Vascular risk of lipid genotype and phenotype in patients with arterial disease

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Vascular risk of lipid genotype and phenotype in patients with arterial disease

Vasculair risico van genotype en fenotype van plasma lipiden in patiënten met arterieel vaatlijden
(met een samenvatting in het Nederlands)

Proefschrift

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Contents

Chapter 1

General introduction

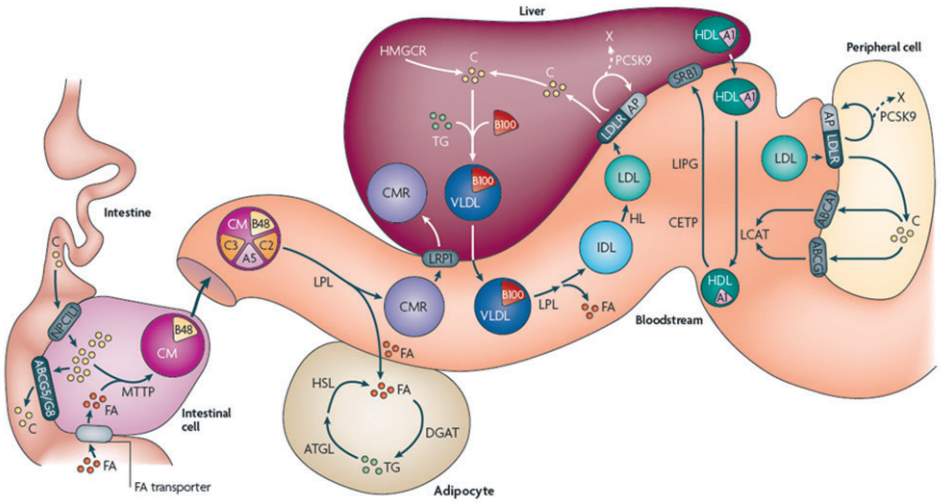
1

Plasma lipids: a (historical) overview

The first research into plasma lipid levels dates back to the 18th century. Albrecht von Haller described aorta plaques in 1755 and noted that a yellow mush effused between the muscular fibre and the intima of the vascular wall(1). Cholesterol was given its name in 1816, and was subsequently shown to be present in blood and in the atherosclerotic plaques(1). In the 1920s, researchers found that patients who encountered a myocardial infarction had higher mean serum cholesterol levels than controls(1). Since then, the knowledge about plasma lipids has increased enormously.

The two most relevant plasma lipids are cholesterol and triglycerides. These lipids are transported in lipoproteins with a hydrophilic coat to facilitate solubility in the plasma(2)(see Figure 1). Lipoproteins can be segregated into several subfractions based on density, varying from large chylomicrons and very low density lipoprotein

Figure 1. Overview of lipoprotein metabolism



In the intestine, cholesterol (C) and fatty acids (FA) are taken up and chylomicrons (CM) are formed, with the apolipoprotein B48 (B48). After action of lipoprotein lipase (LPL), chylomicron remnants (CMR) remain. In the liver, very low density lipoprotein (VLDL) particles are produced, these include the apolipoprotein B100 (B100). Action of LPL results in intermediate density lipoprotein (IDL), after which hepatic lipase (HL) hydrolyses it to low density lipoprotein (LDL). In the liver, also nascent HDL particles are produced, which include the apolipoprotein A1 (A1).
For a further discussion of the mechanisms shown in this figure see the main text or the explanation in reference 2.
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(VLDL) particles transporting mainly triglycerides to low density lipoprotein (LDL) and high density lipoprotein (HDL) transporting mainly cholesterol(3). Chylomicrons are produced in the intestinal wall to transport the triglycerides derived from dietary fats to their destination in the body. The apolipoproteins in chylomicrons include apolipoprotein C2 (apoC2), apoC3 and apoA5 and most importantly apoB48, the structural protein of chylomicrons(2;3). Chylomicrons are drained directly into the systemic circulation via thoracic lymph(4). In the peripheral circulation, the enzyme lipoprotein lipase (LPL)is responsible for lipolysis of the triglycerides from chylomicrons, after which the free fatty acids can enter the peripheral cells. The triglyceride-depleted chylomicron remnants are taken up by the liver(4). The liver on its turn produces VLDL, which consists of triglycerides and cholesterol and apoB100. LPL hydrolyses TG in VLDL particles, resulting in fatty acids that are taken up in peripheral cells, and remnant VLDL. Remnant VLDL is further hydrolyzed by hepatic lipase to leave LDL. The LDL receptor on hepatic and peripheral cells facilitates uptake of LDL particles, providing cholesterol for the peripheral cells(2;3). In contrast to LDL, which mainly transports cholesterol from the liver to peripheral tissues, HDL plays an important role in reverse cholesterol transport from the peripheral tissues to the liver, although this is still matter of debate(2;5). ApoA1, the structural protein of HDL, is secreted by the liver and the intestine and is lipid-poor. These apoA1 particles acquire phospholipids and cholesterol from the liver and from chylomicrons and VLDL to form nascent HDL particles. In the circulation, more cholesterol and phospholipids are taken up from peripheral tissues and triglyceride-rich lipoproteins, resulting in mature HDL particles. Ultimately, HDL particles are again selectively taken up by the liver(3;5).

In clinical practice, the cholesterol content in plasma is determined (total cholesterol) usually in a fasting state, as well as the triglyceride content in the plasma (plasma triglyceride). Furthermore, the amount of plasma cholesterol that is present in HDL particles is determined (HDL-cholesterol) and the amount of plasma cholesterol in LDL particles is determined or calculated (LDL-cholesterol) (6). Total cholesterol level and in particular LDL-cholesterol level is strongly associated with the risk of vascular events, whereas HDL-cholesterol has a strong inverse association with the risk of vascular events(3;7).

Genetics of plasma lipids

Plasma lipid levels are determined by genetic factors and secondary factors. The first heritable plasma lipid disorder was described in 1938 (8), a condition called Familial Hypercholesterolemia. In patients with heterozygous Familial Hypercholesterolemia, plasma LDL cholesterol levels usually range between 5 and 10 mmol/L(9), whereas the

treatment target is <2.5 mmol/L(10). In 1976 the underlying genetic cause was identified as being a defect in the LDL-receptor gene, which is responsible for a large part of the cases of Familial Hypercholesterolemia (11). Also other genetic causes of Familial Hypercholesterolemia and genetic defects causing disturbances in plasma TG and HDL-c have been identified(12). However, these monogenetic defects with large effects on plasma lipid levels represent only the extreme ends of the spectrum of plasma lipids. Most of the genetically determined variation in plasma lipids is due to numerous combinations of variants with small effects on plasma lipid levels(2). Recently, genome-wide association studies (GWAS) have been used as a new method to study associations between genetic markers and specific traits such as plasma lipids. The genetic markers used for these GWAS are single nucleotide polymorphisms (SNPs), a genetic variant in which only one nucleotide is different. SNPs are defined as variants occurring in >5% of the population and are therefore most common type of genetic variants(13). GWAS study more than million SNPs simultaneously(13) and these GWAS have identified many SNPs associated with plasma lipids(14-16). A part of these SNPs is located in or near genes with a known function in lipid metabolism. However, also many SNPs have been identified in parts of the genome where no known genes involved in lipid metabolism are located. These SNPs may lead to the discovery of new genes involved in lipid metabolism, but this is complicated by the fact that these SNPs are unlikely to be the causative genetic defects, but only linked to the true causative genetic defect. Studies targeted at the regions in which SNPs associated with a trait are located are necessary to more precisely map the gene affecting the trait(13). In addition, these SNPs can be used to study the causal effect of a trait on an outcome, for example the causal effect of LDL-cholesterol on vascular events. Since SNPs only related with LDL-cholesterol can be selected, the true causal effect can be studied without confounding effect of diet or other variables affecting LDL-cholesterol and the risk of vascular events(17). So far, GWAS and the identified SNPs are exclusively an area of research, and there is as yet no clinical role for the identified SNPs.

Current treatment of plasma lipid levels

The identification of plasma lipid levels as important determinants for vascular risk has led to development of therapies influencing plasma lipid levels. Therapies reducing LDL-cholesterol have thus far been most successful, largely owing to statin therapy, the mainstay in the treatment of plasma lipids. Statins are inhibitors of the enzyme 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, which is the rate-limiting enzyme in the mevalonate pathway, the pathway producing among others cholesterol(18). Statin treatment therefore results in a marked decrease in LDL-cholesterol (depending on type and dose up to 40-

50% reduction)(19), while also having a small beneficial effect on plasma triglycerides and plasma HDL-cholesterol levels. This translates into a reduction in major vascular events of 22% per 1 mmol/L decrease in LDL-cholesterol(20). Current guidelines advise statin therapy for all patients at high risk of vascular events to reduce LDL-cholesterol to <2.5 mmol/L, or even to <1.8 mmol/L in patients at very high risk(21;22). If statin therapy is not sufficient to lower LDL-cholesterol to this level, add-on therapy with ezetimibe or bile acid sequestrants could be added to further lower LDL-cholesterol(10;21;22).

For plasma triglycerides, the guidelines define levels >1.7 mmol/L as elevated(10;21;22), but so far this has few implications for treatment. The association with vascular events is less strong than for LDL-c, but moreover, few effective therapies are available to reduce the risk associated with elevated triglyceride levels. The level of >1.7 mmol/L is not a treatment target, but instead when triglycerides are >1.7 mmol/L, nonHDL-cholesterol should be the treatment target instead of LDL-cholesterol, since nonHDL-cholesterol also includes triglyceride-rich remnant particles(10;21;22).

For plasma HDL-cholesterol, the same applies as for triglycerides, although the inverse association with vascular events is stronger than the association between plasma triglycerides and vascular events. No treatment target is available for plasma HDL-cholesterol, since there is no clinical trial evidence that increasing HDL-cholesterol will decrease the risk of vascular events(10;21;22).

Current clinical challenges related to plasma lipids

Although our understanding of the impact of plasma lipid levels in the pathogenesis of atherosclerosis has increased and effective lipid-lowering therapies have been developed during the last decades, several challenges remain. Despite effective LDL-c reducing therapy, residual risk of vascular events remains at low LDL-c levels(23). Plasma levels of HDL-c and TG could explain a part of this residual risk(23), but determining their exact contribution to vascular risk and whether this is a causal association has proven to be complicated (24). This is underscored by the fact that development of HDL-c increasing or TG lowering therapy has yielded disappointing results in terms of effective lowering of the risk of vascular events(25-32). Another question is whether using fasting lipid levels are the most appropriate lipid measure to assess vascular risk. Non-fasting plasma TG levels are more strongly associated with vascular events than fasting TG levels(33-35), and other lipid levels show little variation with differing fasting time(36), but whether to change a long-standing clinical practice remains a debate.

In addition, clinician should stay critical as to which patients will benefit most from which specific established therapy. Although statins are safe and effective, they

may increase the risk of type 2 diabetes mellitus(37;38). Identifying the patients at risk and identifying the patients who benefit most from (intensive) statin therapy in terms of vascular event reduction still needs further study.

Genotyping of genetic variants with large effects on plasma lipids has entered clinical practice, for example in patients with familiar hypercholesterolemia(9). The clinical relevance of variants with smaller effects is still unclear(13). So far, the large amount of data gathered by GWAS has few implications for clinical practice. A question is still whether SNPs themselves do add any meaningful information to measured plasma lipid levels, and whether genotyping these SNPs could have implications for treatment of patients with or without clinical manifestations of vascular disease(13).

Genetic information carries also information about lifetime exposure to risk factors and a lifetime exposure to elevated risk factor levels have large effects(17). Conversely, treating patients at an early stage will lead to large reductions in lifetime risk of vascular disease. Therefore, development of models to estimate lifetime risk could add valuable information beyond short-term risk prediction models and could guide lifestyle changes and possibly medical therapy. Currently, lifetime risk models are still under development, and only available for the primary prevention setting(39;40). For the time being, no lifetime risk models are available for patients with established vascular disease, whereas this information will be very relevant for young patients who encountered a vascular event.

Objectives

The general objective of this thesis is to investigate the relation between plasma lipid levels and recurrent vascular events with specific focus on genetics, plasma TG and HDL-c levels, and treatment of lipids.

The objectives are:

- To determine the relation between SNPs associated with LDL-cholesterol and myocardial infarction in the general population and (treated) LDL-c and vascular events in patients with established arterial disease.
- To determine the relation between a SNP associated with plasma triglyceride level and myocardial infarction in the general population and plasma triglyceride level and vascular events in patients with established arterial disease.
- To determine the relation between plasma triglyceride level and vascular events in patients with various clinical manifestations of vascular disease, independent of LDL-cholesterol or nonHDL-cholesterol levels.

- To assess the association of HDL-cholesterol and vascular events in patients treated with moderate or intensive lipid-lowering medication or in patients at different LDL-cholesterol levels.
- To determine whether statins are related to incident type 2 diabetes mellitus and whether this is dependent on insulin resistance or metabolic syndrome criteria.
- To develop and validate a model to predict lifetime risk of recurrent vascular events in patients with established arterial disease and to assess whether using this lifetime risk model is useful to identify high risk patients in addition to a 10-year risk model.

Outline of this thesis

Obesity is a growing health problem in the Western countries, which has its impact also on plasma lipids. In **chapter 2**, the literature on obesity and the resulting adipose tissue dysfunction and plasma levels of triglycerides is reviewed. We also review the evidence for a causal role of plasma triglyceride levels in the occurrence of vascular events and the implications for treatment.

Since plasma lipid levels are also influenced by genetics, we study several SNPS that are associated with plasma lipid levels and myocardial infarction in the general population. In **chapter 3**, we study the relation between four LDL-cholesterol related SNPs and LDL-cholesterol levels in patients with various manifestations of vascular disease. We investigate whether the relation with LDL-cholesterol is independent of use of lipid-lowering medication, and whether these SNPs are still associated with vascular events in a population of patients with clinically manifest vascular disease. In **chapter 4**, the relation between a triglyceride associated SNP and plasma triglyceride levels is investigated in a population of patients with clinically manifest vascular disease, and whether this relation depends on body mass index. Also the relation with clinical parameters such as the prevalence of the metabolic syndrome and the proportion of patients at treatment target is studied, as is the relation with recurrent vascular events.

In patients with established arterial disease, overweight and obesity are common and therefore also the concomitant increase in plasma triglyceride level and decrease in plasma HDL-cholesterol level are common. In **chapter 5** we investigate the effect of plasma trigyceride levels on recurrent vascular events. These analyses are also performed in strata of different localizations of vascular disease and in strata of patients who are on treatment target for LDL-cholesterol, nonHDL-cholesterol or HDL-cholesterol. In **chapter 6** we proceed by assessing the effect of plasma HDL-cholesterol level on recurrent vascular

events. Since the effect of plasma HDL-cholesterol on (recurrent) vascular event could possibly differ for patients treated with (intensive) LDL-cholesterol lowering therapy, we stratify these analyses by use of moderate or intensive lipid-lowering treatment and by LDL-cholesterol level. In addition, we focus on adverse effects of lipid-lowering therapy in **chapter 7**. This chapter quantifies the risk of incident type 2 diabetes mellitus with statin therapy. We investigate the effect of different types or intensities and whether the increase in risk is different for patients at different levels of insulin resistance and with different numbers of metabolic syndrome criteria.

