg (Fontaine II and III)). At time of enrollment, patients underwent a standardized vascular screening, including a standardized health questionnaire, laboratory assessment (fasting lipid, serum glucose, creatinine, insulin and adiponectin levels) and anthropometric measurements.
For the present case-cohort study, all patients who had had a new vascular event (n = 220) during follow-up between enrolment and March 2003 were selected and considered as "cases". At the same moment, a random sample of 240 patients (10%) was drawn from the total cohort (2398) and served as control group. Of these 240 control patients, 16 had already been selected because of an outcome event. Hence, the current case-cohort study consisted of 444 patients. Of 13 patients (4 cases and 9 controls) blood samples were missing; therefore, analyses for the present study were carried out on the data of 431 patients with clinical evident vascular disease.

Definitions

Metabolic syndrome was defined according to the Adult Treatment Panel III criteria of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults [20]. Diabetes mellitus was defined as the use of glucose-lowering agents. Smoking was defined as the use of tobacco within the last 12 months. Creatinine clearance (ml/min) was estimated by the MDRD formula (GFR = 186 x [(serum creatinine x 0.0113)-1.154] x (age-0.203) x F; where F = 1 for males, 0.742 for females). Waist circumference was not measured in participants entering SMART before January 1, 1999. Cutoff values for obesity were waist circumference for males >102 cm and for females >88 cm [20]. If waist circumference was not available, a BMI cutoff point of 30 kg/m^2 was used as determinant of obesity (195 patients). Homeostasis model assessment of insulin resistance (HOMA-IR) was used as quantitative estimates of insulin resistance (=fasting glucose x fasting serum insulin / 22.5) [21].

Laboratory assessment

Insulin was measured with an immunometric assay (Diagnostic Product Corporatian, Los Angelos, USA). Plasma adiponectin levels were determined by using a commercially available kit (Quantitative enzyme immunoassay technique, R&D Systems Inc, USA).

Vascular endpoints

During follow-up, patients were twice yearly asked to fill in a questionnaire on hospitalizations and outpatient clinic visits. When a possible event was reported by a participant, correspondence and relevant data were collected. Vascular death was reported by relatives of the participant, the general practitioner or by the vascular specialist. Based on information of the questionnaire and/or family, all events were audited by 3 members of the SMART study Endpoint Committee, composed of physicians from different departments [19]. In case of disagreement, the opinion of other

members of the end point committee was sought, and final adjudication was based on most of the classifications obtained. The end points of interest were (acute) vascular death, cerebrovascular events, and myocardial infarction or the composite of these vascular events (**Table 1**). If a patient had multiple events, the first recorded event was used in the analysis.

Table 1 *Cardiovascular endpoints definitions*

Vascular death	Acute death: unexpected cardiac death occurring within 1 hour after onset of symptoms or within 24 hours given convincing circumstantial evidence.
	Death from ischemic stroke.
	Death from intracerebral hemorrhage (hemorrhage on CT-scan).
	Death from myocardial infarction.
	Death from congestive heart failure.
	Death from rupture of aneurysm of abdominal aorta.
	Vascular death from others causes such as sepsis after stent placement.
Cerebrovascular events	Definite: relevant clinical features that have caused an increase in impairment of at least one grade on the modified Rankin scale accompanied by a fresh ischemic infarction on a repeat brain scan.
	Probable: clinical features that have caused an increase in impairment of at least one grade on the modified Rankin scale without a fresh ischemic infarction on a repeat brain scan.
Myocardial infarction	At least two of the following criteria:
	1. Chest pain for over 20 minutes, not disappearing after administration of nitrates
	2. ST elevation more than 1 mm in 2 following leads or a left bundle branch block on the electrocardiogram.
	3. CK elevation of at least 2 times the normal value of CK and a MB fraction more than 5% of the total CK.

CT; computer tomography. CK; creatinine kinase. MB; myocardial band

Data analyses

Data are presented as percentages with number of patients in parentheses, as mean ± standard deviation for normally distributed variables and as median with interquartile range for nonnormally distributed variables. Differences between cases and controls at baseline were tested with unpaired *t*-test for continuous variables and with χ^2 for categorical variables. Mean levels of adiponectin were calculated for patients with and without metabolic syndrome and according to the number of metabolic syndrome

components. Adjustments for age, sex and MDRD were carried out with linear regression. To investigate the relationship between plasma levels of adiponectin and new cardiovascular events, hazard ratios (HRs) with corresponding 95% confidence interval (95% CI) were derived from Cox proportional hazard models with a SAS macro with the unweighted (default) method [22]. For this analysis, adiponectin levels were divided in quartiles, and the highest quartile was considered reference. Based on pathophysiological mechanisms and differences at baseline between cases and controls, effect estimates were adjusted for potential confounders; age, sex, BMI, use of RAS-blocking agents, high sensitive C-Reactive Protein (hsCRP) and renal function. Potential effect modification of metabolic syndrome and impaired renal function on the relationship between adiponectin and future vascular events was investigated by calculating interaction terms. In this specific analysis tertiles instead of quartiles were used because of the size of the created strata. Patients without metabolic syndrome or MDRD > 70 ml, respectively, and adiponectin levels within the highest tertile were considered reference group. Effect estimates were adjusted for age, sex and renal function, if appropriate. Interaction was considered present when the *P* value of the interaction term in the model was < 0.05. All statistical analyses were performed using the Statistical Package for the Social Sciences for Windows 12.1 (SPSS, Chicago, IL), except for the case-cohort analysis, which was performed using SAS 8.

Results

Baseline characteristics

In **Table 2** the baseline characteristics of the study population are displayed for cases (n = 216) and controls (n = 215). Among cases, male sex was more prevalent (80% vs 73%) and the mean age was higher (65 ± 10 vs 59 ± 10 years) compared with controls. Renal function defined by MDRD was lower in cases (67.2 ± 24.4 vs 76.4 ± 19.2 ml/min) compared with controls. The use of RAS-blocking agents was slightly higher among cases than controls (24% vs 28%). Adiponectin plasma levels were higher in cases compared with controls (7.5 ± 5.1 vs. 5.4 ± 3.6 µg/ml). In **Table 3**, baseline characteristics according to quartiles of plasma adiponectin concentrations are shown. As expected, higher levels of plasma adiponectin were associated with lower BMI, lower HOMA-IR, and lower prevalence of metabolic syndrome and its components. Male patients had lower plasma adiponectin levels compared with female patients. Plasma hsCRP levels were highest in patients in the quartile with highest adiponectin levels compared with the others quartiles. For controls the hsCRP levels were 1.7 mg/l (1.0-3.7) in the first quartile and 1.6 mg/l (1.0-3.4) in the highest quartile. For cases hsCRP levels were 3.1 mg/l (1.8-5.2) and 3.4 mg/l (1.6-5.5) for the first and last quartile (data not shown), respectively.

Table 2 *Baseline characteristics of study population*

	Controls n= 215	Cases n= 216	P-value
Adiponectin (µg/ml)	5.4 ± 3.6	7.5 ± 5.1	< 0.001
Male sex (%(n))	73 (156)	80 (173)	0.001
Age (years)	59 ± 10	65 ± 10	0.07
BMI (kg/m^2)	27 ± 4	26 ± 4	0.08
LDL-cholesterol (mmol/l)	3.3 ± 1.0	3.6 ± 1.0	0.002
hsCRP (mg/l)[b]	1.9 (1.0-3.7)	3.2 (1.7-5.5)	< 0.001
MDRD (ml/min)	76.4 ± 19.2	67.2 ± 24.4	< 0.001
Micro-albuminuria (mg/l)[b]	27 (13-69)	31 (13-75)	0.93
HOMA-IR	5.3 ± 11.2	5.1 ± 8.4	0.83
Insulin (mU/l)[b]	11.0 (8.0-17.0)	11.0 (8.0-17.0)	0.76
Homocysteine (mmol/l)	14.0 ± 5.0	17.8 ± 11.7	< 0.001
Diabetes Mellitus type 2* (%(n))	22 (48)	19 (41)	0.08
Blood pressure lowering agents (%(n))	40 (85)	34 (73)	0.72
RAS-blocking agents (%(n))	24 (51)	28 (60)	0.33
Smoking (%(n))	37 (80)	42 (91)	0.24
Clinical evident vascular disease at inclusion			
Cerebrovascular disease (%(n))	30 (65)	39 (83)	0.08
Peripheral arterial disease (%(n))	29 (62)	32 (69)	0.48
Coronary artery disease (%(n))	52 (112)	53 (115)	0.81
Abdominal aortic aneurysm (%(n))	9 (20)	25 (53)	< 0.001
Metabolic syndrome (% (n))	55 (118)	54 (117)	
Waist (cm)	96 ± 12	98 ± 12	0.28
Triglycerides (mmol/l) [b]	1.7 (1.2-2.2)	1.6 (1.2-2.4)	0.73
HDL-cholesterol (mmol/l) [b]	1.0 (0.9-1.3)	1.1 (0.9-1.3)	0.31
Glucose (mmol/l)	7.0 ± 3.0	6.6 ± 2.4	0.06
Systolic blood pressure (mmHg)	143 ± 21	147 ± 23	0.05
Diastolic blood pressure (mmHg)	80 ± 10	80 ± 12	0.64

All values are mean ± SD or as stated; b median (interquartile range)
** Diabetes Mellitus type 2: patients treated with glucose lowering agents*

Table 3 *Baseline characteristics according to quartiles of plasma adiponectin concentrations*

	Q1 (n=108)	Q2 (n=108)	Q3 (n=108)	Q4 (n=107)
Adiponectin, range (µg/ml)	0.2-3.3	3.4-5.0	5.1-8.3	8.4-29.6
Adiponectin (µg/ml)	2.2 ± 0.7	4.2 ± 0.5	6.5 ± 1.0	12.9 ± 4.2
Male sex (%(n))	85 (92)	84 (91)	71 (77)	63 (69)
Age (years)	58 ± 11	62 ± 10	63 ± 10	67± 11
BMI (kg/m^2)	27.7 ± 3.5	27.3 ± 4.1	26.1 ± 3.8	24.5 ± 3.4
LDL-cholesterol (mmol/l)	3.2 ± 1.1	3.4 ± 1.0	3.7 ± 1.0	3.5 ± 0.9
hsCRP (mg/l)[b]	2.5 (1.1-4.3)	2.4 (1.3-3.5)	2.4 (1.2-4.8)	3.0 (1.3-5.6)
MDRD (ml/min)	78.9 ± 18.8	73.1 ± 21.2	70.7 ± 19.5	65.8 ± 28.6
HOMA-IR	7.7 ± 15.66	5.1 ± 6.3	3.6 ± 3.8	4.3 ± 9.1
Insulin (mU/l) [b]	14.0 (9.0-19.0)	12.0 (9.0-19.3)	9.5 (7.0-14.3)	8.0 (6.0-12.3)
Homocysteine (mmol/l)	13.6 ± 4.8	15.3 ± 5.6	15.5 ± 6.2	19.7 ± 16.2
Diabetes Mellitus type 2* (%(n))	20 (21)	22 (24)	24 (26)	17 (18)
Blood pressure lowering agents (% (n))	34 (37)	40 (43)	40 (43)	34 (37)
RAS-blocking agents (% (n))	30 (32)	28 (31)	22 (24)	22 (24)
Smoking (% (n))	37 (40)	37 (40)	47 (49)	39 (42)
Number of events (% (n))	36 (39)	49 (53)	50 (54)	65 (70)
Clinical evident vascular disease at inclusion				
Cerebrovascular disease (%(n))	15 (16)	22 (24)	31 (34)	35 (38)
Peripheral arterial disease (%(n))	20 (25)	19 (21)	24 (26)	28 (30)
Abdominal aortic aneurysm (%(n))	8 (9)	12 (13)	9 (10)	16 (17)
Coronary artery disease (%(n))	48 (52)	28 (33)	26 (28)	6 (6)
Metabolic syndrome (%(n))	74 (80)	58 (63)	52 (57)	32 (35)
Waist (cm)	98 ± 11	100 ± 12	95 ± 10	90 ± 12
Triglycerides (mmol/l) [b]	2.12 (1.53-2.85)	1.69 (1.34-2.16)	1.41 (1.22-2.36)	1.41 (1.06-2.04)
HDL-cholesterol (mmol/l) [b]	0.94 (0.81-1.08)	1.02 (0.89-1.31)	1.12 (0.95-1.30)	1.31 (1.07-1.51)
Glucose (mmol/l)	7.4 ± 3.5	6.7 ± 2.1	6.4 ± 2.1	6.7 ± 3.0
Systolic blood pressure (mm Hg)	140 ± 19	144 ± 21	146 ± 24	148 ± 24
Diastolic blood pressure (mm Hg)	80 ± 11	81 ± 9	81 ± 12	80 ± 12

All values are mean ± SD or as stated, b median and interquartile range.
** Diabetes Mellitus type 2: patients treated with glucose lowering agents*

Adiponectin in relation to metabolic syndrome

The prevalence of metabolic syndrome in this population was 55%, equally distributed between cases and controls. Adiponectin levels were lower in patients with metabolic syndrome compared to patients without metabolic syndrome (5.2 ± 0.3 vs 7.9 ± 0.3 µg/ml) (**Table 4**). After adjustment for age, sex, and renal function according to the MDRD levels of adiponectin slightly changed. Although considered as potential confounders, age, sex, and renal function were equally distributed between patients with and without metabolic syndrome. A gradual decrease of plasma adiponectin concentrations was seen with the increase in the number of metabolic syndrome components, from 1 to 5 components present (from 9.1 ± 0.4 to 4.3 ± 0.7 µg/ml, P value for trend <0.001, after adjustment for confounders). The creatinine clearance was lower in patients with metabolic syndrome compared to patients without (69.6 ± 1.5 vs 74.5 ± 1.6 ml/min).

Table 4 *Adiponectin levels according to the presence of the metabolic syndrome and number of metabolic syndrome components.*

	Number of events (patients)	Adiponectin (µg/ml) Mean ± sem	p-value
Metabolic Syndrome			
No	99 (196)	7.9 ± 0.3	
Yes	117 (235)	5.2 ± 0.3	< 0.001
Adjusted for age, sex, creatinine clearance (MDRD)			
No	99 (196)	8.1± 0.3	
Yes	117 (235)	5.2 ± 0.3	< 0.001
Number of metabolic syndrome components			
Adjusted for age, sex, creatinine clearance (MDRD)			
0	6 (14)	6.9 ± 1.1	
1	39 (73)	9.1 ± 0.4	
2	54 (109)	7.5 ± 0.4	
3	58 (115)	5.6 ± 0.4	
4	44 (82)	5.0 ± 0.5	
5	15 (38)	4.3 ± 0.7	< 0.001

Adiponectin and cardiovascular events in patients with clinical evident vascular disease

During a mean follow-up of 2.3 ± 1.8 years, 216 new vascular events occurred: 6 fatal ruptures of abdominal aortic aneurysms, 64 cerebrovascular events, 66 nonfatal myocardial infarctions, 43 acute vascular deaths and 37 other vascular deaths. Patients

with the lowest plasma adiponectin levels had the lowest risk for new vascular events (HR 0.42, 95% CI 0.23–0.75) compared with patients in the highest quartile of adiponectin levels (**Table 5**). Adjustments for age and sex affected this relationship only slightly. Further adjustments for the potential confounders renal function [18], use of RAS-blocking agents [10], smoking [12] and BMI [9] did not change this association. Finally, introducing hsCRP into the regression model only marginally changed the relationship.

Table 5 *Relationship HR (95% CI) between quartiles of plasma adiponectin and the risk for cardiovascular events*

	Q1	Q2	Q3	Q4
Cases (%(n))	40 (43)	50 (54)	54 (58)	68 (73)
HR crude	0.42 (0.23-0.75)	0.71 (0.40-1.26)	0.59 (0.33-1.03)	Reference
HR adjusted for age and sex	0.49 (0.25-0.95)	0.71 (0.37-1.33)	0.63 (0.35-1.13)	Reference
HR adjusted for age, sex and renal function (MDRD)	0.50 (0.25-0.99)	0.72 (0.38-1.37)	0.61 (0.33-1.13)	Reference
HR adjusted for age, sex, renal function (MDRD) and BMI	0.50 (0.24-1.03)	0.72 (0.37-1.40)	0.61 (0.33-1.12)	Reference
HR adjusted for age, sex, renal function (MDRD), BMI and ACE-inhibition and/or AII antagonist use	0.50 (0.24-1.03)	0.71 (0.36-1.39)	0.60 (0.32-1.11)	Reference
HR adjusted for age, sex, renal function (MDRD), BMI, ACE-inhibition and/or AII antagonist use and hsCRP	0.48 (0.23-1.00)	0.81 (0.41-1.61)	0.63 (0.34-1.17)	Reference

Adiponectin, metabolic syndrome, renal function and new cardiovascular events

The relationship between adiponectin plasma levels and future cardiovascular events was not modified by the presence of metabolic syndrome (*P* values for interaction 0.9 and 0.6). Both in patients with metabolic syndrome and nonmetabolic syndrome, lower levels of adiponectin were associated with a decreased risk for future cardiovascular events. In addition, an impaired renal function (MDRD <70 vs >70ml/min) did not modify the relationship between adiponectin plasma levels and future cardiovascular events (*P* values for interaction 0.6 and 0.3).

Discussion

The results of the present etiological study indicate that in patients with already clinical evident vascular disease, patients with metabolic syndrome have lower plasma adiponectin levels compared with patients without metabolic syndrome. Lower levels of plasma adiponectin were associated with a lower risk for new cardiovascular events. The observed relation persisted after adjustments for established and potential

confounding factors and was not influenced by the presence of metabolic syndrome and impaired renal function.

Adiponectin, solely produced by adipocytes, has in vitro and in animal models an array of metabolic effects. It increases insulin sensitivity by inhibiting hepatic glucose production and increasing fatty acid oxidation in both liver and muscle [23]. Furthermore, adiponectin directly affects atherogenesis. Again from in vitro experiments, adiponectin reduces adhesion molecules expression on endothelial cells by inhibiting AMPK and nuclear factor-kappa-B. Finally, adiponectin inhibits cholesterol uptake in macrophages by inhibition of scavenger receptor class A-1 [2]. Based on these findings, it is hypothesized that decreased adiponectin levels are a causal link between dysfunctional adipocytes in obesity and the development of type 2 diabetes and atherosclerosis. However, a meta-analysis of prospective studies concluded that there is indeed an association between adiponectin plasma levels and atherosclerotic vascular disease, but this may be weaker than previously thought [2,24]. In the present study, we observed that low adiponectin levels were associated with a lower risk for future cardiovascular events compared with higher adiponectin plasma levels despite the fact that lower plasma adiponectin levels were associated with a higher mean BMI, higher levels of plasma insulin, and a higher prevalence of metabolic syndrome, all associated with increased vascular risk. The fact that in the present study plasma levels of adiponectin were higher in patients with a decreased renal function is in accordance with previous observations in different populations [11,18]. In addition, the finding that lower plasma adiponectin levels were associated with an increase in the number of metabolic syndrome components, as shown in the present study, is also in concordance with other studies [11,25]. Nevertheless, the risk for developing new cardiovascular events was lower in patients with low levels of adiponectin compared with patients with higher levels of adiponectin even after adjustment for potential confounding factors such as BMI, smoking, renal function, hsCRP [26] and use of RAS-blocking agents. Renin-angiotensin system suppression by treatment with (ACE) inhibitors and/or AII antagonists led to increased levels of plasma adiponectin [10]. Whether this increase is due to enhanced adipogenesis or to direct effects of angiotensin II on intracellular insulin signalling is not yet clear.

The result of the present study that higher plasma adiponectin levels were associated with higher vascular risk in patients with already clinical manifestation of vascular disease was unexpected. How could this be explained? In patients with chronic heart failure (CHF), higher levels of adiponectin were also associated with increased total mortality [27]. A potential explanation could be that patients with CHF have increased resting energy expenditure leading to weight loss and subsequently increased adiponectin plasma levels. Therefore, in this situation, adiponectin reflects a poor physical condition (wasting) associated with an increased cardiovascular risk. Various diseases (CHF, rheumatoid arthritis, and malignancies) are associated with wasting of body mass, mainly mediated by increased levels of TNF-α and IL-6, and a subsequent increase in plasma adiponectin levels [28,29]. In the present study, all patients had a recent clinical manifestation of a vascular disease. The plasma levels of hsCRP at baseline were higher in patients that have a new vascular event during follow-up and were higher in the quartile with the highest adiponectin levels compared with the other quartiles. These higher hsCRP levels may also be the result of increased TNF-α and IL-6 production

by abdominal adipocytes enhancing hepatic hsCRP synthesis. Therefore, higher hsCRP levels may be another reflection of low-grade inflammation and a poor physical condition. This concept may be most applicable for the highest quartile because these patients had the highest cardiovascular risk, the lowest waist circumference, the highest adiponectin levels, and the highest hsCRP levels. Adjustment for the potential confounding effect of hsCRP on the relationship between adiponectin levels and atherosclerotic events did not change the strength of the relationship. We have no information available on changes in body weight at time of inclusion in the SMART program or during follow-up. Another cause for weight changes leading to higher adiponectin levels but also leading to increased cardiovascular risk could be a depressive mental state after a recent clinical manifestation of a vascular disease [30,31]. This may lead to reduced food intake and neglect leading to loss of body mass and is associated with an increased vascular risk [30.31].

Compared with other studies involving patients with vascular diseases, the plasma adiponectin levels in the present study are lower [15,17] but are comparable with those in patients with acute coronary syndrome or first ischemic stroke [32]. Whether this partly explains the unexpected findings in our studies is uncertain.

Adiponectin circulates in plasma as mono, hexa, and high molecular weight forms and as a proteolytic cleavage product: the globular form. It could be hypothesized that different isoforms of adiponectin have different properties and effects on lipid and carbohydrate metabolism [33,34]. Whether functional differences of isoforms or the relatively low levels of total adiponectin can explain the opposing results reported by previous studies and the present study in patients with clinical evident vascular disease in relation to vascular risk is uncertain because adiponectin isoforms were not measured in the present study. However, it should be noted that most circulating adiponectin are present in the full-length form [8].

A potential limitation of our study may be the use of the metabolic syndrome definition according to Adult Treatment Panel III. This definition of metabolic syndrome is associated with an increased risk for cardiovascular disease [3] is most often used in clinical studies, and can be easily used in clinical practice, but other definitions do exist.