Since an increasing number of patients is surviving a first vascular event, estimation of the risk of a recurrent event in these patients is of increasing importance. Although 10-year risk prediction models are available for these patients, the 10-year period is still a short time horizon for young patients. Therefore, we develop and validate a model for lifetime prediction of recurrent vascular events in **chapter 8**. The estimates from the lifetime risk model are compared with 10-year estimates to specify for which patients lifetime risk estimates may be useful.

In **chapter 9**, the main findings of the studies presented in this thesis are discussed, after which a summary is provided in **chapter 10**.

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Chapter 2

Adipose tissue dysfunction and hypertriglyceridemia: Mechanisms and management

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Obes Rev 2011; 12:829-840

Abstract

Elevated plasma triglyceride levels, as often seen in obese subjects, are independently associated with an increased risk of cardiovascular diseases. By secreting adipokines (such as adiponectin and leptin) and other proteins (such as lipoprotein lipase and cholesteryl ester transferase protein), adipose tissue affects triglyceride metabolism. In obesity, adipocyte hypertrophy leads to many changes in adipocyte function and production of anti- and pro-inflammatory cytokines. Furthermore, free fatty acids are released into the circulation contributing to insulin resistance.

Adipose tissue dysfunction will eventually lead to abnormalities in lipid metabolism; such as hypertriglyceridemia (due to increased hepatic very low density lipoprotein production and decreased triglyceride hydrolysis), small dense low density lipoprotein particles, remnant lipoproteins and low high density lipoprotein cholesterol levels, all associated with a higher risk for the development of cardiovascular diseases.

The clinical implications of elevated plasma triglycerides are still a matter of debate. Understanding the pathophysiology of adipose tissue dysfunction in obesity, which is becoming a pandemic condition, is essential for designing appropriate therapeutic interventions. Lifestyle changes are important to improve adipose tissue function in obese patients. Pharmacological interventions to improve adipose tissue function need further evaluation. Although statins are not very potent in reducing plasma triglycerides, they remain the mainstay of therapy for cardiovascular risk reduction in high-risk patients.

Introduction

The association between hypertriglyceridemia and increased risk of atherosclerosis was described more than 60 years ago(1). Hypertriglyceridemia is now an established risk factor for cardiovascular disease, independent of total-, low density lipoprotein- (LDL) and high density lipoprotein- (HDL) cholesterol(2-4). Non-fasting triglyceride levels are even associated with a higher cardiovascular risk compared to fasting triglyceride levels(5-7). Despite convincing evidence for an independent association between triglyceride levels and cardiovascular disease, the interpretation of triglyceride levels in clinical practice is still much debated or neglected, mainly because the drugs currently available for lowering triglyceride levels have not shown an unequivocal benefit in reducing clinical endpoints. Adipose tissue has a central role in triglyceride metabolism. This is emphasized by the association of both lipodystrophy (deficiency of adipose tissue) and obesity (excess of adipose tissue) with insulin resistance and hypertriglyceridemia, indicating the importance of well-functioning adipose tissue(8;9).Due to continuing increase in the prevalence of obesity, the number of patients with hypertriglyceridemia and consequently higher risk of cardiovascular events is increasing. Therefore, clinicians need clear and clinically applicable guidelines to treat this growing population.

The present review will focus on: 1) the causes of elevated triglyceride levels, 2) adipose tissue dysfunction and plasma lipid abnormalities, 3) atherogenicity of triglycerides and 4) clinical interpretation and management of elevated triglyceride levels.

Causes of elevated plasma triglyceride levels

Pathophysiology of triglyceride metabolism

Triglycerides are transported in lipoprotein particles, and plasma triglyceride levels depend on the production rate, catabolism and clearance of lipoprotein particles, all of which could be disturbed in insulin resistance. Triglyceride rich lipoprotein particles (TRLP) enter the circulation either as very low density lipoprotein (VLDL) particles or chylomicrons produced by the liver or the gut, respectively. Hydrolysis of triglycerides in VLDL–particles and chylomicrons to free fatty acids (FFAs) and glycerol is catalyzed by lipoprotein lipase (LPL), which is secreted by the parenchymal cells (such as adipocytes and myocytes). LPL needs apolipoprotein (apo)CII as a cofactor. LPL exerts its function at the luminal site of vascular endothelial cells, where glycosylphosphatidylinositol-anchored high density lipoprotein binding protein 1 (GPIHBP1) has been suggested to provide a platform for triglyceride hydrolysis. GPIHBP1 is expressed in heart, adipose

tissue and skeletal muscle, the same tissues that also express LPL, highlighting the functional relationship between these proteins(10). Mutations in GPIHBP1 and LPL are both associated with hypertriglyceridemia(10).

Primary disorders in triglyceride metabolism

Primary disorders leading to elevated plasma triglycerides refer to conditions with a genetic basis such as LPL-, GPIHBP1- and apoCII deficiency in chylomicronemia. Triglyceride levels are markedly elevated in chylomicronemia (usually >10 mmol/L, whereas up to 1.7 mmol/L is considered normal), but data about the effect on atherosclerosis in humans are scarce. However, in mice LPL deficiency leads to more atherosclerotic lesion formation (11). In humans, these high triglyceride levels confer an increased risk of pancreatitis(12). Also other lipoprotein particles, such as VLDL or remnant particles, could be affected in primary disorders. Plasma triglyceride levels are lower in these conditions compared to chylomicronemia and these disorders seem to induce atherosclerosis. Table 1 gives an overview of currently known causes of chylomicronemia and summarizes the most important other genes associated with hypertriglyceridemia. A detailed review on primary causes of hypertriglyceridemia is beyond the scope of this review and is provided elsewhere (13).

Secondary disorders in triglyceride metabolism

Secondary disorders are acquired conditions leading to hypertriglyceridemia such as obesity, insulin resistance, alcohol intake and hypothyroidism (Table 2)(12). This review will focus on obesity and adipose tissue dysfunction as a cause of hypertriglyceridemia (Figure 1). Adipose tissue is a key player in the regulation of plasma triglyceride levels, because it serves as storage depot in the fed state and releases FFAs during fasting. Expansion of adipose tissue is strongly correlated with insulin resistance and cardiovascular disease. On the other hand, the dramatic loss of adipose tissue as observed in patients with lipodystrophy triggers a high degree of insulin resistance and hypertriglyceridemia(8;9). This indicates that not only quantity but also proper function of adipose tissue is important for energy metabolism.

Table 1. Primary causes of hypertriglyceridemia

Genetic cause	Affected lipoprotein	Clinical presentation	Prevalence
Monogenic syndromes: - LPL deficiency	Chylomicrons	Eruptive xanthomas, hepatosplenomegaly, lipemia retinalis, pancreatitis	Very rare (97)
- apoC-II deficiency (co-factor for LPL activity)	Chylomicrons	Eruptive xanthomas, hepatosplenomegaly, pancreatitis	Very rare(97)
- LMF-1 deficiency (maturation of LPL)	Chylomicrons	Recurrent pancreatitis and tubulous xanthomas in one case as a result of a complete loss of active LPL.	Only a few case reports(98;99)
- GPIHBP1 deficiency	Chylomicrons	Failure-to-thrive, pancreatitis.	A few case reports (100)
- apoA-V deficiency	Chylomicrons, VLDL	Eruptive xanthomas, hepatosplenomegaly, pancreatitis	Very rare (101)
Other genes influencing TG level: Among others ANGPTL3, APOE, CHREBP, GALNT2, TRIB1	Depending on mutation	Depending on mutation	Mutations in these genes are common (13)

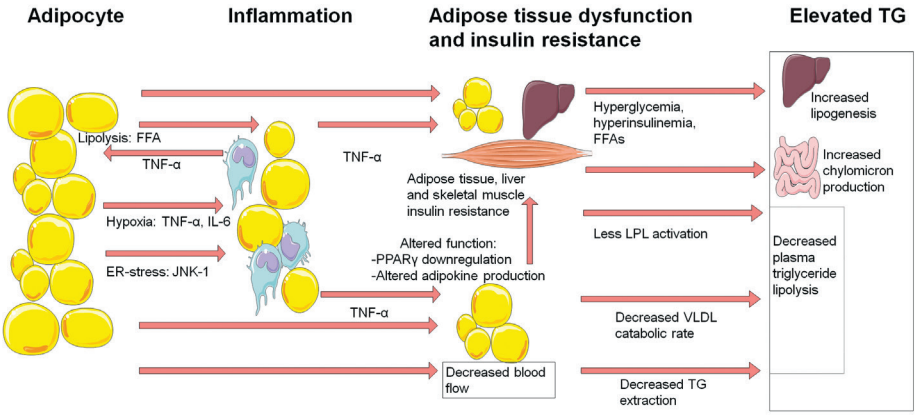
Abbreviations: ANGPTL3: angiopoietin-like-3, apo: apolipoprotein, APOE: apolipoprotein E, CHREBP: carbohydrate response element binding protein, GALNT2: UDP-N-acetylalpha-D-galactosamine:polypeptide N acetylgalactosaminyltransferase, GPIHBP1: glycosylphosphatidylinositol-anchored high density lipoprotein binding protein 1, LMF-1: lipase maturation factor-1, LPL: lipoprotein lipase, TRIB1: tribbles homologue 1, VLDL: very low density lipoprotein.

Table 2. Secondary causes of hypertriglyceridemia

Lifestyle	Physical inactivity	Less activation of LPL, induction of obesity and insulin resistance
	Excessive caloric intake	Stimulation of VLDL production in the liver
	Excessive alcoholic intake	Less oxidation of free fatty acids, leaving more free fatty acids for VLDL synthesis
	High carbohydrate diet	
Diseases and conditions	Obesity	See text/figure 1
	Insulin resistance	See text/figure 1
	Chronic renal disease / nephrotic syndrome	May be due to renal wasting of protein: stimulation of albumin production, which is linked to VLDL production. Impaired lipolysis in uremia.
	Cushing disease	Increased abdominal adipose tissue, insulin resistance, increased peripheral lipolysis
	Glycogen storage disease	Results from derangement of glucose metabolism
	Acromegaly	Lipolysis and insulin resistance
	Hypothyroidism	Indirect by downregulation of LDL receptor.
	Lipodystrophy	Either genetic or acquired (HAART)
	Auto-immune disease	Immune-medicated impaired lipolysis?
	Pregnancy	Physiologic
Medication	Estrogen replacement therapy	Increased VLDL production
	Oral anticonceptives	Mostly due to estrogen
	Tamoxifen	Estrogenic effect on lipids
	Corticosteroids	See Cushing disease
	Beta blockers	Reduced peripheral blood flow à less uptake glucose by skeletal muscle and less availability LPL for triglyceride lipolysis
	Thiazide diuretics	Reduced insulin sensitivity?
	Atypical antipsychotics	Weight gain, insulin resitance, dietary changes?
	HIV protease inhibitors	Insulin resistance, accelerated lipolysis, impaired clearance of TG rich lipoproteins?
	Retinoids	Increase in apoCIII synthesis and other pathways

Abbreviations: apo: apolipoprotein, HAART: highly active anti-retroviral therapy, LDL: low density lipoprotein, LPL: lipoprotein lipase, TG: triglyceride, VLDL: very low density lipoprotein.

Figure 1. Mechanism of hypertriglyceridemia in obesity.



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Inflammation, increased lipolysis, altered adipokine production and insulin resistance all influence each other and ultimately lead to increased plasma TG levels.
Abbreviations: ER: endoplasmic reticulum; FFA: free fatty acids; JNK-1: c-Jun N-terminal kinase; IL-6: interleukin 6; LPL: lipoprotein lipase; PPARγ: peroxisome proliferator-activated receptor γ; TG: triglycerides; TNF-α: tumor necrosis factor α; VLDL: very low density lipoprotein.

Adipose tissue dysfunction and hypertriglyceridemia

Adipose tissue dysfunction due to inflammation

Excessive intake of calories leads to increased storage of triglycerides in adipocytes, facilitated or followed by adipocyte hypertrophy and proliferation (14). Obesity is associated with a chronic low-grade inflammatory state of adipose tissue, as illustrated by increased numbers of macrophages in the adipose tissue(15) and by increased plasma levels of inflammatory cytokines in obese subjects (16). Several hypotheses have been put forward that may explain the inflammatory state of adipose tissue such as hypoxia, endoplasmic reticulum (ER) stress and increased levels of FFA. According to the hypoxia hypothesis, the expanding adipose tissue becomes hypoxic, either due to increased diffusion distance or hypoperfusion, which induces an inflammatory reaction(17;18). Outgrow of hypertrophied adipocytes beyond the oxygen diffusion distance seems unlikely, since also small adipocytes suffer from hypoxia in obese mice(19). In vitro studies have established that hypoxia leads to stabilization of the transcription factor hypoxia

inducible factor 1α (HIF1α)(20), inducing the expression of a wide array of genes, among which are pro-inflammatory cytokines. In cell culture studies of human adipocytes, increased expression of adipokines such as interleukin-6 (IL-6), monocyte migration inhibitory factor (MIF) and plasminogen activator inhibitor-1 (PAI-1) has been observed, and adiponectin expression is decreased (18;21). A lowered oxygen tension has been demonstrated in adipose tissue of obese humans, due to decreased capillary density but this did not increase vascular endothelial growth factor (VEGF) expression(22), which is the theoretical response of hypoxic tissue. Moreover, the lowest oxygen tension observed was 29 mmHg, whereas in cell culture experiments 1% oxygen is used, comparable to an oxygen tension of 7.6 mmHg(22). Although oxygen tension was related to inflammatory changes and increased macrophage infiltration (22), so far there is no convincing evidence that hypoxia underlies inflammation in human adipose tissue, whereas decreased adipose tissue blood flow appears to be an important factor in adipose tissue dysfunction, as will be discussed below.

Another proposed mechanism for the inflammatory changes in obesity is ER stress. The functions of the ER include protein folding and trafficking. Hypoxia, but also excess of nutrients in obesity, may challenge these functions, leading to accumulation of unfolded proteins (23). Accumulation of unfolded proteins upregulates unfolded protein response proteins, which activates inflammatory responses via among others c-Jun N-terminal kinase-1 (JNK-1) and NF-κB. JNK-1 is reported to increase the expression of inflammatory cytokines and to inhibit insulin activation(23;24). So far, most evidence has been obtained from in vitro experiments, but studies in genetically modified mice indicate increased insulin resistance with increased susceptibility to ER stress(25) and decreased insulin resistance with increased expression of protective proteins (26). In humans, increasing BMI is associated with increased ER stress markers (27), and unfolded protein response proteins are upregulated in adipose tissue of obese subjects, as is the downstream effector JNK-1 (28). However, inflammation is reported to increase the unfolded protein response (29), making it difficult to discern the causality.

Obese subjects have increased lipolysis and FFA release by adipocytes. FFAs are reported to bind to Toll-like receptor 4 (TLR4) on both adipocytes and macrophages (30;31) which could lead to a pro-inflammatory state in the adipocyte and TNF-α production in the macrophages. TNF-α in turn also stimulates lipolysis in the adipocyte and increases the expression of pro-inflammatory and monocyte-attracting products, setting up a vicious circle(32). The mechanism through which FFAs may enhance TLR4 signaling has been controversial mainly due to the fact that commercially available FFAs seem to be contaminated with lipopolysaccharides, which by itself can activate TLR4 (33). A recent study suggests that saturated FAs activate TLR4 that in turn stimulates ceramide

synthesis, which is essential for inflammation-induced insulin resistance linking lipids to inflammation and insulin resistance(34).