In conclusion, lower levels of adiponectin are associated with a lower risk for the development of new cardiovascular events in patients with clinical evident vascular disease. Metabolic syndrome and impaired renal function are associated with decreased and increased plasma levels of adiponectin respectively, but do not modify the relationship between adiponectin and cardiovascular risk. To further clarify the relationship between cardiovascular risk and adiponectin, further studies (in vitro and in vivo) are needed in investigating the role of adiponectin isoforms in atherogenesis.

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CHAPTER 5

Low plasma HDL-cholesterol confers an increased cardiovascular risk regardless of LDL-cholesterol levels in patients with various clinical manifestations of vascular disease: the SMART Study

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Submitted

Abstract

Background

To determine the relationship between High Density Lipoprotein-cholesterol (HDL-c) and new (non-) fatal vascular events in patients with various manifestations of clinical evident vascular disease and to evaluate whether this relationship is modified by Low Density Lipo protein-cholesterol (LDL-c) levels.

Methods

Prospective single center, cohort-study of 3837 patients with a history or recent diagnosis of clinical manifest vascular disease (coronary, cerebrovascular, peripheral arterial disease, including Abdominal Aortic Aneurysm) The relationship between HDL-c quintiles and time to a new event (myocardial infarction, ischemic stroke, vascular death) was quantified with Cox regression models and adjusted for potential confounders (age, gender, BMI, type 2 diabetes, triglycerides, smoking, use of alcohol and lipid lowering therapy). Effect modification of LDL-c was assessed with interaction terms.

Results

During a median follow up of 3.3 (range 0.1-9.5) years a total of 465 first new events occurred. Compared to the lowest quintile, the upper quintile of HDL-c levels was associated with a lower risk for new events; HR 0.61 (95%CI 0.43-0.86) irrespective of the localisation of vascular disease and use of lipid-lowering medication. Higher HDL-c levels were associated with comparably lower risks for vascular events in patients with LDL-c levels above and below 2.5 mmol/l (p-values for interaction >0.05).

Conclusions

Higher HDL-c plasma levels are associated with a lower risk for vascular events in patients with clinical evident manifestations of vascular disease at various localisations, independently of the use of lipid lowering therapy. The inverse relationship between high HDL-c levels and future vascular events holds for both patients with low and high LDL-c levels.

Introduction

Patients with clinical manifestations of atherosclerosis in the cerebro-, cardio- or peripheral vasculature are at high risk for developing new vascular events or vascular death in the (near) future compared with patients without clinically evident vascular disease [1]. Despite intensive risk factor management, including Low Density Lipoprotein cholesterol (LDL-c) lowering therapy with statins alone or in combination with a cholesterol-absorption inhibitor, a substantial cardiovascular risk remains present in these patients [2,3]. In European and American cardiovascular prevention guidelines, LDL-c is the primary lipid treatment target, aiming at an LDL-c <2.5 mmol/l [4,5]. Based on anti-atherosclerotic properties of High Density Lipoprotein cholesterol (HDL-c), low plasma HDL-c levels may be one of the factors contributing to the residual risk in statin treated high-risk patients. HDL-c particles presumably play an important role in reverse cholesterol transport by trafficking cholesterol from peripheral cells, including vascular endothelial cells, to the liver for biliary excretion [6]. In addition, HDL-c has anti-atherogenic properties by exerting anti-oxidation of LDL-c particles, reducing monocyte recruitment at endothelial lesions [7, 8] and has anti-thrombotic and anti-inflammatory effects [9, 10].
In the general population low levels of HDL-c have consistently been associated with an increased risk for the development of cardiovascular diseases [11,12]. In patients with coronary artery disease a similar relationship between HDL-c levels and future recurrent cardiovascular events was found [1, 13, 14]. However, it is not yet known whether these results can be extrapolated to patients with symptomatic atherosclerotic disease at other sites of the vascular system, like cerebrovascular or peripheral arterial disease. Studies investigating the relation between HDL-c and cardiovascular risk were performed before cardiovascular disease prevention guidelines were tightened aiming at lower treatment targets for LDL-c and blood pressure resulting in the widespread use of blood pressure-lowering and LDL-lowering medication [4,15]. Recent drug development is directed towards raising HDL-c plasma levels in high-risk patients in addition to intensive LDL-c lowering [8,16,17]. It would be important to know whether HDL-c still is a risk factor for vascular diseases in high risk patients with well treated risk factors.
The aims of the current prospective cohort study were therefore 1) to determine the relationship between HDL-c plasma levels and new vascular events in patients with clinical evident vascular diseases 2) to evaluate whether this relationship is present irrespective of the localisation of vascular disease and the use of lipid-lowering medication and 3) to investigate whether this relation is modified by the plasma LDL-c concentration.

Methods

Study Population

Data were used from patients enrolled in the Second Manifestations of ARterial disease (SMART) study. The SMART study is an ongoing prospective cohort study at the University Medical Center Utrecht, the Netherlands, designed to establish the prevalence of concomitant arterial diseases and risk factors for cardiovascular disease in a high-

risk population [18]. Briefly, patients aged 18 to 79 years newly referred to our medical center with cardiovascular risk factors (hypertension, dyslipidemia, type 2 diabetes) or with already clinically evident vascular disease (cerebrovascular, cardiovascular or peripheral arteries) were asked to participate. Patients who gave their written informed consent underwent a standardized vascular screening including a health questionnaire for clinical information, laborat.ory assessment and anthropometric measurements at enrolment. The Medical Ethics Committee approved the study [18].
For the present prospective cohort study data were used from the first consecutive 3875 patients with either a history or a recent diagnosis of clinical evident vascular disease who were enrolled in SMART between September 1996 and March 2006. Clinical evident vascular disease comprised coronary artery disease (angina pectoris and myocardial infarction); cerebrovascular disease (transient ischemic attack, cerebral infarction, amaurosis fugax or retinal infarction); peripheral artery disease (aneurysm of the abdominal aorta or symptomatic and documented obstruction of distal arteries of the leg; Fontaine II and III). Of 38 patients HDL-c levels were missing, hence the study population consisted of 3837 patients.

Definitions

Diabetes mellitus was defined as the use of glucose lowering agents. Smoking was defined as the use of tobacco within the last 12 months. Creatinine clearance (ml/mim) was estimated by the MDRD formula [19].

Laboratory assessment

Plasma total cholesterol, triglycerides, glucose and creatinine are measured with commercial available enzymatic dry chemistry kits (Johnson and Johnson) [18]. HDL-c in plasma is determined with a commercial enzymatic kit (Boehringer-Mannheim) after precipitation of LDL-c and VLDL-c with sodium phosphotungstate magnesium chloride. LDL-c is calculated by the Friedewald formula [20].

Vascular endpoints

During follow-up, from September 1996 to September 2006, patients were biannually asked to fill out a questionnaire on hospitalizations and outpatient clinic visits. When a possible event was reported by a participant, correspondence and relevant data were collected (discharge letters, laboratory and radiology results). Vascular death was reported by relatives of the participant, the general practitioner or by the vascular specialist. Based on all obtained information, every event was audited by 3 members of the SMART study Endpoint Committee, comprising physicians from different departments [18]. In case of disagreement, the opinion of other members of the end-point committee was sought and final adjudication was based on the majority of the classifications obtained. The endpoints of interest for the present study were (acute) vascular death, (non-) fatal ischemic stroke or (non-) fatal myocardial infarction and the composite of these vascular events (**Table 1**). If a patient had multiple events, the first recorded event was used in the analyses.

Table 1 *Definitions of non-fatal and fatal vascular events.*

Vascular event	Vascular death (as defined below)
	Ischemic stroke (as defined below)
	Myocardial infarction (as defined below)
	Intracerebral hemorrhage: relevant clinical features as in ischemic stroke, accompanied by a hemorrhage on a CT* scan.
	Rupture of a abdominal aortic aneurysm confirmed by ultra-sound, CT-scan or laparotomy
Ischemic stroke	Definite: relevant clinical features that have caused an increase in impairment of at least one grade on the modified Rankin scale, accompanied by a fresh ischemic infarction on a repeat brain-scan
	Probable: clinical features that have caused an increase in impairment of at least one grade on the modified Rankin scale; without a fresh ischemic infarction on a repeat brain-scan
Myocardial infarction	At least two of the following criteria
	1. Chest pain for at least 20 minutes, not disappearing after administration of nitrates
	2. ST-elevation > 1 mm in two following leads or a left bundle branch block on the ECG*
	3. CK elevation of at least two times the normal value of CK* and a MB*-fraction > 5% of the total CK
Vascular death	Sudden death: unexpected cardiac death occurring within 1 hour after onset of symptoms, or within 24 hours given convincing circumstantial evidence
	Death from ischemic stroke
	Death from intracerebral hemorrhage (hemorrhage on CT-scan)
	Death from congestive heart failure
	Death from myocardial infarction
	Death from rupture of abdominal aortic aneurysm
	Vascular death from other cause, such as sepsis following stent placement

**CT: computed tomography, ECG: electrocardiogram, CK: creatinine kinase, MB: myocardial band*

Data analyses

Data are presented as mean ± standard deviation (SD), as percentages or as indicated. For evaluating the relationship between quintiles of HDL-c and time to a first new cardiovascular event (specified to type of event) Cox regression models were used to calculate Hazard Ratios (HR) with corresponding 95% confidence intervals (95%CI). Three models were constructed, in the first model adjustments were made for age and gender. In the second model additional adjustments were made for potential confounding factors: type 2 diabetes mellitus, body mass index (BMI) and plasma triglyceride levels. In the third model additional adjustments were made for smoking, use of alcohol and use of lipid-lowering medication.

For analyzing the relationship of HDL-c on future vascular events in patients with different localizations of vascular diseases, Cox proportional hazard analyses were performed with the combined vascular endpoint (vascular death, non-fatal myocardial infarction and non-fatal stroke). In these analyses patients were grouped according to specific vascular disease localizations at baseline (present or history). Patients could be classified in more than one disease category. In these analyses, additional adjustments were made for the presence of vascular disease at other sites.
To study possible effect modification of LDL-c plasma levels on the relationship between HDL-c and the occurrence of recurrent events, separate strata of LDL-c levels (LDL-c $\geq$2.5 mmol/l and <2.5 mmol/l) were created and corresponding HR (95%CI) calculated. Of 185 patients no valid LDL-c levels could be calculated using the Friedewald formula due to triglyceride plasma levels >4.0 mmol/l at baseline and were excluded for this specific analysis. Interaction was considered present when the p-value of the interaction term in the model was ≤0.05. All statistical analyses were performed with the Statistical package for the Social Sciences for Windows 12.1 (SPSS, Chicago, IL).

Results

Baseline characteristics

The baseline characteristics of the study population (n = 3837) are displayed in **Table 2** according to quintiles of plasma HDL-c. The plasma HDL-c levels ranged between 0.22 mmol/l to 3.92 mmol/l. Cerebrovascular disease was present in 30% (n = 1134) of the patients, in 56% (n = 2139) of the patients coronary artery disease was present and in 35% (n = 1349) of the patients peripheral artery disease was present (including 415 patients (11%) with an aneurysm of the abdominal aorta). Lipid lowering therapy was used by 48% of the patients and was comparable across the quintiles of HDL (99% statins, 2% cholesterol absorption inhibitor, 2% fibrates and 0.3% used bile acid sequestrants).

HDL-c and the occurrence of new vascular events

During a median follow up of 3.3 (range 0.1-9.5) years and a total follow-up of 14.381 person-years, 465 first new vascular events occurred (112 non-fatal strokes, 156 non-fatal myocardial infarctions and 206 fatal events, corresponding to an event rate of 3.2% per year. Overall, 114 ischemic strokes, 273 myocardial infarctions and 277 vascular deaths occurred. Of the patients who died from a vascular origin 13 suffered from a rupture of an aneurysm of the abdominal aorta, 39 from a cerebrovascular event, 19 from a myocardial infarction and 206 patients from other vascular causes (including 24 cases of heart failure and l05 cases of acute vascular death).
Compared with the lowest quintile of HDL-c (≤0.91 mmol/l), patients in the highest quintile of HDL-c at baseline (≥1.50 mmol/l) had a 50% (HR 0.50 (95%CI 0.25-1.00)) lower risk for ischemic stroke, 45% lower risk (HR 0.55 (95%CI 0.35-0.88)) for myocardial infarction, 41% lower risk (HR 0.59 (95%CI 0.38-0.94)) for vascular death and 39% lower risk (HR 0.61 (95%CI 0.43-0.86)) for the combined event (model II) (**Table 3)**.

Table 2 *Baseline characteristics of the study population according to quintiles of HDL-c.*

Total population (n=3837)	**Quintile 1**	**Quintile 2**	**Quintile 3**	**Quintile 4**	**Quintile 5**
N	**750**	**768**	**751**	**781**	**787**
HDL-c range (mmol/l)	≤0.91	0.92 - 1.08	1.09 - 1.25	1.26 - 1.49	≥1.50
HDL-c mean (mmol/l)	0.80 ± 0.10	1.00 ± 0.05	1.16 ± 0.05	1.36 ± 0.07	1.81 ± 0.30
Age (years)	58.2 ± 10.5	59.1 ± 10.4	59.1 ± 10.5	60.6 ± 10.9	60.7 ± 10.2
Male gender (%)	90	85	77	70	54
BMI (kg/m^2)	27.8 ± 4.0	27.2 ± 3.9	26.9 ± 3.7	26.3 ± 3.7	25.4 ± 3.8
Systolic blood pressure (mmHg)	140 ± 22	141 ± 20	142 ± 22	143 ± 22	145 ± 22
Diastolic blood pressure (mmHg)	80 ± 11	81 ± 11	81 ± 11	82 ± 11	82 ± 11
Total cholesterol (mmol/l)	5.1 ± 1.3	5.2 ± 1.2	5.2 ± 1.1	5.2 ± 1.1	5.3 ± 1.1
Triglycerides (mmol/l)	2.7 ± 2.5	2.1 ± 1.3	1.8 ±1.5	1.6 ±1.0	1.3 ± 0.7
LDL-c (mmol/l)	3.2 ± 1.0	3.3 ± 1.0	3.2 ± 1.0	3.1 ± 1.0	2.9 ± 1.0
Non-HDL-c (mmol/l)	4.3 ± 0.5	4.3 ± 0.5	4.0 ± 0.4	3.8 ± 0.4	3.5 ± 0.4
Creatinine (µmol/l)	109 ± 99	99 ± 66	98 ± 64	97 ± 67	93 ± 59
MDRD (ml/min)	75.0 ± 20.6	76.7 ± 19.9	75.7 ± 19.1	75.8 ± 18.9	74.8 ± 18.3
Homocysteine (µmol/l)	14.7 ± 6.6	14.1 ± 5.8	13.6 ± 5.8	13.9 ± 7.2	14.0 ± 13.7
Antiplatelets agents (%)	61	63	64	63	61
Use of blood pressure lowering agents (%)	51	52	53	50	55
Use of lipid lowering agents (%)	47	47	47	48	51
Prevalence of diabetes (%)	30	26	21	18	15
Never smoked (%)	14	17	20	17	22
Smoking (%) $	40	33	27	28	25
Alcohol (%)*	64	67	70	73	76
Localisation of vascular disease #					
Cerebrovascular disease (%)	28	27	26	27	39
Coronary artery disease (%)	59	58	60	56	46
Aneurysm abdominal aorta (%)	12	12	9	11	9
Peripheral artery disease (%)	27	26	21	24	23

Data are expressed as means ± SD or as indicated.
$ Currently smoking or patients who have stopped smoking within the last year
** Use of alcohol within the last year*
 # Patients could be classified in more than 1 disease category

Table 3 *Hazard ratio of HDL-c quintiles on the occurrence of new vascular events.*

			Hazard ratio (95%CI)				
			Quintile 1	**Quintile 2**	**Quintile 3**	**Quintile 4**	**Quintile 5**
HDL-c mmol/l (range)			**≤0.91**	**0.92 - 1.08**	**1.09 - 1.25**	**1.26 - 1.49**	**≥1.50**
N=3837			**750**	**768**	**751**	**781**	**787**
	Events*	Model					
Ischemic stroke	114	I	Reference	0.95 (0.58-1.56)	0.58 (0.32-1.06)	0.71 (0.41-1.23)	0.49 (0.25-0.95)
		II	Reference	0.95 (0.57-1.57)	0.60 (0.33-1.10)	0.72 (0.41-1.27)	0.50 (0.25-1.00)
		III	Reference	0.96 (0.58-1.61)	0.63 (0.35-1.16)	0.75 (0.42-1.33)	0.54 (0.27-1.08)
Myocardial Infarction	273	I	Reference	0.85 (0.60-1.19)	0.82 (0.57-1.18)	0.82 (0.58-1.17)	0.57 (0.37-0.89)
		II	Reference	0.80 (0.57-1.14)	0.81 (0.56-1.17)	0.81 (0.56-1.17)	0.55 (0.35-0.88)
		III	Reference	0.80 (0.56-1.13)	0.83 (0.58-1.21)	0.84 (0.58-1.22)	0.55 (0.34-0.89)
Vascular death	277	I	Re ference	0.82 (0.59-1.14)	0.76 (0.53-1.08)	0.69 (0.48-0.98)	0.54 (0.35-0.84)
		II	Reference	0.84 (0.60-1.18)	0.82 (0.57-1.17)	0.73 (0.50-1.05)	0.59 (0.38-0.94)
		III	Reference	0.86 (0.62-1.22)	0.87 (0.61-1.26)	0.78 (0.53-1.12)	0.64 (0.40-1.02)
All vascular events *	465	I	Reference	0.86 (0.66-1.12)	0.79 (0.60-1.04)	0.78 (0.59-1.02)	0.60 (0.43-0.83)
		II	Reference	0.85 (0.65-1.11)	0.80 (0.61-1.07)	0.79 (0.60-1.05)	0.61 (0.43-0.86)
		III	Reference	0.86 (0.66-1.12)	0.85 (0.64-1.13)	0.84 (0.63-1.12)	0.65 (0.46-0.92)

** Composite of myocardial infarction, ischemic stroke and vascular death*
Model I = adjusted for age and gender
Model II = Model I with additional adjustments for BMI, plasma triglycerides and type 2 diabetes mellitus
Model III = Model II with additional adjustments for smoking, use of alcohol and use of lipid-lowering medication.

HDL-c and new vascular events by primary localisation of vascular disease

In patients with a recent diagnosis or a history of cerebrovascular disease, higher levels of HDL-c were associated with a decreased risk for developing a vascular event; 0.60 (95%CI 0.34-1.05) (model II) (**Table 4**). Also in patients with coronary artery disease, higher HDL-c levels were associated with a lower cardiovascular risk compared with patients with the lowest HDL-c plasma levels HR 0.68 (95%CI 0.43-1.10) (model II). For patients diagnosed with peripheral artery disease and in the highest HDL-c quintile, the HR was 0.39 (95%CI 0.23-0.67).

HDL-c and new vascular events according to LDL-c levels at baseline

Sixty-two percent of the patients with LDL-c <2.5 mmol/l received lipid-lowering therapy while of the patients with an LDL-c ≥2.5 mmol/l 42% received lipid-lowering therapy. In patients with baseline LDL-c plasma levels ≥2.5 mmol/l (n=2599) and LDL-c <2.5 mmol/l (n = 1053), higher HDL-c plasma levels were related to a lower risk for recurrent vascular events compared to those within the lowest HDL-c quintile at baseline (**Table 5**). For patients with low LDL-c and the highest HDL-c levels the HR was 0.64 (95%CI 0.30-1.39) (model II). For patients with LDL-c ≥2.5 mmol/l the vascular risk was 37% lower (model II) for the highest HDL-c levels. Additional adjustments including use of lipid-lowering medication did not change the relationship (model III). P-values for interaction terms were > 0.9 in all three models.

Table 4 *Hazard ratio of HDL-c quintiles on the occurrence of new vascular events (myocardial infarction, ischemic stroke or vascular death), grouped for localisation of vascular disease.*

			Hazard ratio (95%CI)				
			Quintile 1	**Quintile 2**	**Quintile 3**	**Quintile 4**	**Quintile 5**
HDL-c mmol/l (range)			**≤0.91**	**0.92 - 1.08**	**1.09 - 1.25**	**1.26 - 1.49**	**≥1.50**
	Events*	Model					
Cerebrovascular disease (N=1134)	166						
		N	**207**	**208**	**199**	**214**	**306**
		I	Reference	0.90 (0.59-1.39)	0.54 (0.32-0.90)	0.79 (0.50-1.23)	0.55 (0.33-0.93)
		II	Reference	0.93 (0.60-1.44)	0.57 (0.34-0.96)	0.84 (0.52-1.36)	0.60 (0.34-1.05)
		III	Reference	0.92 (0.57-1.13)	0.58 (0.73-1.47)	0.90 (0.60-1.23)	0.69 (0.39-1.22)
Coronary artery disease (N=2139)	246						
		N	**439**	**447**	**452**	**438**	**363**
		I	Reference	0.80 (0.56-1.15)	0.82 (0.57-1.19)	0.73 (0.50-1.07)	0.66 (0.42-1.03)
		II	Reference	0.76 (0.52-1.10)	0.84 (0.58-1.23)	0.75 (0.51-1.12)	0.68 (0.43-1.10)
		III	Reference	0.81 (0.55-1.19)	0.93 (0.63-1.36)	0.85 (0.56-1.27)	0.72 (0.44-1.18)
Peripheral artery disease (N=1273)	235						
		N	**281**	**270**	**222**	**264**	**236**
		I	Reference	0.83 (0.58-1.18)	0.75 (0.50-1.11)	0.82 (0.56-1.19)	0.44 (0.27-0.73)
		II	Reference	0.79 (0.54-1.15)	0.69 (0.46-1.05)	0.78 (0.52-1.16)	0.39 (0.23-0.67)
		III	Reference	0.80 (0.55-1.16)	0.73 (0.48-1.11)	0.84 (0.56-1.26)	0.41 (0.23-0.71)

**Composite of myocardial infarction, ischemic stroke and vascular death*
Patients could be classified in more than 1 vascular disease category.
Model I = adjusted for age and gender
Model II = Model I with additional adjustments for BMI, plasma triglycerides and type 2 diabetes mellitus
Model III = Model II with additional adjustments for smoking, use of alcohol, use of lipid-lowering medication and vascular disease at other sites

Table 5 *Hazard ratio of HDL-c quintiles on the occurrence of new vascular events (myocardial infarction, ischemic stroke, or vascular death) stratified for LDL-c plasma levels at baseline.*

			Hazard ratio (95%CI)				
			Quintile 1	**Quintile 2**	**Quintile 3**	**Quintile 4**	**Quintile 5**
HDL-c mmol/l (range)			**≤0.91**	**0.92 - 1.08**	**1.09 - 1.25**	**1.26 - 1.49**	**≥1.50**
	Events*	Model					
LDL-c <2.5 mmol/l (N=1053)	78						
		N	**170**	**180**	**188**	**213**	**302**
		I	Reference	0.63 (0.32-1.24)	0.76 (0.38-1.50)	0.49 (0.24-1.01)	0.57 (0.29-1.12)
		II	Reference	0.65 (0.32-1.31)	0.82 (0.40-1.69)	0.52 (0.24-1.14)	0.64 (0.30-1.39)
		III	Reference	0.76 (0.37-1.57)	1.00 (0.48-2.12)	0.64 (0.29-1.42)	0.75 (0.33-1.67)
LDL-c ≥2.5 mmol/l (N=2599)	363						
		N	**485**	**548**	**537**	**550**	**479**
		I	Reference	0.92 (0.69-1.24)	0.79 (0.58-1.09)	0.80 (0.59-1.09)	0.59 (0.40-0.87)
		II	Reference	0.93 (0.69-1.26)	0.83 (0.60-1.19)	0.85 (0.61-1.19)	0.63 (0.42-0.97)
		III	Reference	0.93 (0.69-1.26)	0.87 (0.63-1.21)	0.89 (0.64-1.25)	0.67 (0.43-1.02)

**Composite of myocardial infarction, ischemic stroke and vascular death*
Model I = adjusted for age and gender
Model II = Model I with additional adjustments for BMI, plasma triglycerides and type 2 diabetes mellitus
Model III = Model II with additional adjustments for smoking, use of alcohol and use of lipid-lowering medication.

Discussion

In the present prospective cohort study we showed that high HDL-c levels are related to a lower risk for future vascular events in patients with already clinically evident vascular disease. This relationship was present irrespective of the localisation of previous vascular disease and was independent of lipid-lowering therapy. Higher HDL-c plasma concentrations confer an equally lower risk in patients with either low (LDL-c <2.5 mmol/l) or elevated LDL-c (≥2.5 mmol/l) levels.

From previous studies it is known that in patients without clinical evident vascular diseases low HDL-c plasma levels are associated with an increased risk for the occurrence of peripheral artery disease [21], aneurysms of the abdominal aorta [22], coronary artery disease [23] and ischemic stroke [24]. Also, in patients with coronary artery disease high HDL-c levels are associated with a decreased risk for recurrent myocardial infarction [1,13,14,25]. We expand the evidence by showing that also in a cohort of patients, derived from routine clinical practice, with various localisations of clinical evident vascular disease, in which statins were widely used to obtain lower LDL-c levels, higher HDL-c levels confer a lower risk for developing new vascular events.

Plasma HDL-c levels can be regarded as a risk marker and as a risk factor. A lower plasma HDL-c concentration may be a marker of insulin resistance, as seen in obesity, metabolic syndrome and type 2 diabetes mellitus, conditions known to be associated with an increased cardiovascular risk [26, 27]. In the present study, analyses were adjusted for the potential confounding effects of plasma triglycerides, type 2 diabetes and BMI in the relationship between HDL-c and new vascular events. These adjustments did not essentially affect the cardiovascular risk associated with low HDL-c indicating that HDL-c should be viewed as an independent risk factor for the development of vascular events in patients with clinical evident vascular disease, a population in which obesity and metabolic syndrome are highly prevalent. This may imply that HDL-c raising therapy could be beneficial in secondary prevention both in patients with LDL-c levels above and beneath the therapeutic goal of 2.5 mmol/l. In current European and American guidelines, HDL-c is not regarded as a treatment target. Current treatment options to raise HDL-c plasma levels are limited. Statin treatment, being first choice for LDL-c reduction, only marginally increases HDL-c levels by approximately 5-7% [28]. Treatment with fibrates generally results in about 10-30% increased plasma HDL-c concentrations [29,30] and reduced fatal an non-fatal myocardial infarction in patients with coronary artery disease [27]. However, fibrate treatment failed to reduce fatal myocardial infarctions in type 2 diabetes patients [31]. Niacin can increase HDL-c levels up to 30% in patients with dyslipidemia [32], however data on cardiovascular endpoints are scarce. Weight reduction, cessation of smoking and moderate use of alcohol lead to increased HDL-c plasma levels. Diets enriched with omega-3 fatty acids (fish oil) on top of statins [33] and physical exercise have been shown to raise HDL-c and have been linked to a lower incidence of heart disease [34].

In recent trials aiming at a reduction in atherosclerotic development by increasing plasma HDL-c through inhibition of Cholesteryl Ester Transfer Protein in addition to high dose statin treatment, plasma HDL-c concentrations rose up to 60% but were not accompanied with an anti-atherosclerotic effect as measured with intravascular

ultrasound or with carotid intima media thickness [8,16,17]. These results gave rise to speculation on the absence of anti-atherosclerotic effects of rising HDL-c levels in the presence of intensive LDL-c lowering. A possible explanation for these contradicting results between different trials and potential beneficial effect of HDL-c may be sought in the complex metabolism of HDL-c and/or of target drug toxicity [35]. HDL particles are a mixed class of lipoproteins comprising different subspecies which contain different apolipoproteins and vary in lipid composition, densities and which are likely to have different properties regarding reverse cholesterol transport [36] or anti-oxidation [37]. Nevertheless, in the present study a single measurement of HDL-c appeared to be a strong and independent predictor of vascular risk regardless the LDL-c levels. In a trial investigating the effect of moderate compared to intensive LDL-c lowering, lower HDL-c levels were also predictive for major cardiovascular events in patients with stable coronary heart disease and LDL-c levels below 1.8 mmol/l [38].

We acknowledge study limitations. Plasma apolipoproteins (like ApoA1) and HDL-c fractions were not available. However, since the found relationship was present in all analyses and was stable over quintiles of HDL-c it is unlikely that adding this information in the models would influence the results. Also, only fasting baseline HDL-c measurements were available. Therefore it was not possible to determine the influence of changes in plasma lipids and use of lipid-lowering therapy on the relationship between HDL-c and recurrent events. However, since the use of fibrates was low in this study and in the absence of other effective HDL-c raising therapy, it is likely to assume that HDL-c levels will have remained stable during follow-up. The use of lipid-lowering medication at baseline was only 50%. It is likely to assume that a considerable proportion of patients started on lipid-lowering therapy during follow up. This information was not available.

In conclusion, low plasma HDL-c levels confer an increased risk for recurrent non-fatal and fatal vascular events in patients with various clinical manifestations of atherosclerosis. This relationship was present irrespective of the localisation of vascular disease and use of lipid-lowering medication. In addition, this relationship was not modified by plasma LDL-c concentration. Therefore, HDL-c rising therapy for reducing the residual cardiovascular risk in high-risk patients remains worthwhile to be studied in intervention studies.

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CHAPTER 6

Lipid lowering therapy does not affect the postprandial drop in HDL-c plasma levels in obese men with metabolic syndrome

-A randomized double blind crossover trial-

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In revision

Abstract

Introduction

The postprandial lipid metabolism in metabolic syndrome patients is disturbed and may add to the increased cardiovascular risk in these patients. It is not known whether postprandial HDL-c metabolism is also affected and whether this can be influenced by statin and/or ezetimibe treatment.

Methods

Prospective, randomized, double blind, crossover trial comparing simvastatin 80mg with simvastatin/ezetimibe 10mg/10mg treatment for 6 weeks on postprandial HDL-c metabolism in fifteen, non-smoking, male, obese metabolic syndrome patients (ATPIII). HDL-c concentrations, cholesteryl ester transfer (CET), CETP mass and adiponectin were measured before and after oral fat loading. Clinicaltrials.gov NCT00189085.

Results

Plasma HDL-c levels remained stable during continuous fasting following an overnight fast. Pre-fat load HDL-c concentrations without treatment, after simvastatin and simvastatin/ezetimibe treatment were 1.15 ± 0.04, 1.16 ± 0.05 and 1.11 ± 0.04 mmol/l. Fat load induced a 11% drop in HDL-c plasma levels; 1.02 ± 0.05 mmol/l ($p<0.001$) which was not affected by either therapy. Triglyceride levels during fat load were similar after both treatments. Total CET increased from 9.73 ± 0.70 to 12.20 ± 0.67 nmol/ml/hr ($p=0.004$). Four hours after fat loading CETP mass was increased while adiponectin levels were decreased, irrespective of treatment.

Conclusions

HDL-c levels decrease as CET increases after fat loading in obese metabolic syndrome patients. This is not influenced by either simvastatin or simvastatin/ezetimibe treatment. After fat loading, CETP mass and CET increased, and adiponectin decreased pointing towards a potential role for intra-abdominal fat. Decreased postprandial HDL-c levels may contribute to the increased cardiovascular risk in metabolic syndrome patients on top of already low HDL-c levels.

Introduction

The clustering of cardiovascular risk factors associated with abdominal obesity (elevated blood pressure, elevated triglyceride and fasting glucose plasma levels and low plasma levels of high density lipoprotein-cholesterol (HDL-c) is often referred to as metabolic syndrome [1]. The prevalence of metabolic syndrome is increasing and metabolic syndrome subjects are at increased risk for cardiovascular morbidity and mortality and for type 2 diabetes [2,3]. The underlying pathophysiology of metabolic syndrome is not fully understood but insulin resistance and abdominal obesity, with associated alterations in adipocyte metabolism are main characteristics [4-6].

Low plasma HDL-c, associated with abdominal obesity and insulin resistance [7,8], is a strong and independent risk factor for future cardiovascular events and mortality [9]. Apolipoprotein A1 (apoA1), an essential component of HDL particles, is synthesized in both liver and intestine. Interaction of apoA1 with the ABCA1 receptor on the luminal side of the vascular endothelium results in removal of cholesterol from the vessel wall and subsequent maturation of HDL particles [10]. A constant remodelling of HDL particles occurs through the action of both phospholipids transfer protein (PLTP) and cholesteryl ester transfer protein (CETP). CETP is, like adiponectin, visfatin, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) produced by adipocytes [11] and is the main facilitator of cholesteryl ester transfer in exchange of triglycerides between apoA1 (HDL) and apoB containing lipoproteins (VLDL, IDL and LDL) [12]. In metabolic syndrome and insulin resistant subjects, postprandial dyslipidemia is characterized by prolonged presence of atherogenic triglyceride-rich lipoprotein particles (TGR) in the circulation due to increased production of very low density lipoprotein (VLDL-c) and reduced lipolysis as a result of diminished lipoprotein lipase (LPL) activity [13]. Little is known about postprandial HDL metabolism in insulin resistant and obese metabolic syndrome patients. In small studies in healthy subjects [14], in non-overweight patients with coronary artery disease [15] or diabetes [16] and in habitual smoking subjects [17] fat loading induced a decrease in plasma HDL-c levels.

Statins, alone or in combination with ezetimibe, are the most commonly used lipid-lowering therapies for cardiovascular risk reduction. In general, both therapies increase fasting HDL-c plasma levels between 3-9% and apoA1 levels with 6% [18,19]. However, atorvastatin did not affect a decrease in postprandial HDL-c plasma concentrations in male hypertriglyceridemic patients [20]. The effect of statin salone or in combination with ezetimibe on postprandial HDL-c in obese metabolic syndrome patients is not known.

Aim of the present study is 1) to investigate the effects of an oral fat load on HDL-c metabolism in obese patients with metabolic syndrome and 2) to compare the effects of high-dose simvastatin monotherapy with the combination of low-dose simvastatin with ezetimibe on post fat load HDL-c metabolism.

Subjects and methods

Subjects

A total of 32 non-smoking male subjects, aged 18-70 years, were recruited by advertisement in a local newspaper which called for subjects with waist circumference >102 cm. After screening, 15 subjects consented to participate in this HDL-c study and fulfilled the diagnostic criteria for metabolic syndrome according to the Adult Treatment Panel III (ATPIII) criteria of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. [17] Metabolic syndrome was diagnosed if ≥3 of the following abnormalities were present:

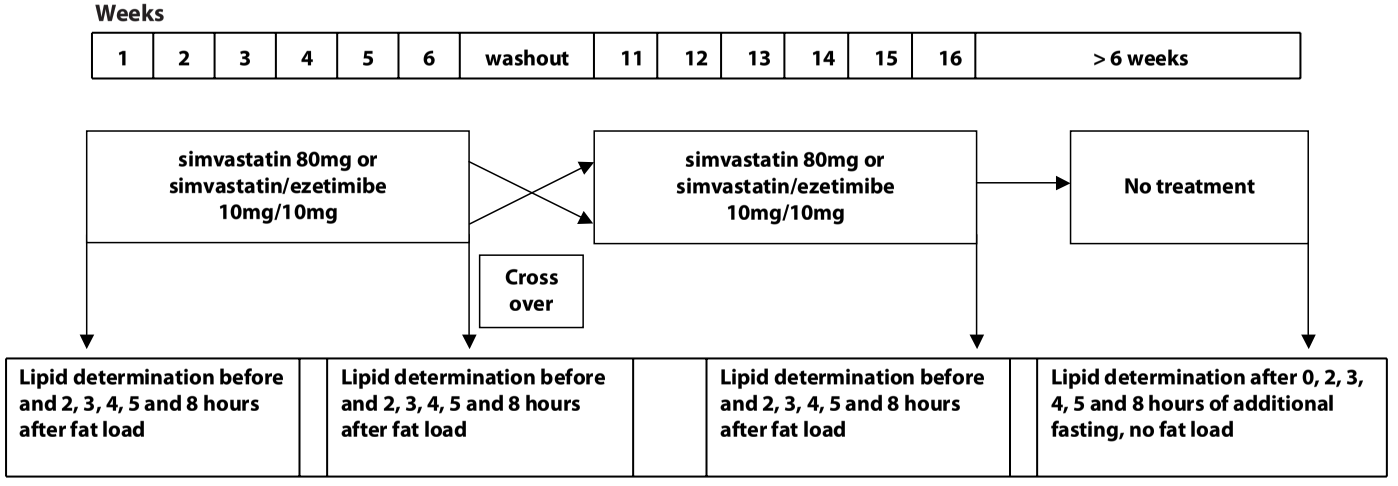
1. abdominal obesity (waist circumference >102 cm)
2. high blood pressure (≥130 mmHg systolic or ≥85 mmHg diastolic)
3. hypertriglyceridemia (serum triglycerides ≥1.70 mmol/l (150 mg/dL))
4. low HDL-c (serum HDL-c <1.04 mmol/l (40 mg/dL)
5. high fasting glucose (fasting serum glucose ≥6.1 mmol/l (110 mg/dL))

Glucose level ≥7.8 mmol/l after a standardized oral glucose tolerance test was also regarded as fulfilling the glucose criterion. Patients with Thyroid Stimulating Hormone (TSH) levels >5.0 mU/L, with clinical symptoms of hypothyroidism, serum aspartate transferase (ASAT) or serum alanine transferase (ALAT) >2 times the upper limit of normal or serum creatinine >1.7 times the upper limit of normal were excluded. Patients with metabolic syndrome and either very high blood pressure (≥180/110 mmHg) or a BMI >35 kg/m^2 were not included in the present study for medical and ethical reasons. These patients were referred to their general practitioner for (immediate) medical attention. Other relevant exclusion criteria were the presence of macrovascular disease, use of lipid-lowering medication and/or use of blood pressure-lowering medication since they may affect insulin sensitivity and adipocyte function[18], HbA1c >6.5 % or plasma triglycerides >8.0 mmol/l. Given the fluctuations in fasting and postprandial HDL-c plasma levels in women due to fluctuating levels of estrogens during menstrual cycles we decided not to include women in the present study.

Study Design

In this prospective, randomized, double blind, crossover trial patients received once daily simvastatin 80mg or the combination of simvastatin 10mg and ezetimibe 10mg during 2 periods of 6 weeks (**Figure 1**). Between both treatment periods patients had a washout period of 4 weeks, after which crossover of therapy occurred. Before the initial therapy and after each treatment period of 6 weeks an oral fat load was performed after an overnight fast of at least 10 hours. Randomization was performed at the pharmacy department of the institute with the use of sub-blocks, with 4 patients per sub-block. Venous blood samples for plasma lipids determination were obtained before and at 2, 3, 4, 5 and 8 hours after the oral fat load. Further, plasma lipid levels were also determined during 8 hours of additional fasting following an overnight fast of at least 10 hours after a period of at least 6 weeks without lipid lowering therapy.

Figure 1 *Study design*



Plasma CETP mass was measured before and 3, 4 and 8 hours after the oral fat load. The study was approved by the Ethical Review Board of the University Medical Center Utrecht (UMCU) and was carried out in a Good Clinical Practice-certified research unit. All subjects gave written informed consent.

Anthropometric measurements

Patients weight and height were measured without heavy clothing and shoes. Body mass index (BMI) was calculated as weight to height squared. Waist circumference was measured halfway between the lower rib and the iliac crest. Total body fat percentage was estimated by using Omron body fat monitor BF306 (Omron Matsusaka Co. LTD., Japan).

Oral fat load

For the fat load, fresh cream was used with a 40% (weight/volume) fat emulsion with a poly-unsaturated/saturated fat ratio of 0.10, containing 0.001% (w/v) cholesterol and 3% (w/v) carbohydrates representing a total energy content of 3700 kCal/L. Cream was ingested at a dose of 50 g fat and 3.75 g glucose per m^2 body surface (with a maximum of 250 ml) within 5 minutes. Participants remained supine during the day and were only allowed to drink mineral water.

Laboratory assessments

At each visit, fasting blood was sampled to determine plasma levels of glucose, creatinine, homocysteine, ALAT, ASAT, total cholesterol, LDL-c and HDL-c, triglycerides and high sensitive C-reactive protein (hs-CRP). ApoE genotyping, TSH and HbA1c were measured only at the first visit. Plasma was isolated by immediate centrifugation for 15 min. at 3000 rpm at 4°C before storage at -80°C. Plasma cholesterol, triglycerides, HDL-c and LDL-c, apoA1 and apoB were measured using commercially available assays (Wako, Osaka, Japan) on a Cobas Mira auto-analyzer (ABX). All other blood samples were measured in a local research facility according to standardized ISO 9001:2000 regulations. Total serum cholesteryl ester transfer (CET) was assayed as described previously [21]. In brief, [^{3}H] cholesterol was equilibrated for 24 h with plasma-free cholesterol at 4°C followed by incubation of the sample at 37°C for 3 h. Subsequently, VLDL and LDL were precipitated by addition of phosphotungstate/$MgCl_2$. Lipids were extracted from the precipitate and the cholesteryl esters were isolated on silica columns and radioactivity was counted. A two-antibody sandwich immunoassay of Cholesterol Ester Transfer Protein (CETP) was set up according to Mezdour et al [22] with major modifications as described below. The coating was performed with a combination of monoclonal antibodies TP1 (5 mg/ml in PBS) and TP2 (2.5 mg/ml) during an overnight incubation at 70°C. To prevent non-specific binding, plates were blocked with 1% BSA at room temperature for 2 h. Samples were tested in 20, 40 and 80-fold dilution whereas standard plasma was diluted from 10- to 160-fold with 0.1% Triton X-100 and 1% BSA in PBS. As a secondary antibody TP20, labelled with digoxigenine, was used. Antidigoxigenine Fab fragments coupled to peroxidase were added. Absorbance, after addition of TMB, was read after 30 min

incubation and termination of the reaction with H_2O_2 at 450 nm. The inter-assay and intra-assay coefficient of variance were 7.8% and 6.0%, respectively. Plasma adiponectin levels were determined by using a commercially available kit (Quantitative enzyme immunoassay technique, R&D Systems Inc, USA).

Statistical analyses

Baseline characteristics are presented as mean ± standard deviation (SD). Fasting and post fat load lipid levels, CET and CETP mass are expressed as mean ± standard error of mean (SEM). The postprandial variations of lipids were integrated as area under the curve (AUC) (mean ± SEM) and were calculated by the trapezoidal rule using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA). Net AUCs (dAUC) were calculated after correction for baseline values. Differences in AUC and dAUC between simvastatin 80mg and combination therapy of simvastatin/ezetimibe 10mg/10mg were analyzed by paired *t*-test; statistical significance was taken at the 5% level. Carryover and period effects were calculated with independent samples *t*-test [23]. Calculations were performed using SPSS for Windows version 12.1. (SPSS Inc. Chicago, IL, USA).

Results

Baseline characteristics of study population

The baseline characteristics of the 15 patients of this study are shown in **Table 1**. Mean age was 55 ± 6 years and levels of metabolic syndrome parameters were: waist 110 ± 7 cm, systolic blood pressure 139 ± 13, diastolic blood pressure 90 ± 6 mmHg, fasting triglycerides 1.59 ± 0.13 mmol/l, glucose 6.2 ± 0.5 mmol/l and fasting HDL-c 1.15 ± 0.04 mmol/l. The BMIs of the study patients ranged between 25.1 and 35.0 kg/m^2. None of the patients used any medication other than the study medication. Patients remained on a stable diet during the study. Weight, waist circumference and body fat percentage remained stable during the study.

HDL-c plasma concentrations during a prolonged fasting state and after oral fat loading

HDL-c plasma levels remained stable during 8 hours of additional fasting after an overnight fast. At t=0 HDL-c was 1.21± 0.06 mmol/l. The AUC was 10.2 ± 0.5 mmol·h/l and after baseline correction 0.2 ± 0.1 mmol·h/l (**Table 2**). HDL-c concentrations dropped 11.3% from 1.15 ± 0.04 to 1.02 ± 0.05 mmol/l after fat load reaching the lowest level after 4 hours; p<0.001 (**Figure 2a**). Eight hours after the oral fat load HDL-c levels almost returned to baseline levels. The dAUC of HDL-c after oral fat load was -0.6 ± 0.1 (p<0.001, compared to dAUC after additional fasting). In 8 of the 15 patients the fasting HDL-c plasma concentration was <1.04 mmol/l, the cut off level used in the NCEP ATPIII metabolic syndrome criteria. There was no difference in the post fat load drop in HDL-c in patients with a fasting HDL-c concentration below or above 1.04 mmol/l.