Although the mechanism by which the inflammatory process originates is not unequivocally defined, its consequences are striking. Hypertrophied adipocytes secrete increased amounts of chemoattractant substances such as monocyte chemotactic protein 1 (MCP-1) and IL-8 (35), which could attract monocytes into the adipose tissue where they differentiate into macrophages. This is nicely demonstrated by number of macrophages in adipose tissue, which is 5-10% of the total number of cells in lean individuals and increases to up to 50% in obese subjects (15). Moreover, in obese individuals an increased proportion of these macrophages has a pro-inflammatory phenotype(36). Macrophages exhibit either a 'classically activated' M1 phenotype, secreting inflammatory cytokines such as TNF-α and IL-1, or an 'alternatively activated' M2 phenotype, secreting anti-inflammatory cytokines such as IL-10(37). Obesity leads to an increased influx of M1 macrophages in the adipose tissue, attracted by the released chemoattractant products, and a to phenotype switch from M2 to M1 macrophages, possibly due to action of FFAs on TLR4 (38). The pro-inflammatory cytokines released by the M1 macrophages increase lipolysis and insulin resistance in the adipocyte, creating a positive feedback loop with increasing inflammation and tissue dysfunction. However, in humans macrophages with M2 surface markers have been observed to have both pro-inflammatory and anti-inflammatory properties(39), suggesting that the situation in humans may be more complex.

Adipose tissue dysfunction, adipokines and insulin resistance

The pro-inflammatory action of the macrophages has profound influence on the function of adipose tissue that results in increased lipolysis, altered endocrine function and insulin resistance.

In normal functioning adipose tissue, storage of lipids in adipocytes and lipolysis of these lipids are balanced. Peroxisome proliferator-activated receptor gamma (PPARγ) is a transcription factor essential for adipocyte development and function and its activation induces many target genes promoting storage of lipids in adipocytes and reducing FFAs in the circulation, among which are LPL and fatty acid transport protein(40). Lipolysis, on the other hand, is coordinated by hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL)(41). The access of these lipases to the lipid droplets in which the lipids are stored is regulated by perilipin. Perilipin is the most abundant protein coating the lipid droplets and it normally forms a barrier around the lipid droplets(42). Lipolysis is stimulated by catecholamines and natriuretic peptides, and inhibited by insulin (43). This

mechanism keeps basal lipolysis low, but allows it to increase when necessary. Adipose tissue inflammation with overproduction of TNF-α causes downregulation of PPARγ(44), reducing its effect on triglyceride storage. However, PPARγ also stimulates expression of HSL and ATGL (41), therefore PPARγ downregulation, possibly together with direct effects of TNF-α (45) lead to decreased production of HSL and ATGL. This does not result in decreased lipolysis, since perilipin is also downregulated by inflammation (45), making lipid droplets more accessible for lipases and increasing lipolysis. Therefore, although stimulated lipolysis decreases due to downregulation of lipases, basal lipolysis is increased by the inflammatory changes in obesity(45), resulting in an increased FFA release from the adipose tissue in in vitro situations. However, in vivo FFA release per mass unit of adipose tissue is decreased in obesity due to hyperinsulinemia(46), although plasma levels are increased due to increased adipose tissue mass.

Adipose tissue inflammation also influences the endocrine function of the tissue. The concept of adipose tissue as an active endocrine organ has emerged recently but has already gained wide acceptance. Next to the role in inflammatory response, adipose tissue-derived secreted proteins, known as adipokines, have paracrine and endocrine actions in metabolic processes. Two of the best studied adipokines are leptin and adiponectin.

The central action of leptin is to increase energy expenditure and to decrease food intake. Leptin deficiency in mice leads to morbid obesity. The peripheral action of leptin is to stimulate AMP activated protein kinase (AMPK), thereby increasing fatty acid oxidation and glucose transport in muscle, maintaining insulin sensitivity (9). Furthermore, leptin reduces the activity of lipogenic genes such as PPARγ. The importance of leptin in the regulation of lipid levels is exemplified by patients with lipodystrophy, who have less subcutaneous adipose tissue with typical central adipose tissue redistribution and low levels of leptin. These patients suffer from hypertriglyceridemia and insulin resistance and leptin replacement therapy improves both lipid and glucose levels (9). However, most obese patients have elevated leptin levels, but due to leptin resistance they show signs of metabolic syndrome(47).

In contrast to leptin, adiponectin production is decreased in obesity(48). It has autocrine effects by promotion of adipocyte differentiation and increasing adipose tissue insulin sensitivity. An endocrine function of adiponectin is to increase insulin sensitivity in the liver, adipose tissue and skeletal muscle. It influences lipid metabolism by increasing skeletal muscle LPL production, and adiponectin levels have been shown to be correlated with VLDL catabolic rate(49)

Adipose tissue inflammation also induces insulin resistance, via multiple pathways. The increased lipolysis in the hypertrophied adipose tissue increases circulating FFAs, impairing insulin sensitivity and inducing hyperglycemia. In skeletal muscle, high levels

of FFAs lead to decreasing uptake of glucose. High plasma FFA levels lead to increased intracellular diacylglycerol concentrations, inducing activation of protein kinase C family serine/threonine kinases. These kinases inhibit phosphorylation of insulin receptor substrate (IRS) 1 or 2, important mediators of insulin action, thus reducing in particular the translocation of glucose transporter (GLUT) 4 to the cell membrane. In a similar way, FFAs reduce hepatic insulin sensitivity, promoting glucose production (50).

The inflammatory cytokines also have a direct effect on insulin resistance. TNF-α induces insulin resistance in skeletal muscle(51) and liver(52) by decreasing insulin signaling through serine kinase activation. In adipocytes, TNF-α inhibits insulin signaling as well(53), and downregulates PPARγ activity that eventually leads to an unfavorable change in adipokine release, in particular a decreased adiponectin secretion(54). This decrease in adiponectin production may be the mechanism via which PPARγ downregulation causes insulin resistance. This is supported by impairment of the insulin-sensitizing effect of PPARγ agonists in adiponectin knockout mice (48). These changes in the endocrine and autocrine function of adipose tissue will eventually translate into unfavorable changes in plasma lipid concentrations.

Adipose tissue dysfunction and plasma lipids

Obese, insulin resistant subjects often have an atherogenic lipid profile, characterized by elevated triglyceride levels, 'small dense' LDL and low HDL cholesterol levels. Adipose tissue dysfunction plays a central role in inducing this lipid triad by increased lipolysis and FFA release, decreased LPL expression and increased CETP expression.

The increased adipose tissue triglyceride lipolysis due to the increased adipose tissue mass increases circulating FFA. FFA availability drives hepatic production of VLDL particles, increased availability therefore leads to overproduction, predominantly of the triglyceride-rich VLDL-1 particles (55). Furthermore, the insulin resistant state leads to increased intestinal production of triglycerides, cholesteryl esters and apoB48, resulting in raised chylomicron production and elevated postprandial triglyceride levels(56).

Beside increased production, TRLP clearance is reduced in insulin resistant subjects, which is clearly illustrated by increased postprandial triglyceride levels (57;58). This reduced clearance of TRLP could be explained by two different mechanisms; i) decreased postprandial response in LPL activity or ii) decreased adipose tissue blood flow (58). Adipocytes produce LPL mainly in the fed state, due to the stimulatory action of insulin. In obesity, the basal LPL production is probably increased but the postprandial LPL-rise is diminished (59). Although LPL activity is difficult to assess in humans and some studies showed conflicting results, insulin resistance has been shown to be

inversely correlated with adipose tissue LPL activity, heparin-releasable LPL activity and LPL mRNA levels in humans (60). However, insulin sensitization with a PPARγ agonist in type 2 diabetic patients had no effect on LPL activity, whereas it increased LPL mRNA (61). Another explanation for reduced TRLP clearance is decreased adipose tissue blood flow, since adipose tissue is less perfused in obesity, as has been discussed earlier. In vivo adipose tissue blood flow in humans seems to be mainly regulated by nitric oxide in the fasting state(62). Postprandial increase in blood flow is related to insulin, although insulin itself does not directly affect blood flow(63), but may do so via sympathetic activation. In insulin resistance, this postprandial increase in adipose tissue blood flow is diminished (64). This may in itself explain increased postprandial triglyceride levels, since adipose tissue blood flow is associated with triglyceride clearance(65).

Lipid metabolism is also influenced by cholesteryl ester transfer protein (CETP), another product of adipocytes(66). Through the action of CETP, triglycerides from TRLP are transferred to HDL in exchange for cholesteryl esters. These triglyceride rich HDL particles are cleared rapidly from the circulation resulting in lower HDL-cholesterol plasma levels(66). It seems likely that the same mechanism transfers triglycerides to LDL, after which these triglycerides are hydrolyzed rapidly by hepatic lipase, leaving small dense LDL particles(66). Adipose tissue seems to be the major contributor of plasma CETP levels. It has been shown that plasma CETP activity in humans correlates with the degree of adiposity, and weight reduction is associated with a decrease in plasma CETP activity(67). Furthermore, plasma CETP concentrations in human subjects are closely related to the abundance of CETP mRNA in adipose tissue(68). Transgenic mice with adipose tissue-specific CETP expression showed a major contribution of adipose tissue CETP to the CETP mass and activity in the circulation, again demonstrating the importance of adipose tissue in plasma CETP levels(69).

In summary, adipocyte hypertrophy and insulin resistance increase FFA release and production of TRLP, whereas decreased LPL activity leads to reduced clearance of TRLP. Furthermore, CETP production by adipose tissue is increased in obesity and induces lower HDL cholesterol levels and the emergence of small dense LDL particles. Adipose tissue dysfunction can be viewed as a major contributor to changes in plasma lipids in obesity; i.e. elevated triglyceride levels, low HDL cholesterol levels and the presence of small dense LDL particles.

Involvement of plasma triglycerides in atherogenesis

Epidemiology

The plasma lipid changes in obesity and insulin resistance are all risk factors for atherosclerosis. Plasma triglyceride levels are well known to increase the risk for vascular events. Although this association has been known for a long time (1), the first meta-analysis to substantiate this hypothesis and to show the effect of plasma TG on cardiovascular events was published in 1996 (2). In this meta-analysis, the risk of cardiovascular disease was increased by 14% for each mmol/L increase of triglycerides in males, and by 37% in females after adjustment for confounding factors(2). The most recent meta-analysis compared the upper tertile of triglyceride levels to the lowest tertile, revealing an adjusted odds ratio for fatal or non-fatal coronary disease of 1.7 (1.6-1.9)(4). These results are in accordance with results from a meta-analysis in the Asia-Pacific region, which showed a relative risk of 1.80 (1.49-2.19) for fatal or non-fatal coronary disease for triglyceride levels in the highest quintile compared to the lowest quintile(3).

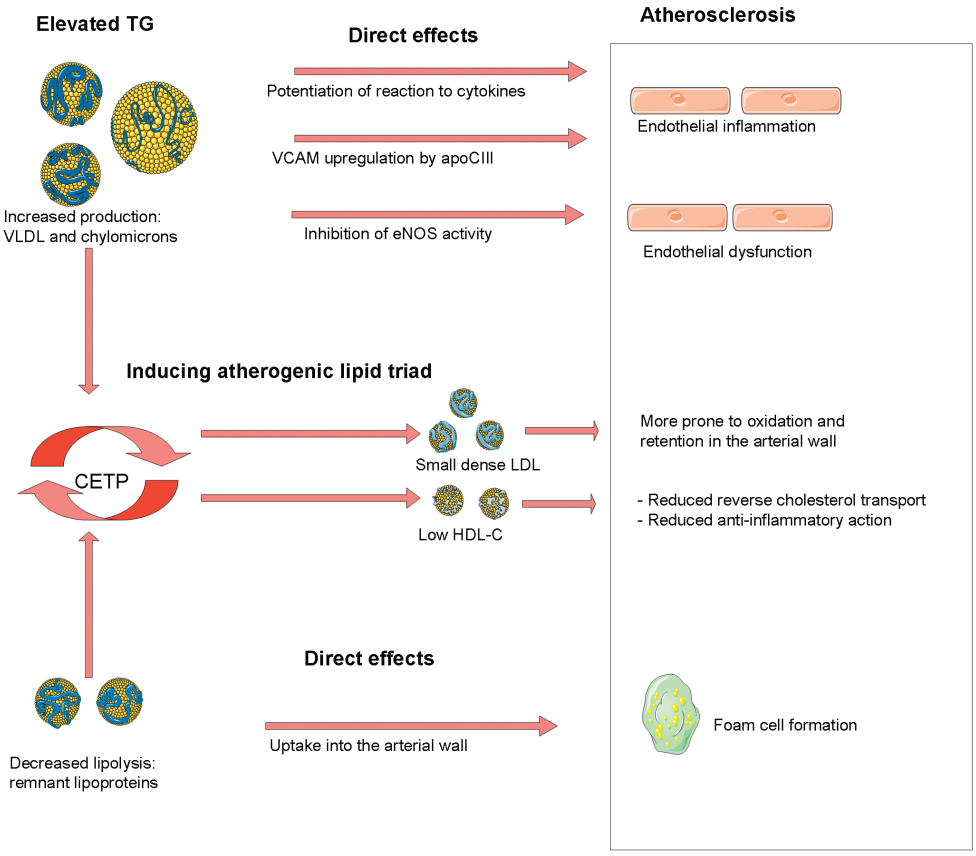
Non-fasting triglyceride levels may be more strongly associated with cardiovascular disease than fasting triglyceride levels(5;6). The Copenhagen City Heart Study(5) followed 7587 women and 6394 men for a mean of 26 years and showed that non-fasting triglyceride levels were associated with myocardial infarction, ischemic heart disease, total death(5;6) and stroke(7). The Women's Health Study(6) followed 26509 women for a median of 11.4 years. Increasing tertiles of fasting triglyceride levels showed no clear trend with hazard ratios of 1.21 (0.96-1.52) and 1.09 (0.85-1.41) compared to the lowest tertile. The corresponding hazard ratios for non-fasting triglycerides were 1.44 (0.90-2.29) and 1.98 (1.21-3.25) compared to the lowest tertile(6).

Mechanisms

Hypertriglyceridemia could influence cardiovascular risk by either directly affecting the vascular wall via TRLP or indirectly by inducing an atherogenic lipid profile; i.e. small dense LDL particles, increased level of remnant particles and low HDL-cholesterol levels (Figure 2).

Direct effects of TRLP may be (partly) mediated through the incorporated apoCIII present in these particles. Priming with TRLP has been shown to upregulate the reaction to inflammatory stimuli in human aortic endothelium (70). ApoCIII leads to a downregulation of the activity of endothelial nitric oxide synthase, thereby reduced

Figure 2. Mechanism of atherosclerosis in hypertriglyceridemia.