Table 1 *Baseline characteristics of the study population*

	No treatment (n=15)	After 6 weeks simvastatin 80 mg (n=15)	After 6 weeks simvastatin/ezetimibe 10mg/10mg (n=15)
Age (years)	55 ± 6	x	x
Height (m)	1.83 ± 0.06	x	x
Weight (kg)	99.6 ± 12.1	100.1 ± 11.9	99.8 ± 12.0
Body mass index (kg/m^2)	29.7 ± 2.8	29.9 ± 2.7	29.8 ± 2.7
Body fat (%)	31 ± 3	31 ± 3	30 ± 3
Laboratory parameters			
ASAT (U/l)	34 ± 5	36 ± 9	36 ± 13
ALAT (U/l)	44 ± 15	48 ± 18	51 ± 19
Creatinin kinase (U/l)	102 ± 40	132 ± 66	108 ± 52
Creatinine Clearance (ml/min)*	110 ± 20	120 ± 23	124 ± 21
TSH (mIE/l)	1.6 ± 0.8	x	x
HbA1c (%)	5.7 ± 0.4	x	x
Hs-CRP (mg/l)	3.1 ± 1.9	5.9 ± 3.6	5.4 ± 2.9
Total cholesterol (mmol/l)$	5.7 ± 0.2	3.8 ± 0.2‡	3.9 ± 0.2‡
LDL-cholesterol (mmol/l)$	3.8 ± 0.2	2.2 ± 0.1‡	2.2 ± 0.1‡
VLDL-cholesterol (mmol/l)$	0.70 ± 0.06	0.47 ± 0.07‡	0.53 ± 0.09
Apolipoprotein B (mg/dl)$	100 ± 4	70 ± 4‡	73 ± 5‡
Apolipoprotein A1 (mg/dl)$	114 ± 2	112 ± 4	107 ± 3
Insulin (mU/l)	18 ± 8	16 ± 9	18 ± 8
Homocysteine (µmol/l)	9.5 ± 1.6	8.9 ± 2.2	9.0 ± 2.4
HOMA-IR	5.1 ± 2.4	4.5 ± 2.4	4.9 ± 2.4
Components of the metabolic syndrome			
Glucose (mmol/l)	6.2 ± 0.5	6.2 ± 0.6	6.2 ± 0.7
Waist (cm)	110 ± 7	110 ± 5	110 ± 7
Systolic blood pressure (mmHg)	139 ± 13	139 ± 15	132 ± 9
Diastolic blood pressure (mmHg)	90 ± 6	88 ± 8	87 ± 5
Triglycerides (mmol/l)$	1.59 ± 0.13	1.12 ± 0.12†	1.28 ± 0.10‡
HDL-c (mmol/l)$	1.15 ± 0.04	1.16 ± 0.05	1.11 ± 0.04

Mean ± SD
$ Mean ± SEM
** Cockcroft-Gault formula*
† p<0.05 versus no treatment
‡ p<0.01 versus no treatment

Table 2 *Area under the curve (AUC) of plasma lipids parameters after an overnight fast, with and without fat load and after oral fat loading with and without treatment.*

	Additional fasting, no fat load	No treatment, fat load	p-value#	After 6 weeks simvastatin 80mg	After 6 weeks simvastatin/ ezetimibe 10mg/10mg	p-value$
	(n=15)	(n=15)		(n=15)	(n=15)	
AUC for lipid parameters						
Total cholesterol	48.2 ± 1.8	46.1 ± 1.8	0.06	30.1 ± 1.3	31.2 ± 1.6	0.47
Triglycerides	14.2 ± 1.4	19.3 ± 1.5	0.03	14.2 ± 0.9	16.3 ± 1.2	0.06
HDL-c	10.2 ± 0.5	8.6 ± 0.4	< 0.001	8.5 ± 0.3	8.4 ± 0.4	0.73
ApoA1	1047 ± 27	906 ± 19	< 0.001	880 ± 25	850 ± 24	0.35
Baseline corrected AUC for lipid parameters						
Total cholesterol	0.3 ± 0.4	0.6 ± 0.5	0.48	-0.5 ± 0.6	0.4 ± 0.5	0.08
Triglycerides	0.0 ± 0.5	6.6 ± 0.8	< 0.001	5.3 ± 0.6	6.0 ± 0.7	0.40
HDL-c	0.2 ± 0.1	-0.6 ± 0.1	< 0.001	-0.7 ± 0.2	-0.4 ± 0.2	0.37
ApoA1	6.2 ± 8.1	-5.1 ± 8.1	0.36	-20.9 ± 16.3	-5.9 ± 11.0	0.69

Mean ± SEM
 # p-value; additional fasting, no fat load vs. no treatment, fat load.
$ p-value; simvastatin 80mg vs. simvastatin/ezetimibe 10mg/10 mg

During prolonged fasting plasma apoA1 concentrations remained stable. The dAUC was 6.2 ± 8.1 mg·h/dl. ApoA1 plasma levels decreased to a minimum at 5 hours post fat load (from 114.2 ± 2.0 to 111.8 ± 2.2 mg/dl), dAUC -5.1 ± 8.1 (p= 0.36, compared to dAUC after prolonged fasting) (**Table 2**). As expected, plasma triglyceride levels were significantly higher after fat load compared with prolonged fasting (dAUC 6.6 ± 0.8 compared to 0.0 ± 0.5, p<0.001). The plasma concentrations of apoB containing lipoproteins remained also stable during 8 hours of additional fasting following an overnight fast.

Effect of simvastatin 80mg or combination of simvastatin/ezetimibe 10mg/10mg on fasting and post fat load HDL-c plasma concentrations

The fasting HDL-c plasma level was 1.15 ± 0.04 mmol/l before the study and changed marginally after simvastatin (1.16 ± 0.05 mmol/l) or simvastatin/ezetimibe therapy (1.11 ± 0.04 mmol/l). Neither treatment regime did affect post fat load plasma HDL-c or apoA1 concentrations (**Table 2**). Fasting plasma LDL-c concentrations after both treatments decreased to exactly the same level (2.2 ± 0.1 mmol/l) from baseline (-43%). The increase of plasma triglyceride levels was not affected by either simvastatin 80mg or simvastatin/ezetimibe 10mg/10mg (dAUC 5.3 ± 0.6 vs 6.0 ± 0.7 mmol·h/l, p=0.40). No carryover or periods effects between the two treatments periods were observed for fasting lipid concentrations or post fat load lipid profiles.

Figure 2a *HDL-c baseline corrected concentrations (mean ± SEM) after an overnight fast, with and without fat loading and after oral fat loading with and without treatment.*

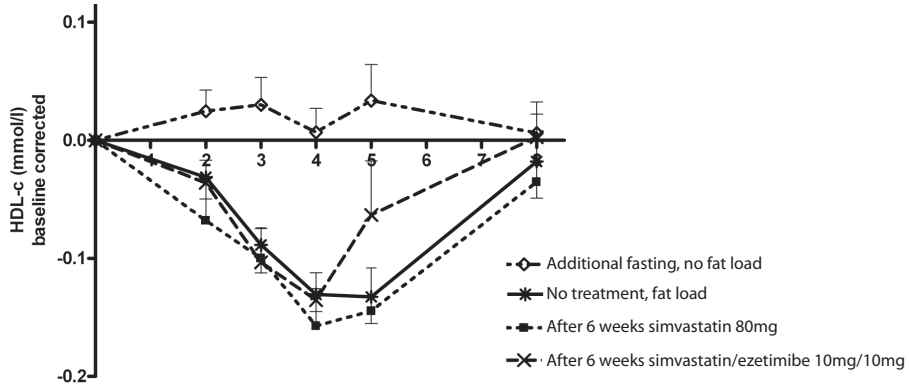
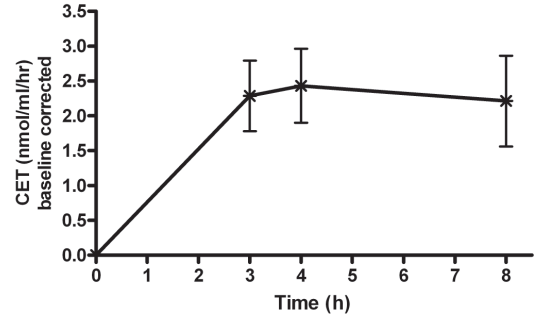


Figure 2b *Total cholesteryl ester transfer (mean ± SEM) after oral fat loading without treatment*



Cholesteryl ester transfer and CETP mass after oral fat loading

Plasma cholesteryl ester transfer in patients without study medication, reached a maximum at 4 hours post fat load (from 9.73 ± 0.70 to 12.20 ± 0.67 nmol/ml/hr, an increase of 25.4% (p=0.004). Eight hours after the fat load plasma CET was 11.98 ± 0.80 nmol/ml/hr (**Figure 2b**). Plasma CETP mass concentrations were comparable after treatment with simvastatin 80mg (1.38 ± 0.12µg/ml) and simvastatin/ezetimibe 10mg/10mg (1.30 ± 0.11 µg/ml) and were lower compared to fasting plasma levels without treatment (1.65 ± 0.19 µg/ml) (**Table 3**). Ingestion of a fat load in subjects without treatment and after simvastatin 80mg mono-therapy resulted in an increase of 16% in plasma CETP mass, three and four hours postprandial respectively. CETP mass increased 10% after fat loading, compared to fasting levels, after simvastatin/ezetimibe 10mg/10mg treatment.

Adiponectin plasma concentrations after oral fat loading

No significant differences in baseline plasma adiponectin levels were observed between the control period and the two treatment regimens. However, adiponectin plasma concentrations decreased after oral fat loading. In subjects without treatment a decrease from 5.0 ± 0.6 to 4.5 ± 0.5 µg/ml (-10%) (**Table 3**) was found 8 hours postprandially, whereas after treatment with simvastatin 80mg and simvastatin/ezetimibe 10mg/10mg, adiponectin plasma levels were also decreased 8 hours after the oral fat load (-8.4% and -6.4% respectively).

Table 3 *Fasting and post fat load CETP and adiponectin levels.*

Hours after fat load	No treatment, fat load	After 6 weeks simvastatin 80mg	After 6 weeks simvastatin/ezetimibe 10mg/10mg
	(n=15)	(n=15)	(n=15)
CETP mass (µg/ml)			
0	1.65 ± 0.19	1.38 ± 0.12	1.30 ± 0.11
3	1.91 ± 0.20	1.61 ± 0.14	1.42 ± 0.13
4	1.59 ± 0.12	1.43 ± 0.12	1.32 ± 0.13
8	1.55 ± 0.16	1.48 ± .012	1.39 ± 0.14
Adiponectin (µg/ml)			
0	5.0 ± 0.6	4.8 ± 0.5	4.7 ± 0.5
3	5.0 ± 0.6	4.7 ± 0.5	4.3 ± 0.5
4	4.7 ± 0.6	4.5 ± 0.5	4.2 ± 0.5
8	4.5 ± 0.5	4.4 ± .05	4.4 ± 0.5

Mean ± SEM

Discussion

In the present randomized, double blind, crossover trial, HDL-c plasma levels decreased after a standardized oral fat load as a model of acute hypertriglyceridemia due to increased cholesterol ester transfer (CET) in male obese metabolic syndrome patients. Treatment with high-dose simvastatin monotherapy or low-dose simvastatin in combination with ezetimibe did not prevent the acute decrease in post fat load HDL-c concentrations.

HDL has anti-atherogenic properties by facilitating cholesteryl ester transport to the liver from peripheral tissues, including the vessel wall, and further by exerting anti-oxidant, anti-thrombotic and anti-inflammatory effects [24,25]. A low fasting plasma HDL-c concentration is, besides a component of metabolic syndrome, an independent risk factor for the development of cardiovascular diseases [8,9]. The present study in non-smoking, obese metabolic syndrome subjects, using oral fat loading as an acute model of increased plasma triglyceride levels, showed that HDL-c plasma levels decreased after an oral fat load on top of already low fasting HDL-c plasma concentrations. This decrease occurred regardless of lipid lowering therapy and is greatly dependent upon an induction of CET in the presence of elevated levels of its substrate: triglycerides. In another study in habitual smoking subjects and in 6 normolipidemic patients with coronary artery disease, HDL-c plasma levels decreased after an oral fat load compared to healthy controls [15,17]. Both in type 2 diabetes patients and healthy controls, HDL-c levels declined after a mixed meal during 6 hours [16]. Since humans are most part of the day in a postprandial state, it may be hypothesised that the increased risk associated with metabolic syndrome is partly caused by further decreased postprandial HDL-c plasma levels in these patients [26]. Postprandial triglyceridesmetabolism strongly influences HDL-c metabolism and lipoprotein composition through the action of CET. CET in plasma is modulated by CETP plasma levels and activity, plasma triglyceride concentrations, smoking and alcohol consumption [27]. Given the fact that adipose tissue is a major site of CETP production and obesity is associated with elevated CETP activity and CETP protein mass [11,28], it is thought that elevated CETP plasma levels are a link between (visceral) obesity, insulin resistance and low HDL-c levels, all features present in metabolic syndrome. Secondly, CETP gene expression in large adipocytes may be even more enhanced in response to dietary cholesterol and saturated fatty acids leading to elevated CETP mRNA levels and increased plasma activity in animal models and humans [11,29]. In accordance, in the present study, CETP mass increased directly after the fat load intake, indicating that the increased CET may in part be the result of higher CETP mass. The observed decline in adiponectin plasma levels supports the hypothesis of an altered adipocyte secretion rate after an oral fat load.

Using an acute model for plasma triglycerides increase, the decrease in post fat load HDL-c plasma concentrations was not affected by treatment with high-dose simvastatin or the combination therapy of low-dose simvastatin/ezetimibe. Statins may decrease plasma CET indirectly by reducing hepatic VLDL synthesis, following HMG-CoA-reductase inhibition and LDL-receptor up-regulation leading to a reduced number of circulating donor particles for CET [30], which might be accompanied by an increase in fasting plasma HDL-c concentrations [31]. The post fat load increase of plasma

triglycerides was similar after both treatments, despite the fact that both treatments significantly reduced baseline plasma triglyceride levels. This opens up the question whether additional treatment with a CETP-inhibitor blocks the decrease in HDL-c levels due to enhanced CET after acute hypertriglyceridemia.
Given the altered lipidmetabolism associated with insulin resistance and obesity, the results of the present study can not be automatically extrapolated to patients without metabolic syndrome. Other potential limitations of our current study comprise the definition used for metabolic syndrome. Although other diagnostic criteria do exist, the ATP III definition is most commonly used and is easily applicable in clinical practice. Also, only obese male metabolic syndrome patients, without a history of vascular diseases or diabetes were included in the present study. Therefore, it is not known whether the results can be generalized to female patients with known differences in lipidmetabolism, patients with diabetes mellitus or patients with clinically manifest vascular diseases. Crossover and carryover effects can not completely be ruled out but are unlikely to have affected the results considering the half-life time of the study drugs and the 4 weeks washout period. Given the 6 week treatment period leads this to a 10 week time lag between the last dose of one treatment and measuring the effects of the other treatment. Finally, it should be noted that the oral fat load consisted of a non-physiological load of triglycerides.
In conclusion, plasma HDL-c levels decreased after an oral fat load in male obese patients with metabolic syndrome which is likely to be the result of increased CET, in part initiated by elevated plasma CETP mass and elevated plasma triglycerides. Treatment with high-dose simvastatin or with the combination of low-dose simvastatin and ezetimibe did not influence the acute increase in triglycerides nor the decrease in postprandial HDL-c levels. Postprandial decrease in HDL-c plasma levels may contribute to the increased cardiovascular risk in metabolic syndrome patients already having low HDL-c plasma concentrations.

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CHAPTER

The effect of statin alone or in combination with ezetimibe on postprandial lipoprotein composition in obese metabolic syndrome patients

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In revision

Abstract

Introduction

Fasting and postprandial hypertriglyceridemia are essential features of metabolic syndrome. Statins decrease fasting lipid levels but fail to reduce fat load induced hypertriglyceridemia. We determined whether ezetimibe combined with simvastatin influences post fat load lipid levels and lipoprotein composition differently as compared to simvastatin 80mg in obese male metabolic syndrome patients.

Methods

Prospective, randomized, double blind, crossover trial. Male obese metabolic syndrome (ATPIII) patients (n=19) were treated with simvastatin 80mg and simvastatin/ezetimibe 10mg/10mg for 6 weeks. At the start of the study and after each treatment period oral fat loading tests were performed. Lipoprotein fractions (triglyceride-rich lipoproteins, IDL, LDL, and HDL) were isolated by density gradient ultracentrifugation. Postprandial changes in lipid levels were integrated as areas under the curve (AUCs).

Results

Fasting LDL-c, RLP-c and triglycerides were lowered equally by both simvastatin 80mg and simvastatin/ezetimibe 10mg/10mg. Postprandial plasma triglyceride levels (net AUC-TG) were equally lowered after both treatments (5.16 ± 0.50 mmol·h/l after simvastatin/ezetimibe 10mg/10mg and 6.09 ± 0.71 mmol·h/l after simvastatin 80mg) compared to fat loading without treatment (6.64 ± 0.86 mmol·h/l). Triglyceride-content in lipoprotein fractions after fat load (net AUCs) were also equally reduced after both treatments. Similarly, cholesterol and apoB content in isolated lipoprotein fractions was lowered equally by both treatment regimens, leading to a reduced number of circulating particles.

Conclusion

Simvastatin 80mg and simvastatin/ezetimibe 10mg/10mg are equally effective in reducing fasting and post fat load LDL-c, triglycerides and apoB plasma concentrations. During acute hypertriglyceridemia fasting and post fat load triglyceride and cholesterol content in TRL, IDL and LDL fractions were not influenced by simvastatin 80 mg or simvastatin/ezetimibe 10mg/10mg in obese metabolic syndrome patients.

Introduction

Abdominal obesity is frequently associated with a clustering of cardiovascular risk factors such as fasting and postprandial dyslipidemia, hyperglycemia, elevated blood pressure, low grade inflammation and altered hemostatics [1, 2]. This clustering is often referred to as metabolic syndrome and is associated with an increased risk for cardiovascular morbidity and mortality as well as for type 2 diabetes [3]. The atherogenic lipoprotein profile observed in these patients is characterized by elevated fasting plasma levels of triglyceride-rich lipoproteins (TRLs) in combination with low high density lipoprotein-cholesterol (HDL-c) levels and increased concentrations of small-dense low density lipoprotein particles [3]. Insulin resistance, a main characteristic of metabolic syndrome, underlies this dyslipidemia by reducing lipoprotein lipase activity resulting in inhibited hydrolysis of triglycerides into free fatty acids (FFAs) from TRLs and reduced uptake of FFAs into adipocytes during the postprandial phase [4]. In addition, more FFAs are released from enlarged adipocytes into the (portal) circulation since hormone sensitive lipase, in concerted action with adipose triglyceride lipase, is no longer inhibited by insulin [5]. These combined metabolic alterations, in addition to decreased apoB degradation due to insulin resistance, result in an increased substrate for hepatic TRL synthesis (VLDL-1) and a prolonged residence time of hepatic and intestinal derived TRL-remnants during the postprandial phase [6]. Increased levels of TRLs (VLDL, chylomicrons and their remnants) contribute to the development of atherosclerosis since these particles are able to invade the subendothelial space providing substrate for foam cell formation [7]. The presumed importance of postprandial lipid metabolism is further illustrated by the fact that humans are in a non-fasting condition most time of the day. Recently it has been shown that non-fasting plasma triglyceride concentrations are associated with an increased cardiovascular risk in both men and women, independently of other lipids [8,9]. Ezetimbe lowers LDL-c plasma levels by inhibiting the Niemann-Pick C1 Like 1 protein dependent absorption of dietary and biliary cholesterol [10]. Simvastatin, a HMG-CoA reductase inhibitor, at high dose (80 mg) and the combination of low dose simvastatin combined with ezetimibe 10mg have proven to be safe and effective in reducing fasting LDL-c, RLP-c and triglyceride levels and to slightly raise HDL-c plasma concentrations [11,12]. Although statins decrease fasting lipid levels, fat load induced hypertriglyceridemia is unaffected [13, 14]. Whether ezetimibe influences postprandial lipid metabolism is not yet clear.
The aim of the present randomized, double blind, crossover study is therefore to evaluate post fat load lipid concentrations and the lipid content of lipoprotein fractions after 6 weeks treatment with simvastatin 10mg combined with ezetimibe 10mg compared to simvastatin 80mg alone in obese male patients with metabolic syndrome.

Subjects and Methods

Subjects

Nineteen, non-smoking obese male subjects, aged 18-70 years, were recruited by advertisement which called for subjects with waist circumference >102 cm. All subjects were screened for the presence of metabolic syndrome according to the Adult Treatment

Panel III criteria including three or more of the following metabolic abnormalities [3]:

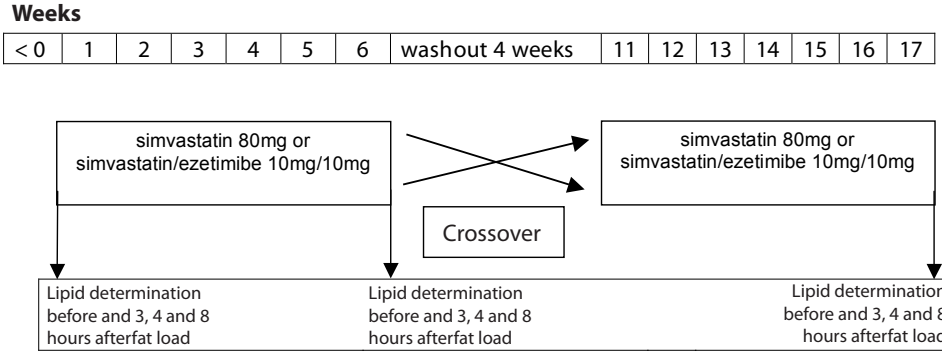
1. abdominal obesity (waist circumference >102 cm)
2. high blood pressure (≥130 mmHg systolic or ≥85 mmHg diastolic)
3. hypertriglyceridemia (serum triglycerides ≥1.70 mmol/L (150 mg/dL))
4. low HDL-c (serum HDL-c <1.04 mmol/L (40 mg/dL)
5. high fasting glucose (fasting serum glucose ≥6.1 mmol/L (110 mg/dL)).

A glucose level ≥7.8 mmol/l after a standardized oral glucose tolerance test was also regarded as fulfilling the glucose criterion. Patients with thyroid- (Thyroid Stimulating Hormone (TSH) levels >5 mU/L with clinical symptoms of hypothyroidism), hepatic- (serum aspartate transferase (ASAT) or serum alanine transferase (ALAT) >2 times the upper limit of normal) or renal diseases (serum creatinine >1.7 times the upper limit of normal) diseases were excluded. Also, patients with the E2/E2 genotype were excluded. Other exclusion criteria were the presence of macrovascular disease, use of vasoactive medication (e.g. beta-blockers, calcium antagonists, ACE-inhibitors, angiotensin type 1 receptor blockers, statins, aspirin, non-steroidal inflammatory drugs), blood pressure ≥180/110 mmHg, Body Mass Index >35, HbA1c >6.5 % and plasma triglycerides >8.0 mmol/L.

Study design

In this prospective, randomized, double blind, crossover trial patients received once daily simvastatin 80mg or the combination of simvastatin 10mg with ezetimibe 10mg during 2 periods of 6 weeks. Between each treatment period patients had a washout period of 4 weeks, after which crossover of therapy occurred. Before the initial therapy and after each treatment period of 6 weeks an oral fat load was performed after an overnight fast of at least 10 hours (**Figure 1**). The study was approved by the Ethical Review Board of the University Medical Center Utrecht (UMCU) and was carried out in a Good Clinical Practice-certified research unit. All subjects gave written informed consent.

Figure 1 *Crossover study design*



Methods

Anthropometric measurements

Before and after each treatment period, patient's weight and height were measured without heavy clothing and shoes. Body mass index (BMI) was calculated as weight to height squared. Waist circumference was measured each visit twice halfway between the lower rib and the iliac crest. Total body fat percentage was estimated by using Omron body fat monitor BF306 (Omron Matsusaka Co. LTD., Japan).

Oral fat load

Fresh cream was used which is a 40% (weight/volume) fat emulsion with a poly-unsaturated/saturated fat ratio of 0.10, containing 0.001% (w/v) cholesterol and 3% (w/v) carbohydrates representing a total energy content of 3700 kCal/L. Cream was ingested at a dose of 50 g fat and 3.75 g glucose per m^2 body surface (with a maximum of 250 ml) within 5 minutes. Participants remained supine during the day and were only allowed to drink mineral water. Venous blood samples were obtained before and at 3, 4 and 8 hours after ingestion and immediately centrifuged for 15 min at 3000 rpm at 4°C before the plasma was stored at -80°C.

Laboratory assessment

At each visit, fasting blood was sampled to determine plasma levels of glucose, creatinine, homocysteine, ALAT, ASAT, total cholesterol, LDL-c, HDL-c, VLDL and triglycerides. ApoE genotyping, TSH and HbA1c, were measured only at the first visit. Plasma cholesterol, HDL-c and LDL-c, apoA-1 and apoB were measured using commercially available assays (Wako, Osaka, Japan) on a Cobas Mira auto-analyzer (ABX). Triglycerides were measured with an assay from Randox laboratories, Crumlin, United Kingdom. All other measurements were analyzed in a local research facility according to standardized ISO 9001:2000 regulations. HOMA-IR: Homeostasis model assessment determined insulin resistance (fasting serum glucose x fasting serum insulin)/22.5 [15]. For conversion of apoB from g/l to mmol/l divide by 550000.
At all time points lipoproteins fractions were isolated by density gradient ultracentrifugation according to Redgrave using a SW40.Ti rotor in a Beckman ultracentrifuge [16]. Briefly, a discontinuous density gradient using the following densities ranges: d=1.21 g/ml, d=1.063 g/ml, d= 1.019 g/ml and d= 1.006 g/ml of KBr solutions was prepared. Lipoprotein fractions were isolated after centrifugation at 40.000 rpm, 40C, for 22 hours. Fractions of interest were TRL (VLDL and apoB containing remnants), IDL, LDL and HDL. Cholesterol, triglyceride and apoB (B100 and B48) content of each fraction (if applicable) were measured by commercially available assays (Wako, Osaka, Japan) on a Cobas Mira auto-analyzer (ABX).

Statistical analyses

All values were expressed as mean ± standard deviation (SD), except for lipid parameters which are expressed as mean ± standard error of mean (SEM). The postprandial change

in lipid plasma levels and content were integrated as area under the curve (AUC) and were calculated by the trapezoidal rule using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA). Net integrated AUCs (net AUC) were calculated after correction for baseline values and display the net change in lipid levels during the 8 hours post fat load phase on top of fasting levels. Differences in parameters between simvastatin 80mg and combination therapy of simvastatin/ezetimibe 10mg/10mg were analyzed by 2-sided paired *t*-test; statistical significance was taken at the of level 5%. Carryover and period effects were calculated with independent samples *t*-test [17]. Calculations were performed using SPSS for Windows version 12.1 (SPSS Inc. Chicago, IL, USA).

Results

Baseline characteristics.

In total 37 subjects were screened, of whom 19 patients were eligible and included in the study. The clinical and laboratory characteristics of the study subjects at baseline are show in **Table 1**. Weight (100.3 ± 11.5 kg), waist circumference (111 ± 7 cm) and body fat percentage (31 ± 3%) remained stable during the study. Fasting triglycerides were 1.66 ± 0.13 mmol/l and HDL-c was 1.14 ± 0.06 mmol/l at baseline. Other components of metabolic syndrome at baseline were blood pressure 138 ± 13 (systolic) and 89 ± 6 (diastolic) mmHg and fasting glucose level was 6.2 ± 0.7 mmol/l and remained stable during the course of the study. No carryover effects or period effects between the two treatments periods were observed for the postprandial lipid profiles.

Effects of simvastatin 80mg and simvastatin/ezetimibe 10mg/10mg on lipoprotein plasma concentrations

The effect of both treatment strategies on plasma lipids is presented in **Table 2**. Fasting plasma LDL-c levels were equally lowered by both treatment regimens to a concentration of 2.1 mmol/l. Plasma apoB levels were significantly reduced by both treatments (simvastatin 80mg: -34%; simvastatin/ezetimibe 10mg/10mg: -28%). Fasting plasma triglycerides were strongly reduced by simvastatin 80mg (-35%). After treatment with the combination of simvastatin with ezetemibe the reduction in plasma triglyceride levels was less prominent (-23%) although this difference did not reach the level of statistical significance (p=0.07). The reduction in plasma RLP-c levels however was similar in both treatment arms.

Table 3 displays the net AUC for post fat load lipid and lipoprotein levels during the 8 hours fat load test. The net AUC-total triglycerides was reduced by 22% after simvastatin 80mg (5.16 ± 0.50 mmol·h/l) and by 8% (6.09 ± 0.71 mmol·h/l) after simvastatin/ezetimibe 10mg/10mg, compared to baseline (6.64 ± 0.86 mmol·h/l). There was no difference between both treatments, p=0.42. The net AUC-apoB and net AUC-RLP-c during fat load were similar after both treatments. In the present study, fasting and postprandial triglyceride plasma levels were comparable among the different genotypes (E2/E3 (n=3), E3/E3 (n=12), E3/E4 n=4)) after both treatment regimes.

Table 1 *Baseline characteristics of study subjects*

	Baseline (n=19)
Age (years)	54 ± 7
Height (m)	1.83 ± 0.06
Weight (kg)	100.3 ± 11.5
Body mass index (kg/m^2)	30 ± 3
Body fat (%)	31 ± 3
Laboratory parameters	
Haemoglobin (mmol/l)	9.9 ± 0.6
Haematocrite (l/l)	0.47 ± 0.03
Thrombocytes (exp9/l)	201 ± 30
Leucocytes (exp9/l)	4.7 ± 0.7
ASAT (U/l)	35 ± 6
ALAT (U/l)	45 ± 16
Creatinin kinase (U/l)	116 ± 53
Creatinine Clearance (Cockcroft) (ml/min)	111 ± 19
TSH (mU/l)	1.7 ± 0.9
HbA1c (%)	5.7 ± 0.4
Homocysteine (µmol/l)	9.9 ± 1.7
Insulin (mU/l)	19 ± 9
HOMA-IR	5.1 ± 2.3
ApoE genotyping	
E2/E3 % (n)	16 (3)
E3/E3 % (n)	63 (12)
E3/E4 % (n)	21 (4)
Components of metabolic syndrome	
Triglycerides (mmol/l)$	1.66 ± 0.13
HDL-cholesterol (mmol/l)$	1.14 ± 0.06
Glucose (mmol/l)	6.2 ± 0.7
Waist circumference (cm)	110.6 ± 6.8
Systolic blood pressure (mmHg)	138 ± 13
Diastolic blood pressure (mmHg)	89 ± 6

Mean ± SD or as indicated.
$ All lipid parameters mean ± SEM

Table 2 *Fasting lipid parameters without treatment and after both treatment periods.*

	Baseline	After 6 weeks simvastatin 80mg	After 6 weeks simvastatin/ezetimibe 10mg/10mg	p-value
Total lipid parameters levels				
Total cholesterol (mmol/l)	5.6 ± 0.2	3.7 ± 0.2	3.8 ± 0.2	0.77
VLDL-c (mmol/l)	0.7 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.36
LDL-c (mmol/l)	3.7 ± 0.2	2.1 ± 0.1	2.1 ± 0.1	0.85
ApoB (mg/dl)	0.98 ± 0.04	0.65 ± 0.03	0.71 ± 0.04	0.51
RLP-c (mmol/l)	0.71 ± 0.08	0.36 ± 0.03	0.35 ± 0.03	0.40
Triglycerides (mmol/l)	1.66 ± 0.13	1.10 ± 0.10	1.27 ± 0.10	0.07
Contents of lipoprotein fractions Triglyceride content (mmol/l)				
TRL	1.06 ± 0.12	0.49 ± 0.06	0.60 ± 0.05	0.77
IDL	0.13 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.68
LDL	0.28 ± 0.02	0.15 ± 0.01	0.20 ± 0.02	0.03
HDL	0.15 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.86
Cholesterol content (mmol/l)				
TRL	0.46 ± 0.06	0.16 ± 0.02	0.18 ± 0.02	0.14
IDL	0.29 ± 0.04	0.14 ± 0.02	0.14 ± 0.02	0.95
LDL	3.98 ± 0.26	1.83 ± 0.17	1.90 ± 0.16	0.76
HDL	0.90 ± 0.08	1.09 ± 0.07	1.05 ± 0.08	0.60
ApoB content (mg/dl)				
TRL	3.60 ± 0.47	2.88 ± 0.24	3.10 ± 0.36	0.59
IDL	4.46 ± 0.44	3.39 ± 0.38	3.02 ± 0.28	0.35
LDL	64.83 ± 2.97	34.00 ± 2.04	37.23 ± 2.46	0.18
Cholesterol / ApoB (mol/mol)				
TRL	10887 ± 2942	2947 ± 278	3452 ± 415	0.30
IDL	3787 ± 616	2289 ± 195	2464 ± 236	0.62
LDL	3413 ± 204	2933 ± 139	2791 ± 174	0.57
Triglycerides / ApoB (mol/mol)				
TRL	23860 ± 5264	9975 ± 1161	12797 ± 1995	0.29
IDL	1855 ± 297	1374 ± 133	1567 ± 193	0.47
LDL	251 ± 30	254 ± 12	320 ± 44	0.11

Mean ± SEM
p-values: simvastatin 80mg compared to simvastatin/ezetimibe 10mg/10mg

Table 3 *Change of lipid levels in plasma and lipid fractions during 8 hours after oral fat load.*

	No treatment, after oral fat load	After 6 weeks simvastatin 80mg	After 6 weeks simvastatin/ezetimibe 10mg/10mg	p-value
Net AUC plasma lipids (mmol•h/l)				
Total Cholesterol	0.28 ± 0.48	-0.42 ± 0.47	-0.19 ± 0.36	0.54
Total triglycerides	6.64 ± 0.86	5.16 ± 0.50	6.09 ± 0.71	0.42
Apo B direct (mg·h /dl)	-2.28 ± 6.27	-10.70 ± 7.00	4.61 ± 9.23	0.21
RLP-c	48.68 ± 8.59	29.01 ± 4.66	26.34 ± 5.00	0.45
Net AUC Triglycerides (mmol·h/l)				
TRL	1.32 ± 0.93	1.66 ± 0.38	1.30 ± 0.32	0.42
IDL	-0.08 ± 0.05	-0.04 ± 0.02	-0.07 ± 0.05	0.50
LDL	0.03 ± 0.17	0.07 ± 0.04	-0.18 ± 0.11	0.06
HDL	0.21 ± 0.15	0.11 ± 0.04	0.10 ± 0.04	0.96
Net AUC Cholesterol (mmol·h/l)				
TRL	-0.86 ± 0.23	-0.16 ± 0.09	-0.50 ± 0.09	0.01
IDL	-0.29 ± 0.11	-0.10 ± 0.07	-0.19 ± 0.07	0.68
LDL	-1.23 ± 0.87	0.72 ± 0.54	-0.30 ± 0.78	0.36
HDL	0.59 ± 0.37	0.12 ± 0.24	0.29 ± 0.32	0.64
Net AUC ApoB (mg·h/dl)				
TRL	6.92 ± 4.32	1.91 ± 1.30	-0.69 ± 1.77	0.28
IDL	-1.12 ± 2.64	-1.16 ± 1.28	0.50 ± 2.20	0.46
LDL	35.35 ± 36.48	7.53 ± 6.07	-20.11 ± 9.69	0.07

Mean ± SEM
p-values: simvastatin 80mg compared to simvastatin/ezetimibe 10mg/10mg

Effects of simvastatin 80mg and simvastatin/ezetimibe 10mg/10mg on lipoprotein composition.

Both simvastatin 80mg and simvastatin/ezetimibe 10mg/10mg led to a similar reduction in fasting levels of cholesterol and apoB in TRL, IDL and LDL fractions compared to baseline (**Table 2**). The fasting triglyceride concentration without treatment in LDL was 0.28 ± 0.02 mmol/l. After 6 weeks of simvastatin 80mg treatment the fasting triglyceride concentration in LDL was lower (0.15 ± 0.01 mmol/l) compared to simvastatin/ezetimibe 10mg/10mg (0.20 ± 0.02 mmol/l). This difference between the 2 treatment arms was statistically significant: p=0.03. **Figures 2-4** display the post fat load triglyceride, cholesterol and apoB content in the different lipoprotein fractions. A peak in the triglyceride concentration in the TRL fraction was seen at 4 hours post fat load (**Figure 2**).

During the oral fat load test no differences were observed between the 2 treatment regimens regarding improvement of net AUC-apoB in TRL, and IDL, although there is a tendency towards a better improvement in net-AUC-apoB in LDL during simvastatin/ezetimibe 10mg/10mg treatment (-20.11 ± 9.69 mg•h/dl) in comparison to simvastatin alone (+7.53 ± 6.07 mg•h/dl, p=0.07). The net AUC-TG, in TRL, IDL, and HDL was similar between both treatments (**Table 3**). However the net AUC-TG in LDL was significantly smaller during simvastatin/ezetimibe 10mg/10mg treatment (-0.18 ± 0.11 mmol·h/l) versus simvastatin alone (0.07 ± 0.04 mmol·h/l, p=0.06). Net-AUC-cholesterol in TRL is significantly more reduced on statin treatment alone (-0.16 ± 0.09 mmol·h/l) versus eze/simva combi (-0.50 ± 0.09 mmol·h/l; p=0.01

Figure 2 *Postprandial triglyceride content in lipoprotein fractions*

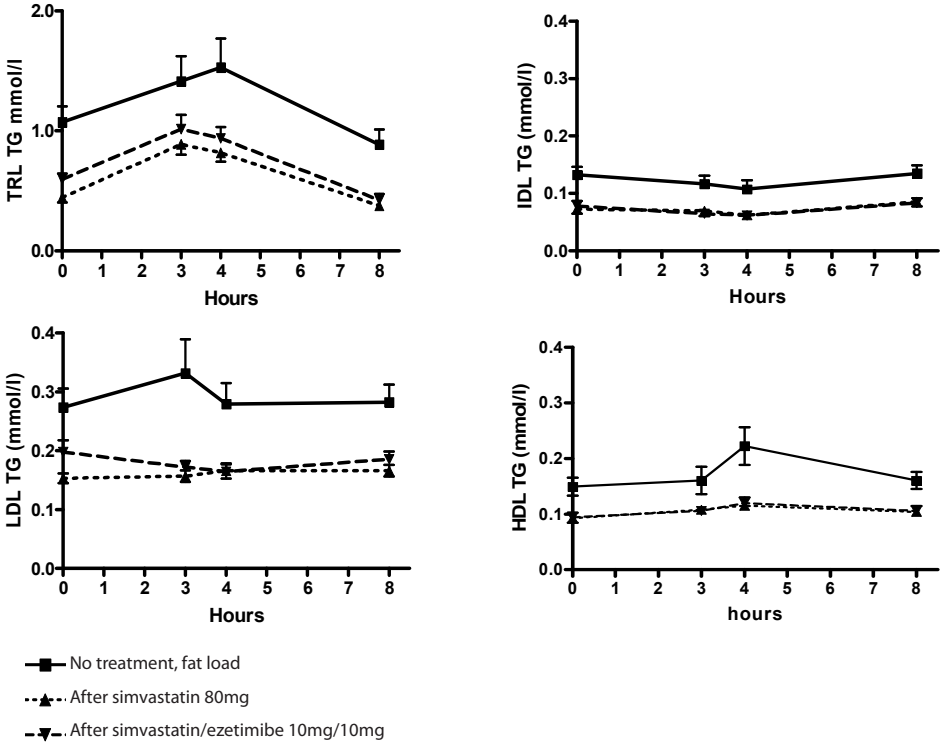


Figure 3 *Postprandial cholesterol content in lipoprotein fractions*

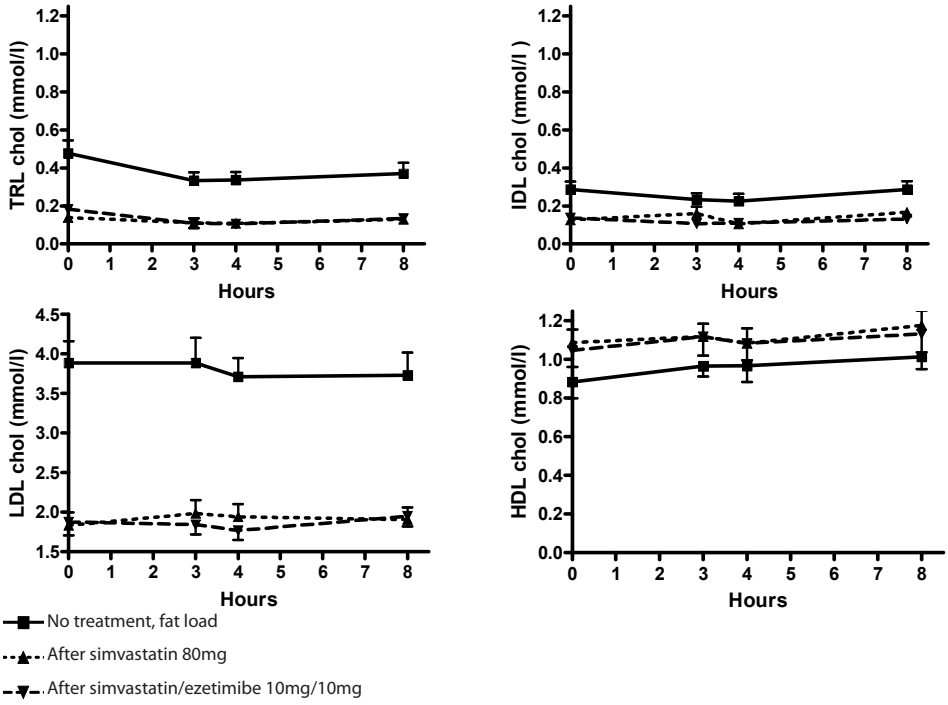
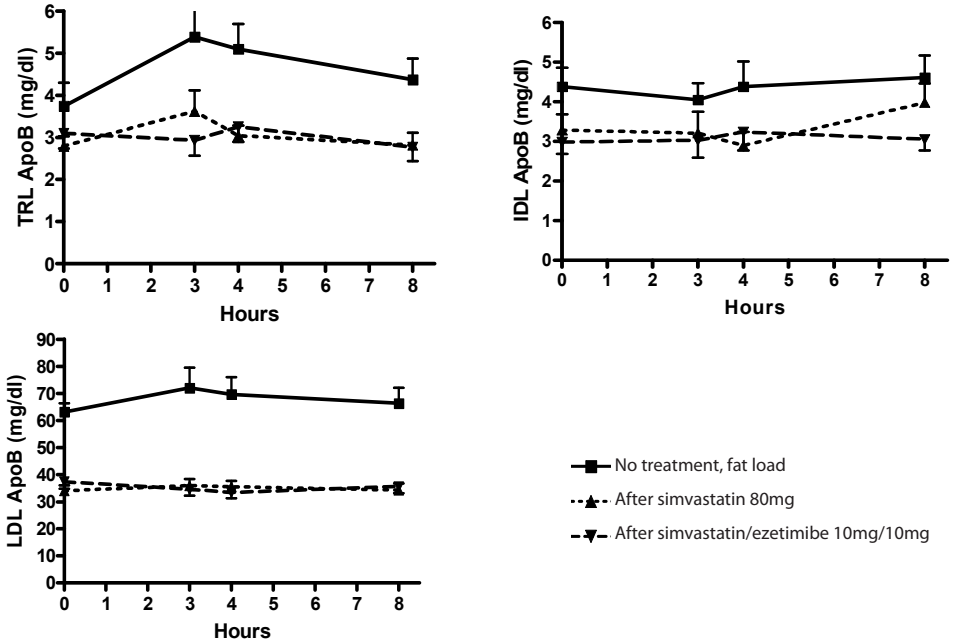


Figure 4 *Postprandial ApoB content in lipoprotein fractions.*



Discussion

In the present double blind, crossover trial it is shown that treatment with low dose simvastatin (10mg) in combination with ezetimibe (10mg) did not result in lower post fat load lipoprotein plasma concentrations nor in differences in lipoprotein particle content when compared to high dose simvastatin (80mg) monotherapy in obese male metabolic syndrome patients.

In metabolic syndrome subjects an atherogenic lipid profile is highly prevalent and associated with central obesity, endothelial dysfunction, low adiponectin plasma levels, insulin resistance, low grade inflammation and fatty liver [3, 6, 18]. However, whether high levels of (postprandial) TRLs are a risk factor for cardiovascular events by itself or a marker of risk reflecting underlying risk factors associated insulin resistance and abdominal obesity is not yet clear. Two recent large cohort studies indicate that non-fasting hypertriglycerdemia is associated with an increased vascular risk, independently of other lipids [8, 9]. Of note, in the analyses the concentration of apoB particles and lipoprotein composition were not taken into account. Postprandial dyslipidemia may not only be a better indicator of vascular risk, treatment of postprandial lipid profiles by lowering the exposure of atherogenic lipoproteins to the endothelium may be a treatment target.