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Both direct effects of TG-rich lipoproteins and remnant lipoproteins and indirect effects via the emergence of small dense LDL and low HDL-cholesterol play a role. Abbreviations: apoCIII: apolipoprotein CIII; CETP: cholesteryl ester transfer protein; eNOS: endothelial nitric oxide syntase; HDL: high density lipoprotein; LDL: low density lipoprotein; VCAM: vascular cell adhesion molecule; VLDL: very low density lipoprotein.

endothelial relaxation(71), and upregulation of vascular cell adhesion molecule-1 (VCAM-1), increasing monocyte binding to the endothelium (72).

LDL particle size is mainly determined by triglyceride levels(66). Small dense LDL particles are more atherogenic than normal size LDL particles. This atherogenicity could arise because small dense LDL particles are less rapidly taken up by the LDL receptor and bind more strongly to arterial wall proteoglycans. Furthermore, small dense LDL

particles are predisposed to arterial wall uptake and they are more subjected to oxidative modification than larger LDL particles, enhancing uptake in macrophages(66).

The atherogenic lipoprotein phenotype is also accompanied by the presence of elevated lipoprotein remnant levels. The Copenhagen City Heart Study showed a strong correlation between non-fasting triglyceride levels and remnant lipoprotein levels(5). Hydrolysis of chylomicrons and VLDL produces remnant lipoproteins, which can enter the arterial wall and have been found in atherosclerotic plaques(73). Remnant lipoprotein cholesterol is an independent risk factor for cardiovascular disease(74). However, as remnant lipoprotein levels are associated with small dense LDL in the atherogenic lipoprotein phenotype, the individual contribution of small dense LDL particles and remnant lipoprotein cholesterol cannot be separated.

Besides reduced LDL particle size and elevated lipoprotein remnant levels, reduced HDL-cholesterol levels are a well-known risk factor for cardiovascular disease(75). Increased catabolism of HDL seems to result directly from elevated triglyceride levels(76). Nonetheless, triglyceride levels are still a risk factor for cardiovascular disease after correction for HDL-cholesterol, but the association with cardiovascular events is considerably weakened(2-4).

Clinical interpretation and management of triglyceride levels

Measurement

Several factors still limit the clinical use of plasma triglyceride measurements.

Hypertriglyceridemia is a heterogeneous disorder in which, depending on the underlying cause, different lipoprotein particle types can be elevated. Routine laboratory measurement of triglycerides is insufficient to identify the underlying mechanism, because the type of particles carrying the triglycerides remains unrevealed. Next to that, fasting levels are the worldwide standard and are used to calculate LDL cholesterol, but since non-fasting triglyceride levels have been shown to be more predictive for cardiovascular disease(5;6), the question arises whether triglyceride levels should be measured in the fasting state. Furthermore, no clear cut-point for plasma triglyceride concentration has been defined at which cardiovascular risk is increased. According to NCEP ATPIII guidelines(77), triglyceride levels <1.7 mmol/L (150 mg/dL) are normal, but even levels < 1.7 mmol/L have been shown to confer cardiovascular risk(4;5). Furthermore, large within-individual variability in triglyceride concentrations impairs the use of absolute TG levels to predict cardiovascular risk(78).

However, the evidence for increased vascular risk with increased plasma TG is clear, and attention should be paid to lowering TG levels in obese hypertriglyceridemic patients.

Management of obesity-induced hypertriglyceridemia

Treatment of high-risk obese hypertriglyceridemic patients should be focussed on the cause of the problem, which is adipose tissue dysfunction. Yet, pharmacological options are limited. Therefore, non-pharmacological treatment, i.e. lifestyle factors such as diet, physical activity and weight reduction, is of vital importance in these patients. Beside lifestyle measures, anti-obesity agents and bariatric surgery have all been reported to reduce inflammatory markers, indicating improved adipose tissue function(79).

With regard to diet, caloric intake, refined carbohydrates and (saturated) fat should be reduced. Physical activity and weight loss increase insulin sensitivity(80;81) and thereby decrease lipolysis in adipose tissue. Obesity, physical inactivity and insulin resistance are associated with a reduction in LPL activity(82;83), and weight loss and physical activity lead to activation of LPL(82;83). A rapid weight loss program resulting in 15% weight loss reduced triglyceride levels by 45%(84). However, sustainable weight loss remains difficult to achieve even with addition of pharmacological therapy such as orlistat or sibutramine(85). Regular exercise participation leads to a mean reduction in plasma triglyceride level of 24%(86), and physical activity even improves the lipid profile in the absence of weight loss(87). A 2-year clinical lifestyle modification program combining diet and physical activity, while achieving only a small weight loss of 3%, reduced triglyceride levels by 20%(88).

Thiazolidinediones are insulin-sensitizing drugs widely used in diabetic patients for glycemic control. Currently, 2 drugs (pioglitazone and rosiglitazone) are available in daily clinical practice. Both act as high-affinity ligands for PPARγ and improve metabolic parameters by directly affecting adipose tissue function. Although thiazolidinediones have been proven beneficial, in terms of glycemic control, its cardiovascular risk reduction has been challenged (89). A controversially discussed meta-analysis reported an increased rate of myocardial infarction with rosiglitazone(90), although this rate was not observed in similar analysis with pioglitazone(91). Furthermore, thiazolidinediones induce weight gain, fluid retention and possibly congestive heart failure, limiting its clinical value in patients with adipose tissue dysfunction (92).

Until more specific drugs are available for improvement of adipose tissue function, statins remain the cornerstone of therapy in patients with increased risk of cardiovascular disease. Although statins reduce plasma triglyceride levels by only 10 to 40%(12), statins are

well tolerated and have a well-known beneficial effect on the incidence of cardiovascular events. Fibrates and niacin induce larger triglyceride reductions (20-50%), but have less effect on clinical outcome(93). Adding fenofibrate to statin therapy seems to be only beneficial for patients with triglyceride levels ≥ 2,3 mmol/L and HDL-cholesterol levels ≤ 0,88 mmol/L(94). Treatment with omega-3 fatty acids 4 gram per day leads to 20-45% reduction in triglyceride levels, however, meta-analyses to the effect on cardiovascular events show conflicting results(95;96).

Conclusion

Adipose tissue dysfunction is likely to play a central role in the development of hypertriglyceridemia. Adipocyte hypertrophy leads to adipose tissue inflammation, contributing to systemic inflammation, and release of FFAs. This causes changes in the production of adipokines, a decrease in insulin sensitivity, increased hepatic VLDL production, lower LPL activity and increased CETP production by adipocytes. All these changes will lead to an atherogenic lipid profile. Elevated triglyceride levels are independently associated with the occurrence of cardiovascular diseases, and this association is even stronger for non-fasting triglycerides. This effect is mediated through different processes such as the presence of small dense LDL particles and remnant lipoproteins, low HDL-cholesterol levels and a direct effect of TRLP on inflammation and the vascular wall.

Treatment of adipose tissue dysfunction remains a challenge in current clinical practice. Whereas pharmacological therapies for weight loss or for improving adipose tissue function have been disappointing, lifestyle changes remain essential and can improve adipose tissue function and lead to a reduction in triglyceride levels and cardiovascular disease. Although statins lower triglyceride levels only slightly, they are proven to be effective in prevention of cardiovascular disease. Future research is needed to elucidate the complex role of adipose tissue dysfunction and hypertriglyceridemia in vascular disease in order to design novel therapeutic approaches and define more specific guidelines for risk assessment and treatment of hypertriglyceridemic patients.

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Chapter 3

Rs964184 (APOA5-A4-C3-A1) is related to elevated plasma triglyceride levels, but not to an increased risk for vascular events in patients with clinically manifest vascular disease

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Submitted

Abstract

Background: SNPs in the APOA5-A4-C3-A1 gene complex are associated with elevated plasma triglycerides (TG) and elevated vascular risk in healthy populations. In patients with clinically manifest vascular disease, hypertriglyceridemia and metabolic syndrome are frequently present, but the contribution of these SNPs to plasma TG, effect modification by obesity and risk of recurrent vascular events is unknown in these patients.

Methods: Prospective cohort study of 5547 patients with vascular disease. Rs964184 (APOA5-A4-C3-A1 gene complex) was genotyped, and we evaluated the relation with plasma lipid levels, presence of metabolic syndrome and the risk for new vascular events.

Results: Rs964186 was strongly associated with log plasma TG (β 0.12; 95%CI 0.10-0.15, $p=1.1*10^{-19}$), and was also associated with 0.03 mmol/L lower high-density lipoprotein-cholesterol (HDL-c) (95%CI 0.01-0.04), and 0.14 mmol/L higher nonHDL-c (95%CI 0.09-0.20). The minor allele frequency increased from 10.9% in patients with plasma TG <1 mmol/L to 24.6% in patients with plasma TG between 4 and 10 mmol/L. The relation between rs964184 and plasma TG was modified by body mass index (BMI) in patients with one minor allele (β 0.02; (95%CI -0.04-0.09) if BMI <24 kg/m2, β 0.17 (95%CI 0.12-0.22) if BMI >27 kg/m2, p for interaction=0.02). The prevalence of the metabolic syndrome increased from 52% for patients with two copies of the major allele to 62% for patients with two copies of the minor allele (p=0.01). Rs964184 was not related with recurrent vascular events (HR 0.99; 95%CI 0.86-1.13).

Conclusion: The SNP rs964184 (APOA5-A4-C3-A1) is associated with elevated plasma TG concentrations in patients with clinically manifest vascular disease. In carriers of one minor allele, the effect on plasma TG was modified by BMI. There is no relation between SNP rs964184 and recurrent vascular events in these patients.

Introduction

High plasma triglyceride (TG) levels and low high-density lipoprotein-cholesterol (HDL-c) levels are common among patients with vascular disease and may contribute to residual risk after treatment of other risk factors (1;2). Both genetic and environmental factors independently influence the plasma TG level (3) and common genetic variants combined with metabolic factors together lead to elevated TG levels(4). Genome-wide association studies (GWAS) have identified multiple single nucleotide polymorphisms (SNPs) associated with plasma TG levels(5-9). Of these SNPs, rs964184(APOA5-A4-C3-A1) was also related with the presence of coronary artery disease in a large GWAS for coronary artery disease(10), although very recently also rs2954029(TRIB1) and rs264(LPL) were identified in a GWAS for coronary artery disease(11). Rs964184 is located in the APOA5-A4-C3-A1 gene complex, a region with several polymorphisms strongly associated with plasma TG levels (5;7-9).

GWAS do not consider effect modification between metabolic factors and SNPs. However, this may be relevant for SNPs associated with plasma TG, since the effect of other SNPs in the APOA5-A4-C3-A1 gene cluster has been shown to depend on secondary factors such as obesity and alcohol (12;13). Interactions between genotype and a second factor could be important to determine the individual metabolic consequences of obesity. Overweight and obesity, the metabolic syndrome and the consequent hypertriglyceridemia are common in patients with clinically manifest vascular disease (14). Therefore, the effect of rs964184 may be different in this population, in which genetically elevated plasma TG are of clinical interest. Furthermore, it is unclear whether a small increase in plasma TG has any effect on the risk for recurrent vascular events in these patients, since all patients with clinically manifest vascular disease are already at high risk for recurrent events and are treated accordingly.

In the present cohort study, we investigated the association between rs964184 and plasma TG levels in patients with vascular disease and whether this association was modified by body mass index. Furthermore, we evaluated the association between rs964184 and clinical parameters such as the metabolic syndrome and being at treatment lipid targets, as well as the risk for occurrence of new vascular events.

Methods

Data were used from the Second Manifestations of ARTerial disease (SMART) cohort, a prospective, ongoing cohort study, designed to establish the presence of concomitant arterial diseases and risk factors for atherosclerosis in patients with known arterial disease or a cardiovascular risk factor. Patients newly referred to the University Medical Center Utrecht with known arterial disease or cardiovascular risk factors (hyperlipidemia, hypertension or diabetes) are eligible for inclusion. All patients gave written informed consent, and the Institutional Review Board of the University Medical Center Utrecht approved the study. After informed consent, patients underwent a vascular screening protocol including a health questionnaire, laboratory measurements and physical examination. A detailed description of this study has been published previously(15).

For the present study, we used data of 5547 patients, enrolled in the SMART study between September 1996 and March 2011, with either a history or recent diagnosis of clinically manifest arterial disease: coronary artery disease (CAD) (n=3348), cerebrovascular disease (n=1597), peripheral artery disease (n=1115) and/or aneurysm of the abdominal aorta (AAA) (n=495). Patients could be classified into more than 1 disease category. Patients with plasma TG ≥10 mmol/L (n=15) were excluded since this most likely results from a rare genetic cause.

Follow-up

Follow-up duration was defined as the period between study inclusion and first cardiovascular event or death from any cause, date of loss to follow-up or the preselected date of 1 March 2011. During follow-up, all study participants received a questionnaire every 6 months to obtain information about hospitalizations and outpatient clinic visits. If a participant reported a possible event, all available relevant data were collected. Death of a participant was reported by relatives, the general practitioner or the specialist who treated the participant, after which all available relevant data were collected regarding the cause of death. All events were classified independently by three members of the SMART Study Endpoint Committee, comprising physicians from different departments. Outcomes of interest for this study were vascular death, myocardial infarction and a composite of myocardial infarction, ischemic stroke and vascular death.

Laboratory assessment

Baseline lipid levels were obtained from fasting patients. Plasma total cholesterol and TG were measured using commercial enzymatic dry chemistry kits (Johnson and Johnson). HDL-c in plasma was determined using a commercial enzymatic kit (Boehringer-Mannheim) after precipitation of LDL-c and VLDL-c with sodium phosphotungstate magnesium chloride. LDL-c was calculated using the Friedewald formula.

Genotyping

Wet-lab genotyping genotyping for SNP analysis was carried out by KBiosciences, Hertfordshire, UK (www.kbioscience.co.uk), whose personnel were blinded to patient status, using their proprietary KASPar PCR technique and Taqman Genotype calling was carried out using an automated system, the results of which were checked manually by study personnel using SNPviewer software. Individuals with a low overall genotyping rate were removed from the study (n=73). Rs964184 was used for the present study, for 112 patients (2.0%), rs964184 genotype was missing, resulting in 5547 patients for the current analyses.

Data analyses

Linear regression models were used to evaluate the association between rs964184 and several lipid parameters (plasma triglycerides, HDL-c, nonHDL-c, apoB), with adjustment for age and sex. Since mainly nonHDL-c and apoB are affected by lipid-lowering therapy, these analyses were also adjusted for lipid-lowering medication. ApoB was only available for patients included from 2006 onwards (2133 patients). Furthermore, to investigate whether the association between rs964184 and plasma TG is modified by metabolic consequences of obesity, we included an interaction term with body mass index in a separate model.

To determine whether rs964184 is associated with metabolic syndrome prevalence, we used a logistic regression model, adjusted for age and sex. Additionally, in patients using lipid-lowering medication, a logistic regression model adjusted for age and sex was used to evaluate whether rs964184 is associated with the proportion of patients who were on treatment target for LDL-c (2.5 mmol/L), nonHDL-c (3.3 mmol/L) or apoB (100 mg/dL)(16).

A Cox proportional hazards model was used to detect an effect of rs964184 on vascular events in patients with known vascular disease, after testing the proportional

hazards assumption. This model was adjusted for age and sex. Furthermore, in a second model additional adjustment for plasma TG was performed, to identify whether a possible association between rs964184 and vascular events could be explained by plasma TG level.

For the analyses, the open source software package R 2.13 was used. A p-value of <0.05 was considered statistically significant for all analyses.