In the present study, the fasting LDL-c and triglyceride plasma concentrations equally decreased after both strategies which is in line with previous studies [19]. Under fasting conditions, the cholesterol and triglyceride content in ApoB containing lipoprotein fractions was reduced compared to baseline, not only by high dose simvastatin but also by the combination of simvastatin with ezetimibe. Both treatment regimes also reduced the number of particles reflected by the plasma apoB concentration. In another study in type 2 diabetes patients, simvastatin 80mg reduced the triglyceride (-39%, -36% and -39%), cholesterol (-53%, -59% and -39%) and apoB (-34%, -55% and -47%) content in fasting VLDL, IDL and LDL fractions, respectively, compared to baseline and is in the range of the effects observed in the present study [20]. The triglyceride content in the fasting LDL fraction was lower after simvastatin treatment compared to combination therapy. However, this tendency was not consistent in the other apoB fractions, thereby making this observation less clinical relevant. Also, the cholesterol content was lower during fat loading in the TRLs fraction after combination therapy without differences in the other fractions.

During the 8 hour post fat load period, simvastatin 80mg and simvastatin/ezetimibe 10mg/10mg treatment resulted in similar reductions in the triglyceride, cholesterol and apoB content in the individual lipoprotein fractions. Statins might increase post fat load lipid particle clearance by reducing the apoC-III plasma levels and stimulating LPL-activity [21, 22]. However, most studies, including the present, showed no influence of statin therapy on postprandial lipoprotein concentration and composition on top of reduced fasting levels [13, 14]. In the few studies that reported an effect of statins on postprandial lipid levels the effect was relatively small compared to the large decrease in fasting lipid levels [23].

Statins and ezetimibe affect lipid homeostasis through different mechanisms. It is plausible that ezetimibe therapy would lead to qualitative or quantitative differences

in apoB-48 containing particles. In enterocytes cholesterol, apoB-48 and triglycerides are assembled into chylomicrons by microsomal triglyceride transfer protein (MTP) [24]. In order to maintain a stable cholesterol homeostasis in the cell, enterocytes have the option to regulate uptake and secretion of cholesterol. Niemann-Pick C1 Like 1 protein is involved in direct uptake of intestinal cholesterol. Regulation of cholesterol homeostasis occurs in the cells is mediated by the ABCG5/G8 cholesterol transporters. Elevated intra-enterocyte cholesterol ester availability, may lead to posttranslational stabilization of apoB-48 molecules leading to increased availability of apoB-48 [25] and may up regulate MTP transcription by activating the positive sterol response element of the MTP-gene [26]. Also *in vitro*, a correlation has been observed between MTP mRNA levels and chylomicrons triglyceride content [27]. Statin monotherapy can decrease the level of intestinal MTP mRNA [27,28] but also increase intestinal cholesterol uptake [29]. Ezetimibe monotherapy is associated with a local intestinal cholesterol synthesis to maintain stable cholesteryl ester levels [10]. Therefore, a reduction in enterocyte cholesterol ester levels by combination therapy may result in lower apoB-48 availability resulting in a decreased secretion of chylomicron particles [28]. When the intracellular cholesterol concentration in intestinal cells is lower, the ABCG5/G8 cholesterol transporters are downregulated to prevent cholesterol loss. This may be the case during statin treatment although direct evidence is still missing [27]. In our model of acute hypertriglyceridemia, simvastatin/ezetimibe did not result in differences in post fat load metabolism compared to simvastatin mono-therapy

Strength of the present study is the double blind, crossover design. We also acknowledge study limitations. A limitation of our study was that we did not individually characterize chylomicron composition. We did however analyze RLP-c as a measure of the presence of apoB-48 containing remnant particles. No differences were observed in plasma RLP-c levels between both treatment arms. ApoB-48 and apoB-100 were not separately measured in the present study. Furthermore, only male metabolic syndrome patients were included without a history of vascular diseases or diabetes. Therefore extrapolating the results to female patients or to non-obese patients should be done with care. Crossover and carryover effects can not completely be ruled out but are unlikely to have affected the results regarding the half-life time of the study drugs, the 4 weeks washout period and a period of 10 weeks between the effect measurements of both treatment regimes.

In conclusion, both simvastatin 80mg and simvastatin/ezetimibe 10mg/10mg are equally effective in reducing fasting cholesterol, triglyceride, RLP-c and apoB lipoprotein concentrations, thereby reducing the absolute post fat load plasma concentrations. The fasting and post fat load concentrations of cholesterol and triglycerides in lipoprotein particles in the TRL, IDL, and LDL fractions were similarly affected by both treatments.

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CHAPTER 8

The effects of low-dose simvastatin and ezetimibe compared to high-dose simvastatin alone on post fat load endothelial function in patients with metabolic syndrome

-A randomized double blind crossover trial-

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Submitted

Abstract

Background

Insulin resistance is associated with postprandial hyperlipidemia and endothelial dysfunction. Patients with the metabolic syndrome, characterized by insulin resistance, are at increased cardiovascular risk. Aim of the present study was to investigate whether a similar LDL-c reduction with either combination therapy of low-dose simvastatin and ezetimibe or with high-dose simvastatin alone has similar effects on (post fat load) endothelial function.

Methods

Randomized, double blind, crossover trial in 19 male obese patients with the metabolic syndrome with high-dose simvastatin 80 mg versus combination therapy of low-dose simvastatin 10 mg with ezetimibe 10 mg. Fasting and post fat load lipids and endothelial function (brachial artery flow-mediated dilation (FMD)) were determined.
Clinicaltrials.gov Identifier NCT00189085

Results

Fasting LDL-c concentrations (2.1±0.5 mmol/l) and fasting endothelial function (6.9±0.8 versus 7.6±1.2%) were the same after both treatments. Although post fat load plasma triglyceride concentrations were higher (3.2±0.4 versus 2.6±0.2 mmol·h/l) with combination therapy compared to monotherapy, ApoB particles were comparable (0.9±3.3 versus -0.2±2.3 g·h/l). Combination therapy did not decrease post fat load endothelial function (7.6±1.2 versus 7.7±1.6%), contrary to high dose simvastatin monotherapy (6.9±0.8 versus 4.3±0.6%).

Conclusions

Combination therapy with low-dose simvastatin and ezetimibe preserved post fat load endothelial function, contrary to treatment with high-dose simvastatin monotherapy in male metabolic syndrome patients while there were no differences in fasting lipid profiles

Introduction

Lowering plasma concentrations of Low Density Lipoprotein cholesterol (LDL-c) is a cornerstone in cardiovascular risk reduction in patients at elevated risk for vascular events [1]. Intensive LDL-c lowering can be achieved by high dose statin treatment or with combination therapy of lower doses statin and ezetimibe [2]. It is unclear whether a similar LDL-c reduction with combination therapy of low-dose simvastatin and ezetimibe or with high-dose simvastatin alone has similar effects on fasting and post fat load endothelial function. Statin therapy may improve fasting and post fat load endothelial function but it is not known whether this is an indirect effect of lipid-lowering or a direct vascular effect of statins influencing the stability of endothelial nitric oxide synthase (eNOS), often referred to as 'pleiotropic effects' [3-4]. Inhibition of cholesterol absorption may influence postprandial lipid metabolism and may therefore have effects on postprandial endothelial function. Ezetimibe inhibits cholesterol absorption but effects on post fat load hyperlipidemia and endothelial function are unknown. Postprandial hyperlipidemia could be regarded as a cardiovascular risk factor, as indicated by the induction of postprandial endothelial dysfunction [5-7]. Chylomicron-remnants and very low density lipoprotein (VLDL) particles may impair endothelial dependent vaso-dilatation [8].

Insulin resistance, often observed in the metabolic syndrome, is associated with elevated triglyceride-rich lipoproteins in the VLDL-1 fraction and their remnants in the postprandial state [9,10]. Insulin resistance causes endothelial dysfunction and decreased nitric oxide bioavailability by several mechanisms including inflammation (as reflected by elevated C-Reactive Protein (CRP) plasma levels), disruption of insulin receptor signalling cascades, increased production of cytokines (among them Interleukin-6 and TNF-α) and activation of the renin angiotensin system [11,12]. Adiponectin, an adipocyte-derived protein, stimulates the production of nitric oxide in vascular endothelial cells *in vitro*, and hypoadiponectinemia observed in insulin resistance is associated with endothelial dysfunction [13-14].

Aims of the present study were to compare the effects of the combination therapy of low-dose simvastatin and ezetimibe with high-dose simvastatin monotherapy, aiming at a similar fasting LDL-c reduction, on fasting and post fat load lipid profiles and endothelial function in obese male patients with metabolic syndrome.

Methods

Subjects

Nineteen non-smoking obese male subjects, aged 18-70 years, were recruited by advertisement which called for subjects with waist circumference >102 cm. All subjects were screened for the presence of the metabolic syndrome according to the Adult Treatment Panel III criteria [15]. Glucose level ≥7.8 mmol/L after a standardized (75 g) oral glucose tolerance test was also regarded as fulfilling the glucose criterion. Patients with thyroid-, hepatic- or renal diseases were excluded. Other exclusion criteria were the history of macrovascular disease, use of vasoactive medication (e.g. beta-blockers,

calcium antagonists, ACE-inhibitors, angiotensin type 1 receptor blockers, statins, aspirin, non-steroidal inflammatory drugs), blood pressure ≥180/110 mmHg, Body Mass Index >35, HbA1c >6.5 % and plasma triglycerides >8.0 mmol/L. The local Ethics Committee approved the study and all participants gave their written informed consent.

Study Design

In this prospective, randomized, crossover, double blind trial patients received once daily simvastatin 80 mg or the combination of simvastatin 10 mg and ezetimibe 10 mg [16]. Vascular function, as determined with flow-mediated dilation measurements, was performed after 6 weeks of treatment. Between the two treatment periods patients had a washout period of 4 weeks. Crossover of therapy occurred after washout followed by reassessment of vascular function after 6 weeks. FMD was measured before and 4 hours after an oral fat load at the end of both treatment periods. At the beginning and at the end of each 6-week treatment period patients underwent physical examination (including height, body weight, waist circumference, body fat and blood pressure measurements) and blood sampling to determine laboratory parameters.

Assessment of vascular function with FMD

This is a non-invasive technique of assessing endothelial function by ultrasonography of the brachial artery. Measurements are made of the vasodilatory responses of the brachial artery to post-ischemic hyperemia, causing endothelium-dependent dilation. As described previously, all measurements were made with a Wall Track System (WTS) (Pie Medical, Maastricht, the Netherlands) which consists of a standard 7.5 MHz linear array transducer connected to a data acquisition system and a personal computer [17]. The first three measurements were averaged to provide a baseline arterial diameter. By inflation of the blood pressure cuff for 5 minutes above a pressure of 250 mmHg, ischemia was applied in the forearm distal to the location of the echo probe. Upon release of the cuff, the brachial artery will dilate through endothelial NO-release (endothelium-dependent vasodilatation). Ultrasonographic measurements were performed 4 times after cuff release at 15 seconds intervals and then 5 times after 30 seconds intervals. Maximal post-ischemic dilation was assessed by the widest lumen diameter. Then nitro-glycerin (0.4 mg) was administrated sublingually to determine endothelium-independent vasodilatation. WTS measurements are stored and analyzed off line by a blinded observer using WTS software analysis. FMD and nitro-glycerin-induced vasodilatation were expressed as percentage change relative to the baseline diameter.

Oral fat load

For the fat load, fresh cream was used which is a 40% (weight/volume) fat emulsion with a poly-unsaturated/saturated fat ratio of 0.10, containing 0.001% (w/v) cholesterol and 3% (w/v) carbohydrates representing a total energy content of 3700 kCal/L. Cream was ingested at a dose of 50 g fat and 3.75 g glucose per m^2 body surface (with a maximum of 250 ml) within 5 minutes. Participants remained supine during the day and were only allowed to drink water. Venous blood samples were obtained before and at 2, 3, and 4 hours after ingestion and were immediately put on ice. Plasma was isolated by

centrifugation for 15 min at 3000 revolutions per minute at 4°C. Plasma samples were stored at -80°C for further analyses.

Laboratory assessment

Buffy coats were sampled for isolation of DNA for apoE genotyping. Insulin was measured with an immunometric assay (Diagnostic Products Corporation, Los Angeles, USA). Plasma cholesterol, HDL-c and LDL-c were measured using commercially available assays (Wako, Osaka, Japan) on a Cobas Mira auto-analyzer (Roche, Basel, Switzerland). VLDL-cholesterol was then calculated (VLDL-c = total cholesterol minus LDL-cholesterol minus HDL-cholesterol). Plasma triglycerides were analyzed using an automated assay (Randox laboratories, Crumlin United Kingdom.) Plasma apoB was analyzed using a nephelometric commercial assay using the Cobas Mira auto analyzer. Plasma remnant-like particle cholesterol (RLP-c) was analyzed using a commercial available assay as extensively described previously [18]. Measurements of plasma adiponectin, interleukin-6 (IL-6) and hs-CRP levels were performed with a commercially available kit (ELISA; R&D Systems Inc., Minneapolis, USA).

Anthropometric measurements

Waist circumference was measured halfway between the lower rib and the iliac crest. Total body fat percentage was estimated by using Omron body fat monitor BF306 (Omron Matsusaka Co. LTD., Japan).

Statistical analyses

Fasting and post fat load FMD measurements were analyzed by an experienced observer blinded to all patients' characteristics and treatment. The post fat load variations of lipids were integrated as area under the curve (AUC) and were calculated by the trapezoidal rule using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA). Incremental integrated AUC (AUIC) was calculated after correction for baseline values. Differences in FMD and in AU(I)Cs for post fat load lipids between simvastatin 80 mg and combination therapy of simvastatin 10 mg with ezetimibe 10 mg were analyzed by paired *t*-test; statistical significance was taken at the 5% level. Carryover and period effects were calculated with independent samples *t*-test [19]. Calculations were performed using SPSS for Windows version 12.1. (SPSS Inc. Chicago, IL, USA)

Results

No carryover effects or period effects between the two treatments periods were observed for fasting and post fat load lipid profiles. Demographic, clinical and laboratory characteristics of the 19 patients at baseline and after the 2 treatment periods are provided in **Table 1**. Mean age was 54±7 years. Weight, waist circumference and body fat remained stable during the study. Creatinin kinase was slightly elevated after treatment (116±53 U/l versus 143±67 U/l and 152±134 U/l).

Table 1 *Clinical characteristics at baseline and after treatment*

	Baseline* Before treatment (n=19)	After 6 weeks simva 80 mg (n=19)	After 6 weeks simva/eze 10/10 mg (n=19)
Age (years)	54 ± 7	x	x
Height (m)	1.83 ± 0.6	x	x
Weight (kg)	100.3 ± 11.5	100.9 ± 11.2	100.3 ± 11.3
Body mass index (kg/m^2)	30.1 ± 2.7	30.2 ± 2.5	30.0 ± 2.6
Body fat (%)	31 ± 3	31 ± 3	30 ± 3
Laboratory parameters			
Haemoglobin (mmol/l)	9.9 ± 0.6	x	x
Haematocrite (l/l)	0.47 ± 0.03	x	x
Thrombocytes (exp9/l)	201 ± 30	x	x
Leucocytes (exp9/l)	4.7 ± 0.7	x	x
ASAT (U/l)	35 ± 6	37 ± 8	41 ± 15
ALAT (U/l)	45 ± 16	49 ± 17	49 ± 18
Creatinin kinase (U/l)	116 ± 53	143 ± 67	152 ± 134
Creatinine Clearance (ml/min)‡	111 ± 19	123 ± 22	127 ± 21
TSH (mIE/l)	1.7 ± 0.9	x	x
HbA1c (%)	5.7 ± 0.4	x	x
Total cholesterol (mmol/l)	5.6 ± 0.9	3.7 ± 0.9	3.8 ± 0.9
LDL-cholesterol (mmol/l)	3.7 ± 0.7	2.1 ± 0.5	2.1 ± 0.5
VLDL-cholesterol (mmol/l)	0.71 ± 0.25	0.46 ± 0.25	0.51 ± 0.33
Apolipoprotein B (g/l)	98 ± 16	65 ± 14	71 ± 18
homocysteine (µmol/l)	9.9 ± 1.7	9.4 ± 2.5	9.5 ± 2.5
Insulin (mU/l)	19 ± 9	17 ± 9.0	17 ± 7
HOMA-IR$	5.1 ± 2.3	4.7 ± 2.4	4.6 ± 2.3
hs-CRP (mg/l)†	2.38 (1.8 - 4.1)	2.32 (1.8 - 4.2)	2.66 (1.5 - 5.1)
Interleukin-6 (pg/ml)	1.36 ± 0.51	1.89 ± 1.39	1.58 ± 0.74
Adiponectin (mg/l)	4.8 ± 2.4	4.8 ± 2.0	4.6 ± 2.2

All data mean ± standard deviation or as indicated
** Values at screening or at baseline visit*
‡ Cockcroft
$ HOMA-IR: Homeostasis model assessment determined insulin resistance (fasting serum glucose x fasting serum insulin)/22.5
† median with interquartiles range

Table 1 *Continued*

	Baseline* Before treatment (n=19)	After 6 weeks simva 80 mg (n=19)	After 6 weeks simva/eze 10/10 mg (n=19)
ApoE genotyping			
E2/E3 % (n)	16 (3)	x	x
E3/E3 % (n)	63 (12)	x	x
E3/E4 % (n)	21 (4)	x	x
Components of metabolic syndrome			
Triglycerides (mmol/l)	1.66 ± 0.29	1.10 ± 0.45	1.27 ± 0.42
HDL-cholesterol (mmol/l)	1.14 ± 0.26	1.14 ± 0.31	1.12 ± 0.26
Glucose (mmol/l)	6.2 ± 0.7	6.1 ± 0.6	6.1 ± 0.8
Waist circumference (cm)	110.6 ± 6.8	110.8 ± 5.5	110.4 ± 7.0
Systolic blood pressure (mmHg)	138 ± 13	135 ± 16	132 ± 8
Diastolic blood pressure (mmHg)	89 ± 6	87 ± 8	87 ± 4

All data mean ± standard deviation or as indicated:
** Values at screening or at baseline visit*

Fasting and post fat load lipid profiles, insulin and interleukin-6 plasma concentrations

Total cholesterol decreased from 5.6±0.9 mmol/l to 3.7±0.9 mmol/l during treatment with simvastatin 80 mg and to 3.8±0.9 mmol/l during treatment with combination therapy. Plasma LDL-c concentration was similarly reduced by both treatment regimes (from 3.7±0.7 mmol/l to 2.1±0.5 mmol/l). In **Table 2** AUCs and AUICs for LDL-cholesterol, VLDL-c, RLP-c, triglycerides and apoB were shown. No differences were observed for the AUICs for post fat load LDL-c (-0.20±0.11 versus -0.14±0.08 mmol·h/l), RLP-c (14±2 versus 13±2 mmol·h/l) and VLDL-c (0.4±0.1 versus 0.6±0.1 mmol·h/l) between both treatment regimes. AUICs for triglycerides were slightly higher after treatment with combination therapy compared to high-dose simvastatin alone (3.2±0.4 versus 2.6±0.2 mmol·h/l), but AUICs for apoB were comparable (0.9 ± 3.3 versus -0.2 ± 2.3 g·h/l).
In **Table 3** it was shown that before as well as 3 and 4 hours after an oral fat load, plasma concentrations of IL-6 were slightly higher during treatment with high dose simvastatin monotherapy compared to combination therapy (4 hours post fat load 3.41±0.67 versus 2.87±0.44 pg/ml). In addition, insulin plasma concentrations were marginally higher after oral fat load during high dose simvastatin monotherapy compared to combination therapy.

Table 2 *Postprandial lipids (area under the curve, baseline not corrected (AUC) and baseline corrected (AUIC))*

	Before treatment	After 6 weeks simva 80 mg	After 6 weeks simva/eze 10/10 mg	p-value*
AUC				
LDL-c (mmol·h/l)[a]	14.8 ± 0.6	8.2 ± 0.5	8.2 ± 0.4	0.99
VLDL-c (mmol·h/l)[a]	3.7 ± 0.3	2.2 ± 0.2	2.6 ± 0.3	0.2
RLP-c (mmol·h/l)[b]	129 ± 13	70 ± 6	67 ± 5	0.4
Triglycerides (mmol·h/l)[a]	9.9 ± 0.7	7.1 ± 0.4	8.3 ± 0.6	0.01
ApoB (g·h/l)[a]	394 ± 16	263 ± 11	285 ± 17	0.2
AUIC				
LDL-c (mmol·h/l)[a]	-0.14± 0.14	-0.20 ± 0.11	-0.14 ± 0.08	0.7
VLDL-c (mmol·h/l)[a]	0.7 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.06
RLP-c (mmol·h/l)[b]	19 ± 3	14 ± 2	13 ± 2	0.5
Triglycerides (mmol·h/l)[a]	3.2 ± 0.3	2.6 ± 0.2	3.2 ± 0.4	0.07
ApoB (g·h/l)[a]	1.1 ± 2.6	-0.2 ± 2.3	0.9 ± 3.3	0.8

All data: mean ± SEM
a measurements before and 2, 3, and 4 hours after oral fat load
b measurements before and 3, and 4 hours after oral fat load
**p-value for differences between treatment with simva 80 mg and simva/Eze 10/10 mg*
RLP-c: Plasma remnant-like particle cholesterol

Table 3 *Fasting and postprandial Insulin and IL-6 plasma concentrations*

	Before treatment		simvastatin 80 mg		After 6 weeks simva/eze 10 mg/10 mg	
	Interleukin-6 (pg/ml)	Insulin (mU/l)	Interleukin-6 (pg/ml)	Insulin (mU/l)	Interleukin-6 (pg/ml)	Insulin (mU/l)
fasting	1.36 ± 0.12	12.6 ± 1.4	1.89 ± 0.32	12.6 ± 1.5	1.58 ± 0.17	11.8 ± 1.3
3 hours after fat load	2.34 ± 0.30	12.0 ± 1.3	3.04 ± 0.70	15.2 ± 1.8	2.52± 0.45	14.9 ± 1.5
4 hours after fat load	2.96 ± 0.44	10.7 ± 1.0	3.41 ± 0.67	15.1 ± 2.0	2.87 ± 0.44	12.1 ± 1.4

All data: mean ± SEM

Fasting and post fat load endothelial function

Fasting FMD was comparable during both treatment periods (6.9%±0.8 versus 7.6%± 1.2) (**Table 4**). However, during simvastatin monotherapy FMD significantly decreased 4 hours after oral fat load ingestion (6.9%±0.8 versus 4.3%±0.6, p= 0.001), whereas no difference in FMD was observed after fat load during combination therapy (7.6%±1.2 versus 7.7%±1.6, p=0.8). Nitro-glycerine-induced endothelium-independent vasodilatation of the brachial artery was comparable between the two treatment periods both fasting, as well as after oral fat load.

Table 4 *(Postprandial) endothelial function according to both treatment regimes*

	After 6 weeks simva 80 mg (n=19)	After 6 weeks simva/eze 10/10 mg (n=19)
Before oral fat load		
Baseline diameter (mm)	5.54 ± 0.15	5.30 ± 0.18
Post-ischemic maximum diameter (mm)	5.91 ± 0.16	5.71 ± 0.19
Endothelial-dependent vasodilatation (%)	6.9 ± 0.8*	7.6† ± 1.2
Nitro-glycerine-induced maximum diameter (mm)	6.22 ± 0.17	5.97 ± 0.15
Nitro-glycerine-induced vasodilatation (%)	12.1 ± 1.1	13.4 ± 1.9
After oral fat load		
Baseline diameter (mm)	5.65 ± 0.17	5.37 ± 0.13
Post-ischemic diameter	5.88 ± 0.16	5.76 ± 0.12
Endothelial-dependent vasodilatation (%)	4.3 ± 0.6*	7.7† ± 1.6
Nitro-glycerine-induced maximum diameter (mm)	6.20 ± 0.18	6.05 ± 0.12
Nitro-glycerine-induced vasodilatation (%)	10.0 ± 1.4	13.1 ± 1.1

All data: mean ± SEM
**p-value 0.001 before versus after oral fat load*
† p-value 0.8 before versus after oral fat load

Discussion

In the present randomized double blind, crossover trial in male patients with the metabolic syndrome combination therapy of low-dose simvastatin and ezetimibe preserved post fat load endothelial function contrary to high dose simvastatin monotherapy, whereas the same reduction in fasting plasma LDL-cholesterol was obtained after 6 weeks treatment. Post fat load plasma triglyceride concentrations were higher during combination therapy compared to monotherapy but apoB concentrations were similar. The post fat load RLP-c concentration was the same after both treatments.
Ezetimibe decreases LDL-c levels by inhibition of uptake of dietary and biliary cholesterol by binding to the Niemann-Pick C1 Like 1 protein at the brush border membrane of enterocytes, a receptor involved in intestinal cholesterol uptake, thereby preventing dietary cholesterol uptake [20-21]. We initially hypothesized that postprandial lipid metabolism would benefit from treatment with a cholesterol uptake inhibitor like ezetimibe in combination with simvastatin compared to high-dose simvastatin. However, in the present study we did not observe differences in RLP-c, during both treatment regimes. In a recent report, no significant effects of ezetimibe on the postprandial kinetics of intestinally derived apoB 48-containing triglyceride-rich lipoprotein particles were observed. It was shown that ezetimibe treatment led to a reduction of plasma LDL-c by increasing the catabolism of hepatic derived apoB-100 containing lipoproteins without reducing chylomicron particle number [22].

Increased postprandial lipoprotein remnant levels are associated with an inflammatory response and with development of atherosclerosis [18,23]. In the present study, the inflammatory response to a fat load might have been different during both treatments resulting in differential effects on post fat load endothelial function. Low-grade inflammation (reflected by elevated concentrations of CRP) is associated with endothelial dysfunction [12] and it was previously shown that at each statin dose level, co administration of ezetimibe induced significantly more hs-CRP reduction compared to monotherapy [24]. In the present study plasma levels of IL-6 were marginally higher during treatment with high-dose simvastatin monotherapy compared to treatment with low-dose simvastatin combined with ezetimibe, also at the time of assessment of endothelial function 4 hours after fat load, although this difference was not statistically significant. Since hepatic CRP release is under influence of plasma levels of IL-6, increased levels of hs-CRP during treatment with simvastatin 80 mg monotherapy can also be expected [25]. After oral fat load, plasma insulin concentrations were higher during treatment with high-dose simvastatin monotherapy compared with combination therapy, most pronounced at the time of FMD measurement. Although insulin enhances eNOS transcription, hyperinsulinemia is associated with endothelial dysfunction by stimulating the release of the potent vasoconstrictor endothelin [12]. It has been shown that compared to placebo, the use of statins improved postprandial insulin sensitivity in patients with the metabolic syndrome [26]. However, the combination of statins with ezetimibe was not investigated. Although no differences were observed in post fat load RLP-c during both treatment regimes, it could be speculated that differences in post fat load lipoprotein composition due to the different treatment regimes lead to differences in postprandial endothelial function [27].

In patients with chronic heart failure 4 weeks of treatment with simvastatin10 mg improved endothelial function contrary to treatment with ezetimibe 10 mg monotherapy, despite a similar decrease in plasma LDL-c concentration [28]. Very recently, in a partially randomized trial in patients with stable coronary artery disease it was shown that both statins and ezetimibe effectively lowered LDL-c plasma concentrations, but only statin therapy was associated with improvement in endothelial function [29]. In both studies it was suggested that pleiotropic, LDL-c -independent effects of statins were involved (i.e. increased vascular nitric oxide bioavailability, reduced oxidant stress, improved endothelial progenitor cell function). Positive effects of LDL-cholesterol lowering on endothelial function have already been described in various studies, as well as endothelial dysfunction after an oral fat load [5-7,30]. In the present study we did not observe a difference in fasting endothelial function between treatment with simvastatin 80 mg and simvastatin 10 mg combined with ezetimibe 10 mg. We could not confirm the existence of pleiotropic effects of high-dose statins in our study cohort during 6 weeks treatment. In addition, combination therapy of low-dose simvastatin with ezetimibe preserved endothelial function after an oral fat load, contrary to high dose simvastatin monotherapy. Strength of our study was that all patients received both treatments in a crossover design, diminishing variation in endothelial function as often seen in studies with parallel groups. The existence of pleiotropic effects of statins can not completely be ruled out by these findings, since it is possible that beyond 10 mg simvastatin the maximal pleiotropic effects are already reached. The effects of

different lipid-lowering regimes on endothelial function in patients with the metabolic syndrome have been investigated in an open-label non-randomized comparison [31]. In that study, in a small number of metabolic syndrome patients combination therapy of atorvastatin 10 mg and ezetimibe 10 mg resulted in more reduction in serum total cholesterol and triglycerides concentrations and better endothelial function compared to atorvastatin 40 mg alone. In contrast to our study, plasma LDL-c reductions were not similar potentially leading to differences in endothelial function. Besides this, post fat load lipid profiles and endothelial function were not assessed.

We acknowledge some limitations of our study. Only male patients with the metabolic syndrome were studied. Therefore, caution should be taken to generalize these results to female patients. Carryover and crossover effects were not observed and are therefore unlikely to have influenced our results but could not be completely ruled out. Considering the elimination half-life of statins and ezetimibe and a washout period of 4 weeks plus 6 weeks treatment carryover effects are unlikely to have occurred. To assess post fat load endothelial function and lipid profiles a standardized, but non-physiological, high fat meal was used.

In conclusion, in male patients with the metabolic syndrome, 6 weeks treatment of low-dose simvastatin combined with ezetimibe preserved postprandial endothelial function contrary to high-dose simvastatin monotherapy, whereas no differences were observed in fasting FMD. Post fat load RLP-c concentration was similar between both treatment regimes. However, the clinical implications of these effects remain to be established.

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CHAPTER 9

Discussion
Summary
Nederlandse samenvatting
Dankwoord
Curriculum Vitae

Insulin resistance and low-grade chronic inflammation play essential roles in the development of endothelial dysfunction and atherosclerosis. Abdominal obesity, in combination with insulin resistance, is highly related to the presence and clustering of common vascular risk factors, e.g. hypertension, dyslipidemia, hyperglycemia, and to the presence of other, less well known, metabolic disturbances like increased inflammation, hyperinsulinemia and altered coagulation.

Etiological role of adipocyte dysfunction in development of type 2 diabetes and atherosclerosis

Research into the origin of the relationship between excessive adipose tissue and insulin resistance, type 2 diabetes and atherosclerosis points to alterations in paracrine and endocrine functions of adipocytes leading to a pro-atherogenic metabolic condition characterized by low grade inflammation and insulin resistance: adipocyte dysfunction (**chapter 2**). The concept of adipocyte dysfunction may improve our understanding of the pathophysiologal processes by which visceral adipose tissue contributes to the presence of vascular risk factors and by which it is involved in the development of insulin resistance and inflammation, locally in adipose tissue as well as in the liver, muscle tissue and the vasculature. In various *in vitro* studies and in animal models it has been shown that interference of intracellular signalling pathways by adipocytokines like interleukin-6, tumor necrosis factor-α, free fatty acids, leptin and adiponectin contribute to the development and maintenance of low-grade inflammation and insulin resistance typically seen in type 2 diabetes and atherosclerotic disease. However, adipose tissue characteristics and metabolic properties are different in rodents compared to humans and may even differ between the different adipose tissue compartments in the human body. The challenge for clinical research investigating adipocyte dysfunction is to translate the results of *in vitro* and animal studies into a patient setting.
Since the discovery of adiponectin an increasing number of studies have been performed to determine the relationship between adiponectin and the development of both type 2 diabetes and atherosclerotic disease. Anti-atherosclerotic and anti-diabetogenic properties (*in vitro*) of adiponectin include inhibition of the expression of vascular adhesion molecules (VCAM, ICAM) and increasing the local availability of NO. In macrophages, adiponectin suppresses TNF-α secretion and reduces the expression of the scavenger receptor resulting in less oxidized LDL-c uptake and less foam cell formation. In addition, hepatic gluconeogenesis is inhibited while fatty acid oxidation is stimulated, thereby ameliorating insulin resistance. Clinical studies show contrasting results. The study presented in this thesis (**chapter 4**), investigating the relationship between adiponectin plasma levels and future vascular events in patients with manifest vascular disease, showed that higher adiponectin concentrations were associated with an increased vascular risk compared to the lowest adiponectin levels. These results are supported by other studies in patients with coronary heart disease [1,2]. Recently a laboratory technique is available to measure adiponectin isoforms. Different isoforms of adiponectin are known to circulate in plasma together forming total adiponectin levels. It is not yet clear what the metabolic properties are of these different isoforms.

It has been postulated that high molecular weight adiponectin and not total adiponectin plasma levels are most closely related to insulin resistance and metabolic syndrome. Even though the presence of insulin resistance and metabolic syndrome are associated with lower levels of adiponectin, it is not yet known which isoform predominantly circulates, in absolute levels or in ratio to others isoforms, in healthy subjects or in patients with manifest vascular disease. However, this might not completely explain the inverse relationship. Sequestering of adiponectin at the site of vascular damage may contribute to lower plasma levels of adiponectin thereby reducing the extent of further vascular damage, however this should be tested *in vivo* [3].

Another aspect in adipocyte dysfunction research is the close interaction between adipocytes and macrophages in the initiation and continuation of chronic low-grade inflammation. Cells from the immune system and adipocytes are evolutionary closely linked as they are proposed to have evolved from the same 'fat body' which is found in insects [4]. There also seems to be a remarkable resemblance between adipocytes in early stages of development and monocytes/macrophages. Both cell types have the ability for phagocytosis, both can secrete pro-inflammatory cytokines (TNF-α, IL-6), can display markers of macrophages (matrix metalloproteinases) and share a number of important genes (PPAR-γ, fatty acid binding protein). It is hypothesised that dysfunctional adipose tissue develops when hypertrophic adipocytes (due to hyperalimentation) attract monocytes by chemotaxis to adipose tissue resulting in inflammatory adipose tissue. There is also a remarkable similarity between adipose tissue and the vascular wall considering local macrophage infiltration and lipid accumulation and similar pathophysiological processes (e.g. inflammation) are active in these tissues. Understanding the cross-talk between monocytes/macrophages and adipocytes in both adipose tissue and vasculature may give directions for future drug development in order to prevent the vascular consequences of adipocyte dysfunction.

How to identify patients with adipocyte dysfunction?

Establishing the presence or the degree of adipocyte dysfunction in clinical practice may be relevant since this condition is associated with a clustering of known vascular risk factors (e.g. hypertension, dyslipidemia, hyperglycemia), and risk factors not often measured in routine clinical practice (e.g. low-grade inflammation, a pro-coagulant condition and postprandial dyslipidemia). Therefore it can be related to an increased vascular risk and risk for the development of diabetes mellitus. However, determining plasma adiponectin, TNF-α, IL-6 levels and assessing the level of insulin resistance by clamp technique is generally only available in research settings. Interpretation of the results in individual patients is difficult since reference values and normal ranges for various patient populations are yet unknown. For clinical practice metabolic syndrome, which is the result of insulin resistance, abdominal obesity and an altered adipocyte metabolism, may be a useful tool to determine the presence of adipocyte dysfunction and insulin resistance. Presence of metabolic syndrome, as a reflection of underlying adipocyte dysfunction, is associated with an increased vascular risk in healthy subjects and in patients with already established cardiovascular disease [5]. More stringent vascular

risk factor treatment with a focus on weight reduction and increasing physical activity is therefore needed. Several studies have shown that physical training leads to enhanced insulin sensitivity in previously sedentary adults [6], and that short-term lifestyle changes augment adiponectin levels [7]. In addition to lifestyle changes, it has been shown that patients with coronary heart disease and metabolic syndrome have incremental benefit from aggressive LDL-c lowering compared to moderate LDL-c reduction [5]. In American treatment guidelines, presence of metabolic syndrome is now explicitly mentioned as one of the risk factors that favour a decision to reduce LDL-c levels below 1.8 mmol/l (if feasible) in already high-risk patients [8]. For HDL-c no specific treatment targets are described. In the European cardiovascular disease guideline HDL-c <1.0 mmol/l in men and <1.2 mmol/l in women only serve as a marker of increased cardiovascular risk, reflecting a detrimental metabolic condition, even though low HDL-c levels are a strong risk factor for vascular events (**chapter 5**).

Dyslipidemia in the context of adipocyte dysfunction

Due to hypertrophic dysfunctional adipocytes the net free fatty acids flux from visceral adipose tissue to the liver is increased resulting in hypersecretion of ApoB100 containing lipoproteins by the liver (VLDL). In addition, triglyceride hydrolysis by lipoprotein lipase is reduced in insulin resistant conditions. During the postprandial phase plasma triglyceride concentrations are further increased due to enhanced assembly of intestinal derived lipoproteins. Hypertriglyceridemia contributes to the formation of small dense LDL particles and low HDL-c plasma concentration, while total LDL-c levels generally remain within the normal range. Based on the results presented in this thesis (**chapter 6**) we propose a direct relationship between dysfunctional adipocytes and HDL-c metabolism during the postprandial phase due to the ability of adipocytes to secrete cholesteryl ester transfer protein. Whether lower postprandial HDL-c plasma concentrations are associated with a higher cardiovascular risk remains to be established. Non-fasting triglyceride plasma concentrations appear to be a better predictor of future cardiovascular events than fasting triglyceride concentrations [9, 10]. Given the close interaction between triglyceride metabolism and HDL-c metabolism it can be speculated that this observation is due to low postprandial HDL-c plasma concentrations.

In addition, by influencing postprandial plasma lipid concentrations and metabolism through the use of statins and/or cholesterol absorption inhibition the cardiovascular risk may be reduced. Especially patients with metabolic syndrome and/or insulin resistance, who are exposed to higher levels of atherogenic lipoproteins during the postprandial phase may benefit from additional therapy specifically for postprandial dyslipidemia. Triglyceride-rich remnants and small dense LDL particles are highly atherogenic by inducing endothelial dysfunction and penetrating the sub endothelial space contributing to the formation of foam cells. In accordance, the absolute number of small dense LDL particles has been shown to be a better predictor of coronary heart disease than plasma LDL-c levels [11]. In this thesis, studies are presented showing that lipid-lowering with high-dose statin or the combination of low-dose statin in combination

with ezetimibe, a cholesterol uptake inhibitor, have similar effects on postprandial lipid metabolism (**chapter 6** and **7**). Both treatments do not affect the increase in the plasma concentrations of atherogenic lipoproteins after an oral fat load in metabolic syndrome patients or modify the lipid content. Non-pharmaceutical improvement of dyslipidemia may be more effective. Weight loss is not only associated with a decrease in insulin and fasting triglyceride levels, the acute response to a fat load was also less intense after weight reduction in formerly obese women [12]. Although both treatment options are equally effective in lipoprotein reduction, it remains to be determined whether non-lipid effects, often called pleiotrophic effects, are relevant in cardiovascular disease prevention. One of these proposed anti-atherosclerotic effects involves, next to stability of atherosclerotic plaques, decrease of oxidative stress and inflammation, the non-lipid dependent improvement of endothelial function [13-15]. Based on results presented in this thesis (**chapter 8**) it seems questionable whether pleiotropic effects of statins are relevant in vascular risk reduction since endothelial function and IL-6 plasma levels did not benefit from either low-dose or high-dose treatment.

Can adipocyte dysfunction be treated?

Since adipocyte dysfunction is likely to relate to an increased risk for development of type 2 diabetes and/or (recurrent) vascular events, diagnosis and treatment of this condition comes into focus.
Increasing physical activity and weight reduction are two important lifestyle changes to reduce visceral obesity and insulin resistance and are therefore central in modifying adipocyte dysfunction. After 12 weeks of restricted caloric intake and increased exercise TNF-α, leptin and IL-6 levels decreased while anti-inflammatory cytokines (adiponectin and IL-10) were significantly increased in obese subjects with metabolic risk factors [16]. Even before weight reduction is obtained, insulin sensitivity is improved and adiponectin plasma levels increase, indicating improved adipocyte function [17,18]. In addition, exercise may enhance lipoprotein lipase activity thereby countering (postprandial) dyslipidemia [19]. However, changes in lifestyle are difficult to achieve and maintain in clinical practice, but are required for a constant improvement of a patients metabolic profile [20]. Statins have anti-inflammatory properties which may point towards an influence of statins on adipocyte function [21]. Nevertheless, short-term treatment with simvastatin and ezetimibe alone or in combination did not result in changes in adiponectin, leptin and resistin levels in healthy subjects [22]. Also lower levels of adiponectin have been observed after simvastatin treatment in patients with manifest vascular diseases [23]. In large randomized statin trials a lower incidence of type 2 diabetes has been observed in patients on a statin compared to the placebo group [24]. It could be hypothesized that statins affect insulin sensitivity through anti-inflammatory effects. In patients with metabolic syndrome insulin sensitivity and adiponectin levels were however not improved after 9 weeks of simvastatin treatment [25]. In our studies, presented in this thesis we did not observe an increase in plasma adiponectin or a decrease in IL-6 plasma concentrations by statin treatment in patients with metabolic syndrome.

Similarly, ACE-inhibitors (ramipril) and angiotensin receptor antagonists (telmisartan) may also have anti-diabetic effects [26,27,28].

In vitro studies suggest that these compounds have peroxisome-proliferator-activated receptor-γ (PPAR-γ) agonistic properties stimulating adiponectin secretion, however it is not yet clear whether this is a class effect or a drug dependent effect [29].

Fibrates lower triglyceride levels and increase HDL-c concentrations, thereby improving the consequences of adipocyte dysfunction on lipid metabolism, particular in insulin resistant subjects. Fibrates activate the transcription factor peroxisome proliferator-activated receptors-α (PPAR-α) leading to induction of apoA-I and apoA-II synthesis. In addition, fibrates stimulate cellular fatty acid uptake, and β-oxidation pathways, which, combined with a reduction in fatty acid and triglyceride synthesis, results in a decrease in VLDL production [30]. Although PPAR-α is predominantly expressed in liver, kidney, heart, and muscle recent research shows that PPAR-α may also be involved in adipocyte gene regulation specific for fatty acid metabolism [15].

Thiazolidinediones (TZDs) are ligands of the nuclear transcription factor, PPAR-γ and have been shown to regulate the expression of numerous genes affecting glycemic control, lipid metabolism and inflammation. TZDs improve insulin sensitivity in type 2 diabetes patients and are proposed to have favourable effects on other metabolic alterations seen in metabolic syndrome and adipocyte dysfunction. PPAR-γ activation stimulates adipocyte differentiation leading to smaller and more insulin sensitive adipocytes, in particular in subcutaneous fat depots. Furthermore; TNF-α, IL-6, CRP plasma levels are decreased and adiponectin plasma levels are increased after TZD treatment in obese and diabetic patients [31, 32]. A similar reduction in cytokine expression was observed in macrophages after incubation with TZD *in vitro*. Although currently available TZDs (rosiglitazone and pioglitazone) have similar effects on insulin resistance there are differential effects on the occurrence of cardiovascular events and carotid artery intima-media thickening in patients with type 2 diabetes [33, 34].

In patients with abdominal obesity there is evidence that the endocannabinoid system is overactive leading to increased food intake and weight gain [35,36]. Rimonabant is an antagonist of the endocannabinoid-1 receptor (CB1), a receptor present on cells of the central nervous system, liver and in adipocytes. Treatment with rimonabant leads to reduction in body weight and waist circumference, 15% reduction in plasma triglycerides and in 25% increased HDL-c levels [37]. Adiponectin, as a marker of adipocyte dysfunction, increased 58% compared to start of treatment. This increase may not only be explained by changes in body weight, leaving the suggestion that direct CB1 receptor blockade in adipocytes results in changes in adipocytes function [37].

Adipocyte dysfunction is not (yet) a primary treatment target in current cardiovascular prevention guidelines, but can be influenced by drugs that are commonly used in patients at elevated risk for development of cardiovascular diseases. The most effective intervention available that beneficially affects adipocyte dysfunction is weight reduction and increasing physical activity.