Results

Baseline characteristics

Baseline characteristics according to rs964184(APOA5-A4-C3-A1) genotype are shown in Table 1. Plasma TG levels are higher and HDL-c levels are lower for the heterozygotes and the homozygotes for the G allele. Furthermore, coronary artery disease is slightly more present in carriers of the G allele, whereas cerebrovascular disease is slightly less present. The overall minor allele frequency was 14.2%.

In figure 1 it is shown that the minor allele frequency increases with increasing levels of plasma TG, being 10.9% in patients with plasma TG levels between 0 and 1 mmol/L and 24.6 % in patients with plasma TG levels between 4 and 10 mmol/L.

Relation between rs964184 and plasma lipids

Rs964184 was strongly associated with plasma TG levels, with log(TG) being 0.12 (95%CI 0.10-0.15, $p=1.1*10^{-19}$) higher per risk allele (Table 2). The association with HDL-cholesterol was less strong, HDL-c levels were 0.03 (95%CI -0.04- -0.01) mmol/L lower per risk allele. The SNP was also associated with increased levels of nonHDL-c (0.14 mmol/L; 95%CI 0.09 - 0.20) and apoB (0.03 g/L, 95% CI 0.01 - 0.05).

The relation between rs964184 and plasma TG was modified by BMI (p for interaction=0.03, Figure 2). When stratified for genotype, only the effect of the CG genotype was modified by BMI (p for interaction=0.02). Including a quadratic term for BMI did not change these results. In the patients heterozygous for the risk allele with a BMI<24 kg/m^2, log(TG) was increased with 0.02 (95%CI -0.04 - 0.09) compared with patients without the minor allele, whereas the log(TG) was 0.13 (95%CI 0.08-0.18) higher in patients with a BMI≥24 kg/m2 and <27 kg/m2 and 0.17 higher (95%CI 0.12-0.22) in patients with a BMI ≥ 27 kg/m2. Conversely, per 1 kg/m^2 increase in BMI, log(TG) increased with 0.038 (95%CI 0.031-0.045) in heterozygous patients compared with 0.028 (95%CI 0.024-0.032) and 0.30 (95%CI 0.006-0.054) in patients homozygous for the major or the minor allele, respectively.

Table 1. Baseline characteristics stratified per rs964184 genotype

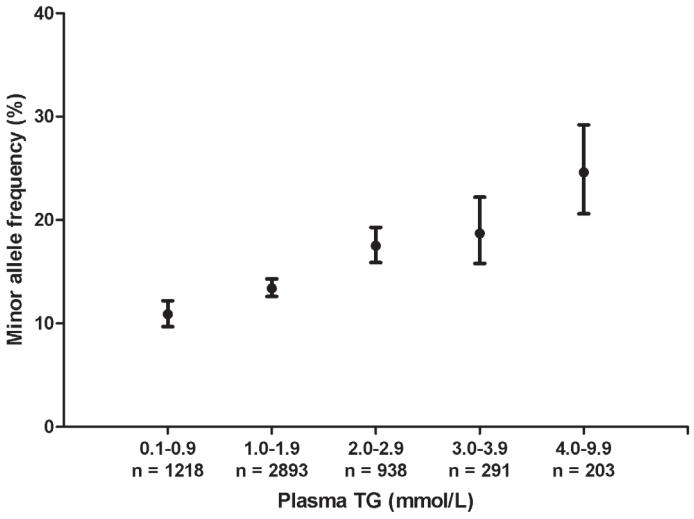
	CC (n=4100)	CG (n=1313)	GG (n=134)
Age (years)	60.0 ± 10.4	60.1 ± 10.3	61.0 ± 9.7
Male sex, n (%)	3048 (74)	982 (75)	101 (75)
BMI (kg/m^2)	26.8 ± 3.9	26.7 ± 4.0	27.1 ± 4.0
Total cholesterol (mmol/L)	4.9 ± 1.2	5.0 ± 1.3	5.2 ± 1.3
Triglycerides (mmol/L)	1.6 ± 1.0	1.9 ± 1.2	2.2 ± 1.3
LDL-c (mmol/L)	3.0 ± 1.0	2.9 ± 1.1	3.0 ± 1.1
HDL-c (mmol/L)	1.23 ± 0.37	1.20 ± 0.35	1.19 ± 0.31
Systolic blood pressure (mmHg)	141 ± 21	141 ± 21	141 ± 21
Diastolic blood pressure (mmHg)	82 ± 11	82 ± 11	81 ± 11
Metabolic Syndrome, n (%)	2139 (52)	718 (55)	83 (62)
Type 2 DM, n (%)	686 (17)	211 (16)	29 (22)
eGFR* (mL/min/1.73 m^2)	76 ± 18	76 ± 18	73 ± 19
Localisation of vascular disease, n (%):			
- coronary artery disease	2449 (60)	813 (62)	86 (64)
- cerebrovascular disease	1198 (29)	365 (28)	34 (26)
- peripheral artery disease	824 (20)	260 (20)	31 (23)
- aneurysm of abdominal aorta	367 (9)	119 (9)	9 (7)
Lipid-lowering medication, n (%)	2596 (63)	869 (66)	90 (67)
Antihypertensive medication, n (%)	3003 (73)	974 (74)	105 (78)
Current smoking, n (%)	1383 (34)	388 (30)	43 (32)
Current alcohol use, n (%)	1993 (49)	661 (50)	61 (46)

* Glomerular Filtration Rate, estimated by the Modification of Diet in Renal Disease (MDRD) equation

Relation between rs964184 and metabolic syndrome and lipid treatment targets

The prevalence of the metabolic syndrome is higher in patients with the minor allele. The prevalence was 52% in patients homozygous for the major allele and 62% in patients homozygous for the minor allele, (Odds Ratio of 1.14 (95%CI 1.03-1.27)) (Table 3). Furthermore, the elevated plasma TG levels also results into a decreased proportion of

Figure 1. Relation between minor allele frequencies and levels of plasma TG



Rs964184 minor allele frequency (95% confidence interval) at increasing levels of TG. N indicates the number of patients with a plasma TG level in this category

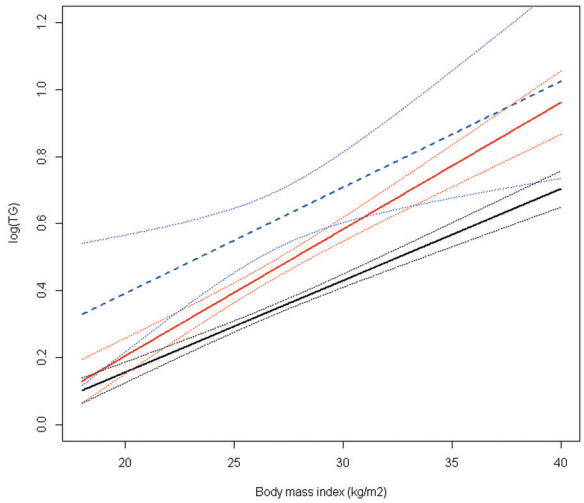
Table 2. Relation between rs964184 (per minor allele) and lipid levels

	Beta (95% CI)	P
Log(TG)	0.12 (0.10 - 0.15)	$1.1*10^{-19}$
HDL-c	-0.03 (-0.04 - -0.01)	0.007
NonHDL-c*:	0.14 (0.09 - 0.20)	$6.3*10^{-7}$
LDL-c*	0.02 (-0.03 - 0.07)	0.34
ApoB*	0.03 (0.01 - 0.05)	0.004

Linear regression adjusted for age and sex
* Additionally adjusted for lipid-lowering medication

patients who were at the nonHDL-c or apoB treatment target (Table 3), whereas it did not influence the proportion of patients reaching the LDL-c target of <2.5 mmol/L. In patients with two copies of the major allele and using lipid-lowering medication 55% were at the nonHDL-c target of <3.3 mmol/L, whereas in patients with 2 copies of the minor allele and using lipid-lowering medication, this was 47%. For the apoB target, 80% of the patients

Figure 2. Association between body mass index and plasma log(TG), stratified by rs964184 genotype



Black line: CC genotype, red line: CG genotype, blue line: GG genotype. Dotted lines indicate 95% confidence interval.

Table 3. Relation between rs964184 genotype and presence of metabolic syndrome or being not at lipid treatment target.

	N (n*)	OR (95% CI)	P
Metabolic syndrome	5547 (2940)	1.14 (1.03 – 1.27)	0.01
Not at LDL-cholesterol target**	3432 (1696)	0.95 (0.83 – 1.08)	0.43
Not at nonHDL-cholesterol target**	3553 (1612)	1.12 (0.98 – 1.27)	0.10
Not at apoB target**	1730 (357)	1.26 (1.01 – 1.57)	0.04

Logistic regression model, adjusted for age and sex
*Number of patients with metabolic syndrome or LDL-cholesterol / nonHDL-cholesterol / apoB not at target level
**Only in patients using lipid lowering medication

with two copies of the major allele were at treatment target as opposed to 67% of the patients with two copies of the minor allele.

Relation between rs964184 and risk for new vascular events

During 32,200 patient-years of follow-up, a total of 825 vascular events occurred. There was no relation between rs964184 and occurrence of new vascular events (HR 0.99; 95%CI 0.86-1.13; Table 4), vascular mortality (HR 1.09; 95%CI 0.92-1.30) or myocardial infarction (HR 1.00; 95%CI 0.83-1.21). After adjustment for plasma TG, the HRs decreased, consistent with an association between both rs964184 and plasma TG, and between plasma TG and vascular events.

Table 4. Relation between rs964184 and risk of new vascular events

	Myocardial infarction HR (95%CI)	Vascular mortality HR (95%CI)	All vascular events HR (95%CI)
N (events)	5547 (434)	5547 (479)	5547 (825)
Model 1	1.00 (0.83-1.21)	1.09 (0.92-1.30)	0.99 (0.86-1.13)
Model 2	0.96 (0.79-1.16)	1.05 (0.88-1.25)	0.95 (0.83-1.09)

Hazard ratio (95% confidence interval) estimated with Cox proportional hazard models
Model 1: adjusted for age and sex
Model 2: Model 1 + triglyceride plasma level

Discussion

The SNP rs964184 in the APOA5-A4-C3-A1 region is associated with plasma TG level in patients with clinically manifest vascular disease. In patients with one minor allele, this association is modified by BMI. Furthermore, rs964184 was related to the presence of metabolic syndrome, but was not related to the risk for new vascular events.

Previous studies have shown that several polymorphisms in or near the APOA5-A4-C3-A1 gene complex, among which rs964184, are associated with plasma TG level(5;7;8) and vascular risk(10) in healthy populations. Among patients with clinically manifest vascular disease, the metabolic syndrome and elevated TG levels are common (14), and the results of the present study show that rs964184 is associated with plasma TG in these patients, as well as with HDL-c, nonHDL-c and apoB. Although in this population of patients with manifest vascular disease the average TG levels may be higher than in the general population, the magnitude of the association is still similar to the association reported in the general population (5;7;8). Due to the higher level of TG-rich particles, the LDL-c levels are less accurate in estimating the atherosclerotic lipoprotein particle burden, since there is an higher concentration of atherogenic TG-rich remnant particles and since higher TG plasma levels lead to development of atherogenic small dense LDL particles(17). Therefore, the nonHDL-c levels or apoB levels may better reflect the cardiovascular risk since they include all atherogenic particles. This is demonstrated in the present study, indicating a smaller chance to be at the apoB treatment target associated with the minor allele, despite a similar proportion of patients being at the LDL-c treatment target.

The prevalence of the metabolic syndrome in carriers of the minor allele was higher and this finding is concordant with results in the general population, although the relation is less strong than in the general population (18). This may be due to the already higher prevalence of the metabolic syndrome among patients with manifest vascular disease, attenuating the contrast between groups with a different genotype. Nevertheless, the association of rs964184 with the metabolic syndrome persists, showing that also in this high risk population rs964184 does contribute to the prevalence of the metabolic syndrome. Since this SNP only selectively influences TG and HDL-c level, whereas the metabolic syndrome is seen as a clustering of risk factors related to obesity and insulin resistance, the contribution of rs964184 to the prevalence of the metabolic syndrome may be regarded as obscuring the real common basis of metabolic syndrome(18). However, since the difference in plasma TG between patients homozygous for the major allele and patients with one minor allele depends on BMI, this will still lead to clustering of obesity and plasma TG, in which obesity is the trigger for expression of increased TG level. Furthermore, these type of interactions may also explain why some obese patients are less

vulnerable to develop the metabolic consequences of obesity, as they may have a favorable genetic background. This could be one of the underlying explanations for the existence of metabolically healthy obese patients(19). Conversely, the TG levels for carriers of one minor allele may be similar to the homozygote wild type patients, provided that their BMI is low. This is in line with the model that common genetic variants combined with modifier variables (genes or other factors such as obesity and insulin resistance) together lead to elevations in plasma TG levels (4) and it is in accordance with the mechanism by which also mutations in the near APOA5 gene cause hypertriglyceridemia, requiring a second factor to express the clinical phenotype (20). Although genotype cannot be changed, the phenotype may be influenced by reducing bodyweight, and our results underscore the importance of advocating weight loss for overweight patients with hypertriglyceridemia.

Rs964184 was not associated with recurrent vascular events in the present study. A reason for the apparent absence of the association between rs964184 and vascular events could be the sample size of the study, since especially patients carrying two copies of the minor allele were infrequent and hence also few events occurred in this group (n=134 with 20 vascular events during follow up). The relatively small increase in risk of vascular events of 13% associated with the presence of rs964184 in the general population, as found in the Cardiogram GWAS, may be too small to be relevant in high-risk patients. Patients with clinically manifest vascular disease not having the risk allele may have a higher burden of other risk factors leading to a first vascular event (21). This can also be observed from the baseline table, as the frequency of the minor allele is increased in patients with coronary artery disease compared to patients with cerebrovascular disease. This may indicate that rs964184 (and hence plasma TG) is a more important risk factor for coronary artery disease than for cerebrovascular disease, and that patients without the minor allele have other risk factors, predisposing more to cerebrovascular disease.

The present results do not justify genotyping TG-associated SNPs in clinical practice, since they do not add clinically significant information to readily available characteristics and the known risk profile. However, once large scale genotyping is available at low costs, these results show that individualized treatment or lifestyle advices could be given since the effects of influencing secondary factors may depend on the genetic background.

Strengths of the study are the observational cohort reflecting clinical reality, and the extensive phenotyping of all patients according to standardized procedures, ascertainment of vascular events in this cohort and low proportion (<4%) lost to follow up. A limitation of this study is the genotyping of only one TG-associated SNP. Furthermore, the use of lipid-lowering medication during follow up may have changed and may thus potentially influence the associations between the SNP and plasma TG or vascular events.

However, the effect of statins on plasma TG is small and adjustment for baseline use of lipid-lowering medication did not influence the results. Drugs specifically targeting plasma TG, such as fibrates, were used by very few patients (0.8%).

In conclusion, rs964184 in the APOA5 region is associated with elevated TG plasma levels and presence of the metabolic syndrome in patients with clinically manifest vascular disease. Although this association translated into a decreased probability to be at apoB treatment target and a trend towards being not at nonHDL-c treatment target, there was no relation between rs964184 and the risk for new vascular events in these patients.

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Chapter 4

LDL-c linked SNPs are associated with LDL-c and myocardial infarction despite lipid-lowering therapy in patients with established vascular disease

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Abstract

Background Several single nucleotide polymorphisms (SNPs) are associated with both plasma low-density lipoprotein cholesterol (LDL-c) level and coronary artery disease in the general population. It is unclear whether these associations also apply to patients with vascular disease, and whether the associations are independent of lipid-lowering therapy.

Design SNPs associated with plasma LDL-c and vascular risk in the general population (rs11206510(*PCSK9*), rs1122608(*LDLR*), rs579459(*ABO*) and rs599839(*SORT1*)) were genotyped in a prospective cohort study of 5482 patients with vascular disease. We determined the association between LDL-c associated alleles and plasma LDL-c levels and risk of new vascular events.

Results All tested SNPs were associated with LDL-c plasma levels with a magnitude between +0.06(95%CI 0.02–0.10) mmol/L and +0.14(95%CI 0.09–0.18) mmol/L per LDL-c increasing allele. The associations were independent of use of lipid-lowering medication, except for rs579459, for which the association was not present in patients using lipid-lowering medication. In patients with 7-8 risk alleles for these SNPs, 59% of the patients treated with lipid-lowering medication did not reach the LDL-c target of <2.5 mmol/L compared with 45% in patients with 3 or fewer risk alleles. LDL-c increasing alleles were not associated with increased risk of vascular events in patients not using lipid-lowering medication (HR 1.01, 95%CI 0.93–1.09). In patients using lipid-lowering medication the risk for myocardial infarction increased with 14%(HR 1.14, 95%CI 1.01-1.28) per allele.

Conclusions In patients with established vascular disease, the studied SNPs increase LDL-c plasma levels. LDL-c increasing alleles may be associated with increased risk of myocardial infarction in patients treated with lipid-lowering medication, but not in patients not treated with lipid-lowering medication.

Introduction

Multiple single nucleotide polymorphisms (SNPs) are associated with low-density lipoprotein cholesterol (LDL-c) plasma levels in genome-wide association studies (GWAS) in the general population(1-5). In the recent meta-analysis of GWAS for coronary artery disease (CAD) from the Cardiogram Consortium, 4 LDL-c related SNPs were associated with CAD at genome wide significance(6): rs11206510, located near *PCSK9*; rs1122608, located near *LDLR*; rs579459, located near *ABO*; and rs599839, located near *SORT1*. Although other LDL-c related SNPs were associated with CAD in GWAS for plasma lipids, this was not at genome-wide significance(3). Individually, LDL-c related SNPs are associated with relatively small changes in LDL-c levels, but collectively, they can contribute to hyperlipidemia in the population at large(3). Moreover, the genetic associations represent lifelong exposure to increased LDL-c levels, which are associated with larger increases in risk of CAD than the corresponding increases in LDL-c(7;8). However, due to treatment to target, the effects of genetic variants on LDL-c could be smaller in patients with established vascular disease, who are mostly treated with lipid-lowering medication. Moreover, also the relation with vascular events may be different, since patients without the risk allele may have other risk factors that caused the first event and also increase the risk for recurrent events.

In the present cohort study in patients with established vascular disease, we evaluated the association between 4 SNPs related to LDL-c and CAD in the general population and LDL-c levels, and whether this relation is independent of treatment with lipid-lowering medication. Furthermore, we analyzed the association between these SNPs and recurrent vascular events in patients with and without lipid-lowering medication.

Methods

Patients

We used data from the Second Manifestation of ARTerial disease (SMART) cohort, an ongoing, prospective cohort study designed to establish the presence of concomitant arterial diseases and risk factors for atherosclerosis in patients with known arterial disease or cardiovascular risk factors (hyperlipidemia, hypertension or diabetes). For the present study, only patients with known arterial disease were selected. The Institutional Review Board of the University Medical Center Utrecht approved the study. Eligible patients newly referred to the University Medical Center Utrecht were asked to participate. Patients with a terminal malignancy, patients not sufficiently fluent in Dutch, patients not independent in daily activities or patients referred back to the referring specialist immediately after one

visit were excluded. After written informed consent was obtained, patients underwent a vascular screening protocol including a health questionnaire, laboratory measurements and physical examination. A detailed description of this study has been published previously(9). Reporting of the study conforms to STROBE statement (10;11)

Follow-up

Follow-up duration was defined as the period between study inclusion and first cardiovascular event or death from any cause, date of loss to follow-up or the preselected date of 1 March 2011. During follow-up, all study participants received a questionnaire every 6 months to obtain information about hospitalizations and outpatient clinic visits. If a participant reported a possible event, all available relevant data were collected. Death of a participant was reported by relatives, the general practitioner or the specialist who treated the participant, after which all available relevant data were collected regarding the cause of death. All events were classified independently by three members of the SMART Study Endpoint Committee, comprising physicians from different departments. Outcomes of interest for this study were myocardial infarction, vascular death and a composite of myocardial infarction, ischemic stroke and vascular death (definitions as shown in Supplementary table 1).From 1996 until March 2011, 198 patients were lost to follow-up (3.6%).

Laboratory measurements

Fasting plasma total cholesterol and triglycerides were measured with commercial enzymatic dry chemistry kits (Johnson and Johnson). High-density lipoprotein cholesterol (HDL-c) in plasma was determined using a commercial enzymatic kit (Boehringer-Mannheim) after precipitation of LDL-c and very low-density lipoprotein cholesterol with sodium phosphotungstate magnesium chloride. LDL-c was calculated with the Friedewald formula. Calculated values of <0.5 mmol/L were regarded as unreliable and not used for analyses.

LDL-c was calculated with the Friedewald formula up to a plasma TG level of 9 mmol/L, in line with data showing that the Friedewald formula may be used up to this level(12), to reduce missing values in the analyses.

Table 1. Baseline characteristics, stratified by the number of LDL-c increasing alleles

Number of risk alleles N	0-3 461	4 1081	5 1797	6 1521	7-8 622
Age (years)	60.8 ± 10.6	59.9 ± 10.3	60.1 ± 10.1	59.9 ± 10.4	59.7 ± 10.6
Male sex, n(%)	329 (71)	817 (76)	1337 (74)	1137 (75)	466 (75)
BMI (kg/m^2)	26.8 ± 3.9	26.8± 4.0	26.8 ± 3.9	26.8 ± 3.9	26.8 ± 3.9
Type 2 DM, n(%)	81 (18)	175 (16)	280 (16)	255 (17)	124 (20)
Total cholesterol (mmol/L)	4.9 ± 1.2	4.8 ± 1.1	4.9 ± 1.2	5.0 ± 1.3	5.1 ± 1.3
LDL-c (mmol/L)	2.9 ± 1.0	2.8 ± 1.0	2.9 ± 1.0	3.0 ± 1.0	3.2 ± 1.1
HDL-c (mmol/L)	1.2 ± 0.4	1.2 ± 0.4	1.2 ± 0.4	1.2 ± 0.4	1.2 ± 0.4
Triglycerides (mmol/L)	1.8 ± 1.3	1.6 ± 1.0	1.7 ± 1.4	1.8 ± 1.8	1.7 ± 1.1
Systolic blood pressure (mmHg)	144 ± 23	141 ± 21	141 ± 21	141 ± 20	141 ± 21
Creatinine (µmol/L)	95 ± 44	95 ± 46	94 ± 40	93 ± 34	96 ± 49
eGFR* (mL / min / 1.73 m^2)	75 ± 20	76 ± 18	75 ± 17	76 ± 18	76 ± 18
Current smoking, n(%)	155 (34)	348 (32)	572 (32)	509 (34)	205 (33)
Localisation of vascular disease, n(%)					
- Coronary arteries	244 (53)	636 (59)	1102 (61)	954 (63)	382 (61)
- Cerebrovascular	159 (35)	332 (31)	493 (27)	421 (28)	163 (26)
- Peripheral arteries	90 (20)	203 (19)	351 (20)	309 (20)	147 (24)
- AAA	47 (10)	93 (9)	144 (8)	130 (9)	67 (11)
Use of lipid-lowering agents, n(%)	241 (52)	668 (62)	1174 (65)	1026 (68)	409 (66)
- Intensive lipid-lowering treatment, n(%)	115 (25)	337 (31)	593 (33)	526 (35)	207 (33)

Data shown as mean ± standard deviation for continuous variables and n (%) for categorical variables.

* Glomerular Filtration Rate, estimated by the Modification of Diet in Renal Disease (MDRD) equation

Abbreviations: AAA: aneurysm of the abdominal aorta; BMI: body mass index; DM: diabetes mellitus; eGFR: estimated glomerular filtration rate; HDL-c: high-density lipoprotein cholesterol; LDL-c: low-density lipoprotein cholesterol

Genotyping

Wet-lab genotyping for single nucleotide polymorphism (SNP) analysis was carried out by KBiosciences, Hertfordshire, UK (www.kbioscience.co.uk), whose personnel were blinded to patient status, using their proprietary KASPar PCR technique and Taqman. Genotype

calling was carried out using an automated system, the results of which were checked manually by study personnel with SNPviewer software.

SNPs that were genotyped and included in analyses were: 1)rs11206510, near *PCSK9*, its minor allele is associated with lower LDL-c levels in several GWAS(1;2;4); 2) rs1122608, adjacent to *LDLR* in intron 30 of *SMARC4A*, its minor allele is associated with lower LDL-c levels(6); 3)rs579459, near *ABO*, its minor allele was associated with increased LDL-c in the Cardiogram consortium GWAS, although rs579459 is also associated with plasma level of P- and E-selectin(6;13) and a different SNP in ABO was found in a GWAS for LDL-c(3); 4)rs599839, near *SORT1,* its minor allele is associated with decreased LDL-c in GWAS(1). Individuals with a low overall genotyping rate were excluded(n=73). The number of missing values was 80(1.4%) for rs11206510, 78(1.4%) for rs1122608, 50(0.9%) for rs579459 and 50(0.9%) for rs599839. Patients for which SNPs were missing were excluded from the analyses, since no total number of LDL-c linked SNPs could be calculated, resulting in 5482 patients available for the analyses.

Use of lipid-lowering medication

At baseline, type and dose of lipid-lowering therapy was registered for all participants. We analyzed the effect of the SNPs also separately in patients using intensive lipid-lowering medication, which was defined as medication lowering LDL-c with ≥40%. Theoretical LDL-c reduction per type and dose of lipid-lowering therapy was determined based on (systematic) reviews and meta-analyses to the efficacy of statins and other lipid-lowering drugs(14-16). This implies that pravastatin and fluvastatin are not considered intensive lipid-lowering medication, whereas rosuvastatin at any dose is considered intensive lipid-lowering therapy. Atorvastatin 20-80mg and simvastatin 40-80mg are also considered intensive lipid-lowering therapy. Furthermore, we accounted for the combined use of lipid-lowering drugs: e.g. simvastatin 10 mg in combination with ezetimibe 10 mg was also considered intensive lipid-lowering therapy.

Data analyses

For every patient, we calculated the number of LDL-c increasing alleles, thus creating a 'genomic LDL-c score' (Supplementary table 1).

The association of separate SNPs with LDL-c was estimated per allele using a linear regression model adjusted for age and sex. To account for lipid-lowering medication, we added a sensible constant to the LDL-c level, which was 1.0 mmol/L, or 1.5 mmol/L if patients used intensive lipid-lowering therapy, since this will induce less bias than adjusting for

medication use in the linear model(17). In a sensitivity analysis, patients with a calculated LDL-c despite a triglyceride level of >4.5 mmol/L were excluded. Furthermore, we checked whether these SNPs also influenced plasma HDL-C or triglycerides. Subsequently, a logistic regression model was used to evaluate whether the number of patients who were at the LDL-c target of 2.5 mmol/L decreased with an increasing number of risk alleles.

To compute hazard ratios (HRs) for the occurrence of vascular events per LDL-c increasing allele, a Cox proportional hazards model was constructed, adjusting for age and sex (model 1). To evaluate whether any observed association with vascular events was due to the association with LDL-c, in model 2 LDL-c was added to model 1. In the Cox proportional hazards model, an interaction term of lipid-lowering medication * number of LDL-c increasing alleles was added to test for effect modification by lipid-lowering therapy and to calculate stratum-specific hazard ratios. For comparison, the association between plasma LDL-c level and vascular events was evaluated, using a Cox proportional hazards model adjusted for age, gender, localization of vascular disease, HDL-c, plasma triglycerides, smoking and body mass index. Patients were censored if lost to follow-up.

The proportional hazards assumption was tested using scaled Schoenfeld residuals, confirming proportional hazards.

The open source software program R 2.13.2 was used for data analyses. For all analyses, $p<0.05$ was considered statistically significant.

Results

Baseline characteristics according to number of LDL-c increasing alleles are shown in Table 1. Only few patients had 0-2 (n=91) LDL-c increasing alleles or 8 LDL-c increasing alleles (n=62). Use of lipid-lowering medication and the proportion of patients with coronary artery disease increased, whereas the proportion of patients with cerebrovascular disease decreased with an increasing number of LDL-c increasing alleles. The overall minor allele frequency was 19% for rs11206510(*PCSK9*), 24% for rs1122608(*LDLR*), 21% for rs579459(*ABO*) and 22% for rs599839(*SORT1*).

Table 2 compares baseline characteristics stratified by use of lipid-lowering therapy. The mean number of LDL-c increasing alleles was 5.0±1.2 in patients not using lipid-lowering medication and 5.2±1.2 in patients using lipid-lowering medication (difference 0.2, 95%CI 0.13-0.27; $p=1.24x10^{-7}$).