Final remarks

In daily clinical practice, the concept of adipocyte dysfunction may provide a pathophysiological framework for understanding the clustering of cardiovascular risk in close relation to abdominal obesity and insulin resistance in individual patients. The concept of adipocyte dysfunction may raise awareness among patients and physicians on the importance of abdominal obesity in vascular risk and risk for developing type 2 diabetes. Also, the construct of adipocyte dysfunction may provide targets for drug development in the future. Metabolic syndrome can be a useful tool to identify patients with adipocytes dysfunction benefiting from treatment of abdominal obesity and vascular risk factor reduction.

In conclusion, studies presented in this thesis showed that:

- Patients with metabolic syndrome have elevated plasma concentrations of homocysteine but these higher levels are not associated with an increased vascular risk
- Low plasma concentrations of adiponectin are associated with a low vascular risk in patients with clinical evident vascular disease
- In patients with manifest vascular disease, low plasma HDL-c levels confer an increased risk for recurrent vascular events, irrespective of the localisation of the disease and LDL-c levels, and independent of the use of lipid-lowering therapy
- In obese metabolic syndrome subjects HDL-c plasma concentrations decrease after fat loading due to increased cholesteryl ester transfer
- High-dose simvastatin and low dose simvastatin in combination with ezetimibe are equally effective in reducing postprandial lipoprotein concentrations and composition but show no effects on top of reduced fasting lipoprotein concentrations
- Endothelial function during fat load is preserved by the combination of low-dose simvastatin and ezetimibe in contrast to high-dose simvastatin monotherapy in obese metabolic syndrome subjects despite similar reductions in LDL-c plasma levels

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CHAPTER

Cardiovascular disease is the most common cause of morbidity and mortality in Western populations. Different mechanisms have been postulated that link obesity as a vascular risk factor to the development of metabolic syndrome, type 2 diabetes, endothelial dysfunction and atherosclerotic vascular disease. The clustering of elevated blood pressure, (postprandial) dyslipidemia and insulin resistance is frequently associated with obesity. In addition, adipose tissue in concordance with insulin resistance also induces a pro-inflammatory and pro-atherogenic condition through the secretion of large quantities of adipocytokines that influence lipid and carbohydrate metabolism, inflammatory and coagulation pathways and directly affect atherosclerosis.
The work presented in this thesis focused on 1] the relationship between metabolic risk factors associated with insulin resistance and adipocyte dysfunction and the occurrence of new cardiovascular events in patients with clinical manifest vascular disease, 2] the evaluation of the effect of lipid lowering therapy on postprandial lipid metabolism and endothelial function in obese patients with metabolic syndrome.

In **chapter 2** of this thesis the concept of adipocyte dysfunction and its relationship with obesity, type 2 diabetes and atherosclerotic vascular disease is presented. The concept of adipocyte dysfunction, a condition of hypersecretion of pro-atherogenic, pro-inflammatory and pro-diabetic adipocytokines which is accompanied by a decreased production of adiponectin, improves our understanding of the underlying pathophysiology by which visceral adipose tissue, in its unique position to the liver, is linked to the clustering of risk factors next to insulin resistance, endothelial dysfunction and atherosclerosis. Based on the potential anti-atherosclerotic properties of adiponectin on the vascular endothelium, and to a lesser degree on fatty acid and glucose metabolism, we evaluated the hypothesis that lower adiponectin plasma levels would also confer an increased cardiovascular risk in a population of high-risk patients (**chapter 4**). In our population of patients with clinically manifest vascular disease lower adiponectin plasma levels were associated with a decreased risk for vascular events, even though the presence of metabolic syndrome was associated with lower adiponectin plasma levels; harzard ratio for the lowest adiponectin plasma levels versus the highest quartile 0.50, 95%CI 0.25 - 0.99.
Patients with metabolic syndrome are at increased risk for cardiovascular disease due to the presence of insulin resistance-associated risk factors. It is unclear whether other metabolic disturbances which are not part of the definition also contribute to this observed risk. The debate whether hyperhomocysteinemia is also causally related to a higher cardiovascular morbidity and mortality is still ongoing and is kept alive by contradicting study results. Since elevated levels of homocysteine might be both the cause and the result of insulin resistance we determined the relationship between homocysteine plasma levels and metabolic syndrome (**chapter 3**). In addition, we evaluated whether higher levels of homocysteine were associated with a higher cardiovascular risk in patients with clinical evident vascular disease. Plasma levels of homocysteine increased with the number of components present. In patients with metabolic syndrome higher homocysteine plasma levels were not associated with an even higher vascular risk. In contrast, in patients without metabolic syndrome higher homocysteine plasma levels marked an increased cardiovascular risk.

Patients at high cardiovascular risk should intensively be treated according to recent multi-disciplinary guidelines. Nevertheless, despite widespread use of anti-hypertensive and LDL-c lowering medication a substantial residual risk remains in these patients. So far, only the effect of low HDL-c levels on vascular outcome was determined in healthy subjects and coronary artery disease patients without treatment aiming at vascular risk reduction. In **chapter 5** we evaluated whether low HDL-c levels are also a vascular risk factor for the development of cardiovascular events in treated, high-risk patients. We have shown that compared to the lowest quintile, the upper quintile of HDL-c levels was associated with a lower risk for new events; HR 0.61 (95%CI 0.43-0.86) irrespective of the localisation of vascular disease. In addition, higher HDL-c levels are related to a reduced vascular risk in patients with LDL-c levels below target (2.5 mmol/l) independently of the use of lipid-lowering therapy.

In insulin resistant and metabolic syndrome subjects postprandial dyslipidemia is characterized by the prolonged presence of ApoB containing lipoproteins in the circulation, which can be regarded as a risk factor for endothelial dysfunction and atherosclerosis.
Less well characterized is the postprandial metabolism of HDL particles, especially in insulin resistant and obese patients. In **chapter 6** we determined HDL-c levels in a model of acute hypertriglyceridemia in obese metabolic syndrome patients. HDL-c levels decreased 11% as cholesteryl ester transfer increased with 25% after fat loading in obese metabolic syndrome patients. We hypothesize that adipocyte function directly affects HDL-c metabolism since cholesteryl ester transfer protein mass increased (16%) while adiponectin levels decreased during fat loading and both are produced in adipocytes. Decreased postprandial HDL-c levels may contribute to the increased cardiovascular risk in metabolic syndrome patients on top of already low HDL-c levels. This is not influenced by either simvastatin or simvastatin/ezetimibe treatment.

Due to the prolonged presence of elevated levels of triglyceride-rich lipoproteins and additional modification of the lipid content by lipoprotein lipase, highly atherogenic small dense LDL particles are formed. Primary and secondary risk prevention is mainly focused on lowering of fasting LDL-c levels by either inhibiting cholesterol synthesis (statin therapy), inhibition of intestinal cholesterol absorption (ezetimibe) or by a combination of these two. Whether additional risk reduction can be achieved by preventing the development of this atherogenic postprandial lipoprotein profile by using ezetimibe in addition to a statin was unknown. In **chapter 7** we determined lipoprotein particle composition during oral fat loading in obese metabolic syndrome patients without treatment and after treatment with both simvastatin 80mg and simvastatin/ezetimibe 10mg/10mg. No difference between either treatment was observed in lipoprotein composition. Both regimes only affected fasting lipid levels, without changing the postprandial metabolism.

Postprandial hyperlipidemia can induce endothelial dysfunction and can clinically be assessed by measuring the vasodilative response of the endothelium to shear stress with flow mediated dilation. Cholesterol absorption may lower postprandial cholesterol levels

and thereby limiting the appalling effects of postprandial dyslipidemia on endothelial function. In **chapter 8** we compared the effects of high-dose simvastatin treatment to the combination of low-dose simvastatin with ezetimibe on endothelial function in a model of acute hypertriglyceridemia in obese metabolic syndrome patients. Although no differences were observed between the postprandial plasma lipid levels after both treatment periods, the post fat load endothelial function was best preserved after combination therapy.

CHAPTER

Introductie

Hart- en vaatziekten ten gevolge van atherosclerose (slagaderverkalking) komen frequent voor in de Westerse samenleving en vormen daarmee de belangrijkste doodsoorzaak. Atherosclerotische vaatvernauwingen kunnen gelijktijdig optreden in perifere (aorta of benen), cerebrale (hersenen) en/of coronair (kransslagaders) arteriën en geven pas in een laat stadium, als de bloedstroom geobstrueerd wordt, klinische klachten.
Bekende risicofactoren voor het ontwikkelen van atherosclerose zijn een verhoogde bloeddruk, roken, dislipidemie (een verhoogd LDL-cholesterol en een verlaagd HDL-cholesterol), type 2 diabetes en obesitas (overgewicht). Cholesterol wordt in het lichaam gebruikt als bouwsteen voor cellen en als substraat voor hormonen. Triglyceriden zijn ook bloedvetten, opgebouwd uit vetzuren die met name afkomstig zijn uit voeding en worden opgeslagen in adipocyten (vetcellen). Hoewel cholesterol dus noodzakelijk is voor normale fysiologie, is een toename van LDL-cholesterol, 'slecht cholesterol', gerelateerd aan een verhoogd risico op hart- en vaatziekten omdat LDL-c in de vaatwand wordt opgeslagen. HDL-c wordt gezien als 'goed cholesterol' vanwege zijn anti-atherosclerotisch eigenschappen waaronder het transport van cholesterol van perifere weefsels, inclusief de vaatwand, terug naar de lever.
Naast de bekende risicofactoren dragen ook andere, minder bekende factoren zoals een chronische ontstekingsreactie van de vaatwand en insulineresistentie, bij aan het ontstaan van atherosclerose, en zijn onderwerp van huidig onderzoek. Insulineresistentie is een fenomeen dat optreedt indien lichaamscellen, zoals spier- en vetcellen, minder gevoelig worden voor de fysiologische werking van insuline met als gevolg een verminderde opname van glucose in cellen.
Manifeste vaatlijders, patiënten die reeds een 'event' hebben gehad (myocard- of herseninfarct of een obstructie van een perifere arterie) vormen een hoogrisicogroep voor het ontwikkelen van een nieuw event, op dezelfde locatie of ergens anders in het lichaam.

Obesitas, metabool syndroom en risicofactoren.

Obesitas is geassocieerd met het gelijktijdig optreden van een karakteristieke combinatie van metabole risicofactoren. De combinatie obesitas, hypertensie, lage HDL-c en hoge triglyceride concentraties is ook bekend onder de verzamelnaam "metabool syndroom" en is geassocieerd met een verhoogd risico op type 2 diabetes en atherosclerotische hart- en vaatziekten (**Tabel 1**). Hierbij moet de kanttekening gemaakt worden dat het met name mensen betreft die lijden aan abdominaal overgewicht, in tegenstelling tot mensen die vet op de heupen hebben: appels versus peren. Hoewel de oorzaak achter deze clustering van risicofactoren niet geheel bekend is, wordt aangenomen dat insulineresistentie en obesitas hierin een belangrijke rol spelen. Daarnaast vervalt door insulineresistentie de rem die ervoor zorgt dat vetzuren in adipocyten opgeslagen blijven. Als gevolg hiervan neemt de aanvoer van vetzuren van adipocyten naar de lever toe waardoor deze meer triglyceriden gaat maken.

Tabel 1. *Criteria van het metabole syndroom.*

Variabele	Drie van de vijf variabelen, ongeacht welke combinatie	
Lichaamsgewicht	♂ buikomvang > 102cm	
	♀ buikomvang > 88cm	
Lipiden	Triglyceriden ≥1.70 mmol/l	
	HDL-c	< 1.04mmol/l (♂)
	of	< 1.29mmol/l (♀)
Bloeddruk	≥ 135/85mmHg	
	en/of het gebruik van medicatie	
Glucose	Nuchter glucose ≥ 6.1mmol/l	
	en/of het gebruik van medicatie	

Adipocyten en metabool syndroom

Overgewicht is het resultaat van een volumetoename van adipocyten. Adipocyten slaan niet alleen triglyceriden op om dit vrij te geven in tijden van energietekort, maar produceren ook tientallen hormonen: "adipocytokines". Sommige adipocytokines zijn betrokken in glucose- en vetmetabolisme, andere zijn onderdeel van de chronisch ontstekingsreactie die geactiveerd is bij artherosclotische ziekten. Adiponectine is een typisch voorbeeld van een adipocytokine omdat het uitsluitend geproduceerd wordt door adipocyten en heeft potentieel anti-atherosclotische eigenschappen zoals aangetoond in dier-expermenteel en *in vitro* onderzoek. Andere voorbeelden van adipocytokines zijn tumor necrosis factor-α, dat betrokken is bij de chronische ontsteking van de vaatwand en leptine dat onderdeel is van de honger-eetregulatie. Indien adipocyten groter worden neemt de secretie van adipocytokines toe maar die van adiponectine juist af.

In **hoofdstuk 2** van dit proefschrift wordt het concept adipocyte disfunctie (vetcel disfunctie) gepresenteerd en wordt de relatie tussen disfunctionele adipocyten en het ontstaan van obesitas, insulineresistentie, type 2 diabetes en atherosclerotische hart- en vaatziekten beschreven. Het concept 'adipocyte disfunctie' is gedefinieerd als een conditie waarin adipocyten een verhoogde secretie vertonen van adipocytokines die kunnen bijdragen aan het ontstaan van atherosclerose en type 2 diabetes in combinatie met een afname van adiponectine. Deze benadering vergroot de kennis en begrip van de onderliggende pathologie achter de geclusterde risicofactoren, insulineresistentie en atherosclerose.
Met de potentiële anti-atherosclerotische en anti-diabetogene eigenschappen van adiponectine op vaatwand, vetzuur en glucose metabolismen als fundament, werd de

hypothese getoetst dat lage adiponectine plasma concentraties gerelateerd zijn aan een verhoogd vasculair risico in vergelijking met hoge adiponectine concentraties bij hoogrisico patiënten (**hoofdstuk 4**). In onze populatie van manifeste vaatlijders zijn lagere concentraties van adiponectine opmerkelijk genoeg geassocieerd met een 50% lager risico voor nieuwe events in vergelijking tot hogere adiponectine concentraties. Dit ondanks het feit dat de aanwezigheid van metabool syndroom, wat een risicofactor op zichzelf is, gerelateerd is aan lagere adiponectine plasma concentraties.
Metabool syndroom patiënten hebben een verhoogd risico op een vasculair event ten gevolge van de aanwezigheid van insulineresistentie geassocieerde risicofactoren. Het is echter niet duidelijk of risicofactoren die geen deel uitmaken van de definitie ook bijdragen aan dit verhoogde risico.
Al jaren is er discussie over de vraag of verhoogde homocysteïne concentraties, een bouwsteen voor eiwitten, bijdragen aan een verhoogd vasculair risico. Omdat hyperhomocysteïnemie zowel het gevolg als de oorzaak kan zijn van insulineresistentie is de relatie onderzocht tussen homocysteïne en metabool syndroom (**hoofdstuk 3**). De plasma concentratie homocysteïne neemt inderdaad toe naarmate patiënten meer componenten van het metabool syndroom hebben. Bij patiënten met zowel manifest vaatlijden als metabool syndroom zijn hogere homocysteïne concentraties echter niet geassocieerd met een hoger risico naast het risico van metabool syndroom. Dit in tegenstelling tot patiënten met manifest vaatlijden zonder metabool syndroom waarbij hogere homocysteïne concentraties wel geassocieerd zijn met een hoger vasculair risico.

Patiënten met een verhoogd risico voor hart- en vaatziekten moeten behandeld worden volgens actuele internationale multidisciplinaire richtlijnen met als doel het verlagen van de kans op (nieuwe) events. Echter, ondanks het veelvuldig gebruik van bloeddruk- en LDL-c verlagende medicatie is er een groot recidief percentage in manifest vaatlijders. Tot nu toe is de relatie tussen HDL-c en toekomstige events alleen onderzocht in niet-behandelde populaties zonder manifest vaatlijden. In **hoofdstuk 5** is onderzocht of een lage HDL-c concentratie ook een risicofactor is op het ontwikkelen van een nieuw vasculair event bij behandelde manifeste vaatlijders en het resterende risico bij deze behandelde patiënten (deels) kan verklaren. In de beschreven studiepopulatie hadden patiënten met de hoogste HDL-c concentraties een 39% lager risico op een nieuw event in vergelijking tot patiënten met de laagste HDL-c waardes, ongeacht de locatie van de eerste atherosclerotische aandoening (hart, hersenen of perifere arteriën). Bij patiënten die al lage LDL-c waardes hebben zijn hogere HDL-c waardes nog steeds geassocieerd met een verlaagd vasculair risico, onafhankelijk van gebruik van cholesterolverlagende middelen.

Bij patiënten met insulineresistentie en metabool syndroom is er niet alleen sprake van dislipidemie onder nuchtere condities. Ook niet-nuchtere (postprandiaal) concentraties zijn afwijkend, gekenmerkt door verhoogde concentraties van triglyceriderijke lipoproteïnen. Lipoproteïnen zijn de deeltjes waarin cholesterol en triglyceriden door het bloed vervoerd worden, maar bevatten ook eiwitten die belangrijk zijn voor herkenning door lichaamscellen. Een verhoogde concentratie van triglyceriderijke deeltjes tijdens

de postprandiale fase is een sterke risicofactor voor het ontwikkelen van atherosclerose. Dit in combinatie met het gegeven dat mensen door hun frequente eetpatroon vrijwel altijd in een postprandiale toestand zijn, maakt het postprandiale lipidenmetabolisme erg belangrijk en een therapeutische optie voor vasculaire risicoreductie. Ten gevolge van insulineresistentie worden lipoproteïnen slechter afgebroken en opgenomen in adipocyten. Doordat deze deeltjes langer in de bloedbaan blijven, wisselen zij onderling inhoud uit met andere cholesteroldeeltjes. De deeltjes die zo ontstaan, zijn gerelateerd aan progressie van atherosclerose. In **hoofdstuk 7** wordt de samenstelling van verschillende lipoproteïnen beschreven gedurende een vetbelasting, zonder behandeling en na twee keer zes weken behandeling met simvastatine 80mg en simvastatine/ezetimibe 10mg/10mg. Na twee keer zes weken waren er geen verschillen in de samenstelling van de verschillende soorten lipoproteïnen. Beide behandelstrategieën verlaagden alleen de nuchtere cholesterol waardes, maar hadden geen gunstig effect op de postprandiale toename van het aantal lipidendeeltjes.

Er is weinig bekend over postprandiaal HDL-c metabolisme in het algemeen en in het bijzonder bij patiënten met metabool syndroom. In **hoofdstuk 6** is onderzocht wat het effect is van een triglyceridenrijke maaltijd op HDL-c concentraties bij patiënten met metabool syndroom en wat de invloed van cholesterolverlagende medicatie hierop is. Vier uur na de start van het experiment waren de HDL-c waardes 11% gedaald. Deze daling kan gedeeltelijk worden verklaard door een toegenomen secretie in obese patiënten van de adipocytokine: 'cholesteryl ester transfer protein'. Deze zorgt voor een daling in HDL-c concentraties. Omdat lage HDL-c concentraties een risicofactor zijn, kunnen nog lagere postprandiale HDL-c concentraties bijdragen aan het verhoogde cardiovasculaire risico bij patiënten met metabool syndroom. Cholesterolverlagende medicatie had geen effect op de geobserveerde daling.
Endotheeldisfunctie, een verminderd relaxerend vermogen van de vaatwand, is een vroeg stadium in de ontwikkeling van atherosclerose en kan geïnduceerd worden door de aanwezigheid van triglyceriderijke lipoproteïnen. Mogelijk kan remming van de cholesterolabsorptie in de darm bijdragen aan een lagere concentratie van deze deeltjes in de postprandiale fase en daarbij leiden tot een betere vaatfunctie.
In **hoofdstuk 8** worden de mogelijke verschillen tussen twee behandelingen (simvastatine 80 mg versus simvastatine 10mg gecombineerd met ezetimibe 10mg) onderzocht op endotheelfunctie door middel van echografisch vaatonderzoek tijdens de postprandiale fase bij patiënten met metabool syndroom. Hoewel er geen verschillen waren in de postprandiale lipoproteïneconcentraties tussen de twee behandelingen, was de endotheelfunctie het best behouden na combinatie therapie.

Conclusies:

- Homocysteïne concentraties zijn hoger bij patiënten met metabool syndroom, maar zijn niet geassocieerd met een hoger vasculair risico.
- Lagere plasma concentraties adiponectine zijn geassocieerd met een lager vasculair risico bij patiënten met manifest vaatlijden.
- Bij patiënten met manifest vaatlijden zijn lage plasma HDL-c concentraties een risicofactor voor het optreden van nieuwe vasculaire events, ongeacht de locatie van de aandoening en onafhankelijk van LDL-c waarden of het gebruik van cholesterolverlagende medicatie.
- Bij obese patiënten met metabool syndroom dalen HDL-c plasma concentraties na vetbelasting, ten dele het gevolg van adipocytedisfunctie.
- Een hoge dosering simvastatine of een lage dosering simvastatine in combinatie met ezetimibe is even effectief in het reduceren van nuchtere lipoproteïne-concentraties en samenstelling maar hebben geen additief reducerend vermogen op nuchtere cholesterolconcentraties.
- De endotheelfunctie wordt bij patiënten met metabool syndroom tijdens vetbelasting beter gespaard door de combinatie van simvastatine en ezetimibe dan door een behandeling met alleen hoge dosering simvastatine, ondanks gelijke reducties in LDL-cholesterol.

CHAPTER

Tot slot waarschijnlijk het meest gelezen deel van dit proefschrift. Het cliché is echter zeker waar: promoveren is geen solo-prestatie. Dus in niet-geheel willekeurige volgorde:

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CHAPTER

Gideon Robertus Hajer was born on the second of June, 1978, Utrecht, the Netherlands. After graduation in 1997 from secondary school (De Breul, Zeist), he studied Fundamental BioMedical Sciences at the University of Utrecht. After two years he was able to switch to Medical School at the same University. As a medical student he performed a research project about the diagnostic criteria for coeliac disease in children under the age of two years old at the Wilhelmina Children's Hospital Utrecht. During his final year he participated in a research project concerning postprandial endothelial function and hyperlipidemia in patients with metabolic syndrome under supervision of Dr. F.L.J.Visseren at the department of Vascular Medicine. In August 2005 he obtained his Medical Degree and started the research described in this thesis at the department of Vascular Medicine of the University Medical Center Utrecht, again under supervision of Dr. F.L.J. Visseren and Prof. dr. Y. van der Graaf (Department of Clinical Epidemiology). On the 1st of November 2007 he started his clinical career as a resident at the department of Internal Medicine at "Ziekenhuis De Gelderse Vallei" in Ede.
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