Table 2. Baseline characteristics, stratified by use of lipid-lowering medication

Lipid-lowering medication N	No 1964	Yes 3518
Age (years)	60.2 ± 11.4	60.0 ± 9.6
Male sex, n(%)	1392 (71)	2694 (77)
BMI (kg/m^2)	26.2 ± 4.0	27.1 ± 3.8
Type 2 DM, n(%)	268 (14)	647 (18)
LDL-c (mmol/L)	3.6 ± 1.0	2.6 ± 0.9
HDL-c (mmol/L)	1.2 ± 0.4	1.2 ± 0.4
Triglycerides (mmol/L)	1.8 ± 1.7	1.7 ± 1.3
Systolic blood pressure (mmHg)	143 ± 22	140 ± 21
eGFR* (mL / min / 1.73 m^2)	76 ± 19	76 ± 18
Current smoking, n(%)	790 (40)	999 (28)
Number of LDL-c associated alleles	5.0 ± 1.2	5.2 ± 1.2
Localisation of disease, n(%)		
- Coronary arteries	739 (38)	2579 (73)
- Cerebrovascular	685 (35)	883 (25)
- Peripheral arteries	603 (31)	497 (14)
- AAA	257 (13)	224 (6)

* Glomerular Filtration Rate, estimated by the Modification of Diet in Renal Disease (MDRD) equation

Abbreviations: AAA: aneurysm of the abdominal aorta; BMI: body mass index; DM: diabetes mellitus; eGFR: estimated glomerular filtration rate; HDL-c: high-density lipoprotein cholesterol; LDL-c: low-density lipoprotein cholesterol;

Association between LDL-c-related SNPs and LDL-c

All SNPs were associated with plasma LDL-c level, accounting for use of lipid-lowering medication (Table 3). The strongest associations were for rs1122608(*LDLR*) and rs599839(*SORT1*), the minor alleles of which were related with 0.11 mmol/L (95%CI 0.07-0.15) and 0.15 mmol/L (95%CI 0.11-0.19) lower LDL-c levels respectively when compared with the major allele. The effects were similar in patients with or without lipid-lowering medication for rs11206510, rs1122608 and rs599839, p for interaction was respectively 0.71, 0.80 and 0.52. For rs579459, there was no association with LDL-c in patients using lipid-lowering medication, whereas this association was present in patients not using lipid

Table 3. Associations between SNPs in/near *PCSK9*, *LDLR*, *ABO* and *SORT1* and plasma LDL-c levels

		All patients* (N=5482)		No lipid-lowering medication (N=1964)		Any lipid-lowering medication (N=3518)		Intensive lipid-lowering medication (N=1778)	
SNP	Major allele / minor allele	Beta (95% CI)	P	Beta (95% CI)	P	Beta (95% CI)	P	Beta (95% CI)	P
rs11206510 (PCSK9)	T/C	-0.09(-0.13 – -0.05)	$5.78*10^{-5}$	-0.07 (-0.15 – 0.01)	0.098	-0.09(-0.14 – -0.04)	$4.95*10^{-4}$	-0.07 (-0.14 – 0.00)	0.052
rs1122608 (LDLR)	G/T	-0.11(-0.15 – -0.07)	$1.12*10^{-7}$	-0.10 (-0.18 – -0.03)	0.006	-0.11(-0.15 – -0.06)	$5.14*10^{-6}$	-0.15 (-0.21 - -0.09)	$1.95*10^{-6}$
rs579459 (ABO)	T/C	0.06(0.02– 0.11)	0.003	0.13 (0.05 – 0.21)	0.002	0.02(-0.02 – 0.07)	0.294	0.01 (-0.05 – 0.08)	0.663
rs599839 (SORT1)	T/C	-0.15(-0.19 – -0.11)	$5.64*10^{-13}$	-0.15 (-0.22 – -0.07)	$1.12*10^{-4}$	-0.13(-0.17 – -0.08)	$1.24*10^{-7}$	-0.17 (-0.23 - -0.10)	$7.16*10^{-7}$

Linear regression, adjusted for age and sex. Beta given for each additional copy of the minor allele (relative to the major allele).
*Additional adjustment for lipid-lowering medication

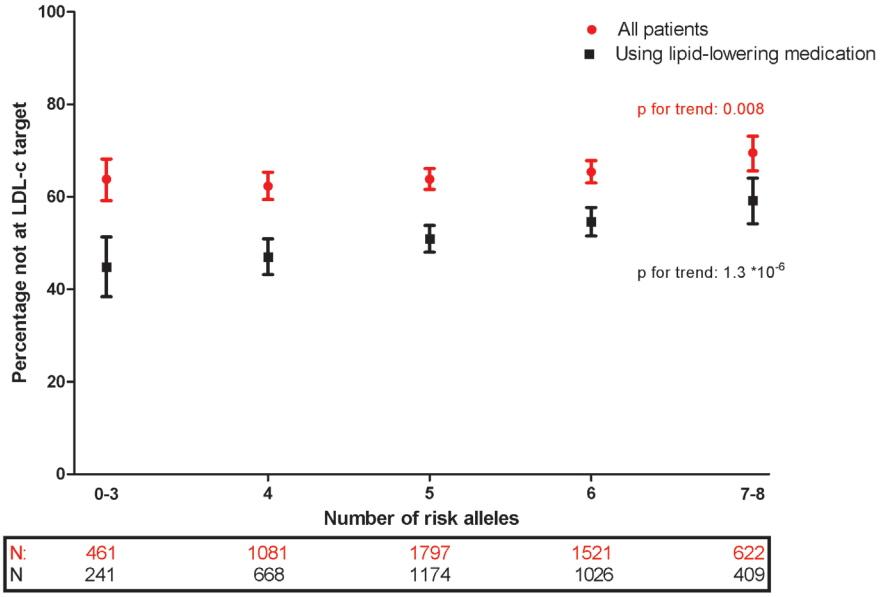
lowering medication, p for interaction was 0.03. The associations between individual SNPs and LDL-c in patients using intensive lipid-lowering therapy were comparable to those in patients using any lipid-lowering medication. Additionally, the minor allele of rs599839 was associated with higher HDL-c plasma level of 0.02 mmol/L (95%CI 0.01-0.04, p=0.008). The other SNPs were not associated with HDL-c and none were associated with plasma triglyceride levels. A sensitivity analysis including patients for whom not all SNPs could be genotyped or excluding calculated LDL-c for patients with plasma triglyceride levels of >4.5 mmol/L did not change these results.

For patients using lipid-lowering medication, the proportion not being at LDL-c treatment target of <2.5 mmol/L increased from 45% for patients with 0-3 LDL-c associated alleles to 59% for patients with 7-8 LDL-c associated alleles (p for trend 1.3 x 10^{-6}), despite a slight increase in use of intensive lipid-lowering medication from 45% to 51% (Figure 1).

Relation between LDL-c-related SNPs and vascular events

During a total of 31800 person-years of follow-up (median follow-up 5.2 years), 807 vascular events occurred. In the 1964 patients not using lipid-lowering medication, a total

Figure 1. Proportion of patients who are not at the desired LDL-c goal as a function of the number of LDL-c increasing alleles present.



of 418 vascular events occurred, in the 3518 patients using lipid-lowering medication, a total of 389 vascular events occurred.

In patients using lipid-lowering medication, there was a trend towards a higher risk of all vascular events (1.07, 95%CI 0.98-1.17)(Table 4). Furthermore, the results suggest an increased the risk of myocardial infarction with 14% per LDL-c increasing allele (HR 1.14; 95%CI 1.01-1.30) and a trend towards a higher risk for vascular death (HR 1.10, 95%CI 0.99-1.24). Additional adjustment for LDL-c, the putative mechanism through which these polymorphisms are related with vascular risk, slightly attenuated the risk. Adjustment for HDL-c, diabetes mellitus, systolic blood pressure, body mass index, smoking, and localisation of vascular disease did not affect the point estimates (data not shown). In contrast, the risk of ischemic stroke was not increased (HR 0.97, 95%CI 0.80 – 1.17). In patients not using lipid-lowering medication, there was no association between LDL-c increasing alleles and vascular events.

Similarly, plasma levels of LDL-c were associated with the composite outcome of all vascular events and with myocardial infarction in patients using lipid-lowering medication (HRs 1.25, 95%CI 1.11-1.40 and 1.30, 95%CI 1.10-1.53 per mmol/L respectively). In patients not using lipid-lowering medication, plasma level of LDL-c was not associated with vascular events (HR for the composite outcome 1.02, 95%CI 0.92-1.13).

Discussion

In patients with established vascular disease, the SNPs rs11206510(*PCSK9*), rs1122608(*LDLR*), rs579459(*ABO*) and rs599839(*SORT1*) are associated with LDL-c plasma level, and for rs11206510, rs1122608 and rs599839 this association is irrespective of lipid-lowering medication. The chance to be at the desired LDL-c target level is reduced for patients carrying more risk alleles, also in patients using lipid-lowering medication. The results suggest that the number of LDL-c increasing alleles present is associated with myocardial infarction in patients treated with lipid-lowering medication, but not in patients not treated with lipid-lowering medication.

Although previous GWAS have identified the present SNPs to be related to LDL-c and vascular risk, these results were obtained in the general population(3;4;6;18). The present study demonstrates a robust effect of these SNPs on plasma LDL-c levels in patients with established vascular disease. The changes in LDL-c levels were comparable with the reported values in the literature(1;6;18), largely independent of use of lipid-lowering medication. Only for rs579459(*ABO*) it could be argued that lipid-lowering therapy abolishes the relationship between the SNP and LDL-c. In addition, the chance of reaching

Table 4. Association between the number of LDL-c increasing alleles and risk of vascular events

Per additional copy of LDL-c increasing allele	Patients not using lipid-lowering medication			Patients using lipid-lowering medication		
	Events/n	Model 1	Model 2	Events/n	Model 1	Model 2
All vascular events	418/1964	1.01 (0.93 – 1.09)	1.00 (0.92 – 1.08)	389/3518	1.07 (0.98 – 1.17)	1.06 (0.97 – 1.16)
Myocardial infarction	207/1964	0.96 (0.86 – 1.07)	0.94 (0.84 – 1.05)	217/3518	1.14 (1.01 – 1.30)	1.13 (1.00 – 1.28)
Ischemic stroke	97/1964	1.12 (0.95 – 1.33)	1.11 (0.94 – 1.32)	101/3518	0.97 (0.80 – 1.17)	0.95 (0.78 – 1.16)
Vascular death	281/1964	0.98 (0.89 – 1.08)	0.97 (0.88 – 1.07)	182/3518	1.10 (0.99 – 1.24)	1.10 (0.99 – 1.23)

Hazard ratio (95% confidence intervals) per 1 increase in LDL-c associated alleles, stratified by use of lipid-lowering medication.
Cox proportional hazards models adjusted for age and sex (model 1) + LDL-c (model 2)
Abbreviation: LDL-c: low-density lipoprotein cholesterol

LDL-c treatment goals decreased with an increasing number of risk alleles. This illustrates the concept that multiple common genetic variants collectively contribute to polygenic dyslipidemia(3), which may impair the realization of LDL-c goals, and underscores that even small effects associated with individual SNPs could be of clinical significance.

Although all patients in our study would qualify for treatment with lipid-lowering treatment, a part of these patients was not treated at baseline. These patients were in general included earlier in this study, which started in 1996, when the use of lipid-lowering therapy was lower. In addition, in patients with coronary artery disease lipid-lowering medication was more commonly used than in patients with vascular disease at other localizations. In patients on lipid-lowering medication, the number of LDL-c increasing alleles seemed related with increased risk for myocardial infarction, and there was a trend towards higher risk for all vascular events. However, in patients not on lipid-lowering medication, there was no increased vascular risk. This could be due to a lack of power in this smaller group of patients compared with the group using lipid-lowering medication, but the number of events was similar, furthermore also plasma LDL-c levels were not associated with vascular events in these patients. Although counterintuitive, the different characteristics of patients not using lipid-lowering medication may explain this difference. In addition to the above-mentioned difference in localization of vascular disease, more patients smoked and the mean blood pressure was higher in patients not using lipid-lowering medication. Furthermore, the number of LDL-c increasing alleles was lower in the group not using lipid-lowering medication, similar to an observation in the general population(19), indicating that this genetic component of LDL-c may have played a less prominent role in the pathophysiology of the first vascular events in this group. Although these patients have a genetic background with a lower number of LDL-c associated alleles, they have developed vascular disease possibly due to risk factors such as smoking and high blood pressure, and these risk factors may drive the occurrence of new vascular events.

The number of LDL-c increasing alleles was primarily related to the occurrence of myocardial infarction, which is understandable since these SNPs were selected based on GWAS for CAD(6;20). Moreover, a recent study demonstrated that 3 out of 4 of these SNPs (rs11206510(*PCSK9*), rs1122608(*LDLR*) and rs646776, which is highly correlated to rs599839(*SORT1*) in this study) were not associated with stroke risk(21). The magnitude of the observed association of LDL-c increasing alleles and vascular events was larger than could be explained by the difference in current LDL-c levels alone, since adjustment for LDL-c only slightly attenuated the risk for vascular events. An explanation could be that the SNPs are associated with other risk factors mediating this association between the SNPs and vascular events. Rs579459(*ABO*) is not located in a known gene involved in lipid

metabolism and may operate also via different mechanisms than LDL-c, but the other SNPs are all located near genes with well-known roles in lipid metabolism. Adjustment for other known risk factors did not change our results. A larger effect of LDL-c increasing alleles than could be explained by current LDL-c levels is in line with previous studies in patients without vascular events(7;8). Most probably, this is because the SNPs are a marker of lifelong exposure to (slightly) elevated LDL-c levels, whereas the measured value gives only a value at one specific point in time(8;22). In the present study, moreover, the LDL-c level before start of the medication is not reflected in the current LDL-c level of the patients using lipid-lowering medication.

The present study was performed in an unselected group of patients with established vascular disease and the results may therefore apply for all patients with vascular disease. The present results imply that these SNPs may be of clinical significance in patients with established vascular disease as these polymorphisms affect the likelihood of realizing the desired LDL-c treatment target. The (lifelong) increase in LDL-c still seems to translate into an increased risk of myocardial infarction in patients using lipid-lowering therapy, despite the already high background risk of recurrent vascular events in patients who all have established vascular disease.

Limitations of this study include the selection of only four SNPs, whereas many more SNPs associated with LDL-c levels have been identified. However, only these four SNPs are also associated with CAD at genome-wide significance in large GWAS, which demonstrates an additional disease burden for these SNPs. A second limitation may be that LDL-c levels in this study were calculated using the Friedewald formula up to a plasma triglyceride level of 9 mmol/L, which may induce some imprecision at higher triglyceride levels. However, excluding these patients did not change the results. Furthermore, lipid-lowering medication was only registered at baseline. During follow-up, the use of lipid-lowering medication will have increased, since treatment advice was part of the screening for this study. However, the registered medication used for these analyses was used at time of measurement of plasma LDL-c level. Finally, due to the small increase in risk of cardiovascular disease per SNP, the association analyses of these SNPs with respect to recurrent vascular events may have only limited power.

In conclusion, LDL-c linked SNPs are associated with LDL-c plasma levels in patients with vascular disease, mostly independent of the use of (intensive) lipid-lowering medication. The number of LDL-c increasing alleles may be related with an increased risk of myocardial infarction in patients using lipid-lowering medication, irrespective of LDL-c plasma levels, but not in patients not using lipid-lowering medication.

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Supplementary table 1. Composition of the genomic risk score based on the number of LDL-c increasing alleles present

SNP	Genotypes	Frequency (n, %)	Score
rs11206510 (PCSK9)	TT	3621 (66%)	2
	TC	1671 (31%)	1
	CC	190 (4%)	0
rs1122608 (LDLR)	GG	3144 (57%)	2
	GT	2023 (37%)	1
	TT	315 (6%)	0
rs579459 (ABO)	TT	3365 (61%)	0
	TC	1888 (34%)	1
	CC	229 (4%)	2
rs599839 (SORT1)	TT	3378 (62%)	2
	TC	1807 (33%)	1
	CC	297 (5%)	0

Chapter 5

Plasma triglyceride levels increase the risk for recurrent vascular events independent of LDL-cholesterol or nonHDL-cholesterol

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Abstract

Background Plasma triglyceride (TG) levels are known to confer an increased risk of vascular disease in healthy populations, but data in high-risk patients are scarce. In this study we evaluated the risk on recurrent vascular events conferred by increased plasma TG levels in patients with various clinical manifestations of vascular disease.

Methods Prospective cohort study of 5731 patients with clinically manifest vascular disease.

Results First new vascular events (myocardial infarction, ischemic stroke, vascular death) occurred in 782 subjects during a median follow-up of 4.9 years (interquartile range 2.5–8.1 years). Patients in the highest plasma TG quintile (>2.24 mmol/L) had a higher risk for recurrent vascular events (HR 1.45; 95%CI 1.13-1.86) compared with the lowest plasma TG quintile (<0.97 mmol/L) after adjustments for age, gender, body mass index, smoking, lipid-lowering medication and low-density lipoprotein-cholesterol. The increased risk associated with increasing plasma TG levels was irrespective of the presence of type 2 diabetes (T2DM), but only present in patients without the metabolic syndrome. Furthermore, the increased risk was particularly present in patients with coronary artery disease (CAD) (HR 1.45; 95%CI 1.02-2.08) and was not modified by other lipid levels (p-value for interaction >0.05). Plasma TG still contributed to vascular risk when other lipid levels were at target level.

Conclusions Higher plasma TG levels are associated with increased risk for recurrent vascular events, in particular in CAD patients. This increased risk is independent of the presence of T2DM and the use of lipid-lowering medication and is not modified by other lipid levels.

Introduction

Patients with clinical manifestations of vascular diseases are at high risk for new vascular events, and due to improved medical treatment and an ageing population, their number is increasing. Although these patients are treated intensively for cardiovascular risk factors, they are still at considerable risk for a recurrent vascular event. Due to an increasing prevalence of obesity and obesity-related insulin resistance, the number of individuals with hypertriglyceridemia is rising(1;2). Elevated plasma triglyceride (TG) levels lead to an atherogenic lipoprotein phenotype consisting of high plasma TG levels, low high-density lipoprotein cholesterol (HDL-C) levels and small dense low-density lipoprotein (LDL) particles(3). Therefore, hypertriglyceridemia could contribute to the residual risk and it would be important to assess the risk associated with plasma TG levels on recurrent vascular events in these high-risk patients against a background of well treated risk factors, including widespread use of statin therapy.

Plasma TG level has been shown to be an independent risk factor for vascular events in healthy populations(4;5) and in trials with patients with coronary artery disease (6;7). However, drugs lowering plasma TG levels have not clearly proven to be effective in preventing vascular events (8;9). Therefore TG levels are not defined as a treatment target in current guidelines, although a TG level of <1.7 mmol/L is propagated as desirable(10;11). Instead, non-high density lipoprotein-cholesterol (nonHDL-C) is advocated as a treatment target in patients with hypertriglyceridemia, as nonHDL-C, contrary to LDL-C, also includes TG-rich VLDL-particles (10;11). However, it is unclear whether plasma TG levels by itself still add to the risk associated with higher nonHDL-C levels.

In the present study the risk of plasma TG levels for recurrent vascular events in patients with different manifestations of arterial vascular disease was determined. Furthermore, it was evaluated whether this association was present irrespective of the presence of type 2 diabetes (T2DM) or the metabolic syndrome, the use of lipid-lowering medication, and the location of vascular disease. Finally, modification of the effect of plasma TG levels on vascular events by the level of several other lipoproteins (LDL-C, nonHDL-C, HDL-C and total cholesterol(TC)/HDL-C ratio) was investigated.

Methods

Patients

For the present study, data were used from the Second Manifestations of ARTerial disease (SMART) cohort. This is a prospective, ongoing cohort study, designed to establish the presence of concomitant arterial diseases and risk factors for atherosclerosis in patients with known arterial disease or a cardiovascular risk factor, who were newly referred to the University Medical Center Utrecht. All patients gave written informed consent, and the Medical Ethics Committee of the University Medical Center Utrecht approved the study. After informed consent, patients underwent a vascular screening protocol including a health questionnaire, laboratory measurements and physical examination. A detailed description of this study has been published previously(12).

For the present study, we used data of 5746 patients, enrolled in the SMART study between September 1996 and March 2010, with either a history or recent diagnosis of clinically manifest arterial disease: coronary artery disease (CAD) (n=3448), cerebrovascular disease (n=1612), peripheral artery disease and/or aneurysm of the abdominal aorta(AAA) (n=1603). Patients could be classified into more than 1 disease category. Patients with plasma TG ≥10 mmol/L (n=15) were excluded since this most likely results from a rare genetic cause. Hence the study population consisted of 5731 patients.

Single imputation methods were used to reduce missing covariate data for smoking (n=23; 0.4%), body mass index (BMI) (n=8; 0.1%), TC (n=3; 0.1%) and HDL-C (n=9; 0.2%), since complete case analysis leads to loss of statistical power and to bias.

Laboratory assessment

Baseline lipid levels were obtained from fasting patients. Plasma TC and TG were measured using commercial enzymatic dry chemistry kits (Johnson and Johnson). HDL-C in plasma was determined using a commercial enzymatic kit (Boehringer-Mannheim) after precipitation of LDL-C and VLDL-C with sodium phosphotungstate magnesium chloride. LDL-C was calculated using the Friedewald formula up to a plasma TG level of 9 mmol/L, which is in line with data showing that the Friedewald formula can be used up to this level(13). Calculated LDL-C levels < 0.5 mmol/L were regarded as unreliable and not used for analyses. As a result, LDL-C levels could be calculated for 5718 of 5731 patients (99.8%).

Follow-up

During follow-up, all study participants received a questionnaire every 6 months to obtain information about hospitalizations and outpatient clinic visits. If the participants reported a possible event, all available relevant data were collected. Death of a participant was reported by relatives, the general practitioner or the specialist who treated the participant. All events were classified independently by three members of the SMART Study Endpoint Committee, comprising physicians from different departments. Outcomes of interest for this study were vascular death, myocardial infarction or ischemic stroke, and the composite of these vascular events. Follow-up duration was defined as the period between study inclusion and first cardiovascular event or death from any cause, date of loss to follow-up or the preselected date of 1 March 2010.

Data analysis

Cox proportional hazard models were used to calculate hazard ratios (HR) and corresponding 95% confidence intervals (CI) for the occurrence of vascular events associated with plasma TG levels. If a patient had multiple events, the first recorded event was used in the analyses. Patients were censored if they were lost to follow-up. Plasma TG levels were divided into quintiles, the lowest quintile served as reference. Three models were built, model I with adjustment for age and gender and model II with additional adjustments for BMI, use of lipid-lowering medication, LDL-C and smoking, all potential confounders in the relationship between plasma TG and vascular events. In order to study the effect of plasma TG on recurrent vascular events independent of plasma HDL-C, additional adjustment for HDL-C was made in model III.

Subgroup analyses were performed stratified for the presence of T2DM and the metabolic syndrome (as defined according to the NCEP criteria (10)) and in patients with CAD, CVD and PAD/AAA separately. Because some patients fell into more than one vascular disease-manifestation category, adjustments were made for the presence of other vascular diseases in each category. For the same reason, effect modification by vascular disease-manifestation category could not be statistically tested. Furthermore, subgroup analyses were performed in strata of low versus high levels of several lipoproteins (LDL-C, nonHDL-C, TC/HDL-C ratio and HDL-C), to assess whether the effect of TG was still present when these lipoprotein levels are optimal according to current guidelines. For LDL-C, a cut-off of <2.5 mmol/L (97 mg/dL) was defined as optimal and for nonHDL-C, a cut-off of <3.3 mmol/L was defined as optimal, according to European guidelines(11). For HDL-C the NCEP ATP III cut-offs for metabolic syndrome were used: >1.03 mmol/L (40 mg/dL) in men

and >1.29 mmol/L (50 mg/dL) in women(10). An optimal TC/HDL-C ratio was defined as <5. For these analyses, TG was log transformed to have a normal distribution and used as a continuous variable.

In order to test for interaction, i.e. whether the relation between plasma TG levels and vascular events was modified by LDL-C, nonHDL-C, HDL-C or TC/HDL category, we included these interaction terms in the Cox model. If the p-value of the interaction term was <0.05, effect-modification was considered present.

Analyses were performed using the statistical package Predictive Analytics SoftWare (PASW) Statistics 18.0. For all analyses, P <0.05 was considered significant.

Results

Baseline characteristics

Baseline characteristics of the study population according to quintiles of plasma TG are presented in Table 1. The prevalence of the metabolic syndrome increases from 14% in quintile 1 to 80% in quintile 5. With increasing quintiles of plasma TG, the proportion of current smokers increased, paralleled by an increase in the incidence of peripheral artery disease.

Plasma TG levels and risk of new vascular events

During a median follow-up of 4.9 years (interquartile range 2.5–8.1 years), 782 first new vascular events occurred. Overall, 193 (non-)fatal ischemic strokes, 451 (non-)fatal myocardial infarctions and 473 vascular deaths occurred.

As shown in Table 2, the risk of vascular events increased across quintiles of plasma TG and was 45% higher in the highest quintile (HR 1.45; 95%CI 1.13-1.86) compared to the lowest quintile (model 2) for the combined endpoint. P for trend across quintiles was 0.002. The risk for ischemic stroke was 47% higher in the highest TG quintile (HR 1.47; 95%CI 0.89–2.42) compared to the lowest TG quintile, the risk for myocardial infarction 56% higher (HR 1.56; 95%CI 1.11-2.18) and the risk for vascular death 48% higher (HR 1.48; 95%CI 1.07-2.05). Additional adjustment for HDL-C (model 3), considerably weakened the effect of plasma TG on all vascular endpoints (HR for all vascular events: 1.22; 95%CI 0.93-1.60, highest quintile compared to lowest quintile). Additional adjustment for nonHDL-C instead of LDL-C in model 2 only slightly attenuated the effect of plasma TG on vascular events (data not shown).

Table 1. Baseline characteristics of the study population according to quintiles of plasma triglycerides (TG).

	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5
Range TG (mmol/L) Median TG (mmol/L)	<0.97 0.80	0.97-1.24 1.10	1.25-1.60 1.41	1.61-2.24 1.88	>2.24 2.90
N	1163	1133	1158	1131	1146
Age (years)	60.5 ± 11.1	60.8 ± 10.2	61.0 ± 10.3	59.5 ± 10.3	58.3 ± 9.8
Male gender, n (%)	843 (73)	820 (72)	855 (74)	813 (72)	914 (80)
BMI (kg/m^2)	25.6 ± 3.7	26.1 ± 3.6	26.9 ± 4.0	27.6 ± 4.0	28.0 ± 4.1
Waist circumference (cm)	91 ± 12	93 ± 11	96 ± 12	98 ± 11	100 ± 11
Systolic blood pressure (mmHg)	141 ± 21	140 ± 2	142 ± 20	140 ± 20	143 ± 20
Diastolic blood pressure (mmHg)	79 ± 10	79 ± 10	80 ± 10	79 ± 10	81 ± 10
Total cholesterol (mmol/L)	4.3 ± 1.0	4.7 ± 1.1	4.9 ± 1.1	5.2 ± 1.1	5.6 ± 1.2
HDL-C (mmol/L)	1.4 ± 0.4	1.3 ± 0.4	1.2 ± 0.3	1.1 ± 0.3	1.0 ± 0.3
LDL-C (mmol/L)	2.5 ± 0.9	2.8 ± 1.0	3.1 ± 1.0	3.2 ± 1.0	3.1 ± 1.2*
NonHDL-C (mmol/L)	2.9 ± 0.9	3.3 ± 1.0	3.7 ± 1.0	4.0 ± 1.0	4.6 ± 1.2
Creatinine (µmol/L)	89 ± 25	92 ± 42	95 ± 39	95 ± 34	100± 55
MDRD-GFR (mL / min / 1.73 m^2)	78 ± 17	77 ± 17	74 ± 17	75 ± 18	75 ± 20
Type 2 diabetes mellitus, n (%)	147 (13)	148 (13)	174 (15)	207 (18)	281 (25)
Homa-IR†	2.1 ± 1.4	2.6 ± 2.0	3.0 ± 2.0	3.3 ± 2.3	4.0 ± 3.1
Metabolic syndrome NCEP ATP III, n (%)	165 (14)	214 (19)	319 (28)	741 (66)	912 (80)
Current smoking, n (%)	312 (27)	328 (29)	387 (33)	399 (35)	475 (41)
Use of alcohol in the last year, n (%)	882 (76)	813 (72)	805 (70)	762 (67)	790 (69)
Antiplatelet/anticoagulant agents, n (%)	986 (85)	929 (82)	953 (82)	916 (81)	890 (78)
Use of blood pressure lowering agents, n (%)	804 (69)	833 (73)	846 (73)	858 (76)	858 (75)
Use of lipid-lowering agents, n (%)	811 (70)	755 (67)	707 (61)	711 (63)	657 (57)
Localisation of vascular disease‡					
Coronary arteries, n (%)	681 (59)	691 (61)	699 (60)	688 (61)	689 (60)
Cerebrovascular, n (%)	390 (34)	315 (28)	331 (29)	283 (25)	305 (27)
Aneurysm abdominal aorta, n (%)	77 (7)	98 (9)	107 (9)	112 (10)	119 (10)
Peripheral arteries, n (%)	164 (14)	208 (18)	233 (20)	264 (23)	312 (27)

Continuous variables are shown as mean ± standard deviation
*Calculated with Friedewald formula up to plasma TG 9 mmol/L
†Only measured from July 2003 onwards
‡Patients could be classified in more than 1 vascular disease category
Abbreviations: BMI: body mass index, HDL-C: high-density lipoprotein-cholesterol, HOMA-IR: homeostatic model assessment-insulin resistance, LDL-C: low-density lipoprotein-cholesterol, MDRD-GFR: modification of diet in renal disease-glomerular filtration rate.

Table 2. Risk of new vascular events in quintiles of plasma TG

TG range (mmol/L) N=5731			Quintile 1 <0.97 1163	Quintile 2 0.97-1.24 1133	Quintile 3 1.25-1.60 1158	Quintile 4 1.61-2.24 1131	Quintile 5 >2.24 1146
		Model					
Ischemic stroke	# events		24	29	39	44	57
		I	Reference	1.04 (0.60-1.78)	1.23 (0.74-2.30)	1.39 (0.85-2.30)	1.75 (1.08-2.84)*
		II	Reference	0.96 (0.56-1.65)	1.06 (0.63-1.79)	1.23 (0.74-2.06)	1.47 (0.89-2.42)
Myocardial infarction	# events		52	75	99	100	125
		I	Reference	1.22 (0.85-1.73)	1.39 (0.99-1.94)	1.43 (1.02-2.00)*	1.67 (1.20-2.31)**
		II	Reference	1.18 (0.83-1.68)	1.31 (0.93-1.84)	1.36 (0.96-1.92)	1.56 (1.11-2.18)*
Vascular death	# events		55	80	112	98	128
		I	Reference	1.18 (0.84-1.67)	1.35 (0.97-1.86)	1.27 (0.91-1.77)	1.62 (1.18-1.77)**
		II	Reference	1.13 (0.80-1.59)	1.24 (0.89-1.72)	1.19 (0.85-1.67)	1.48 (1.07-2.05)*
All vascular events	# events		96	134	170	169	213
		I	Reference	1.17 (0.90-1.52)	1.29 (1.00-1.65)	1.32 (1.02-1.69)*	1.59 (1.25-2.03)**
		II	Reference	1.11 (0.86-1.45)	1.18 (0.91-1.52)	1.22 (0.94-1.58)	1.45 (1.13-1.86)*

Hazard ratio (95% confidence interval), adjusted for:
Model I: age and gender
Model II: Model I + smoking, lipid-lowering medication, BMI and LDL-C
*: $p < 0.05$; **: $p < 0.01$
Abbreviations: as in Table 1.

In patients without T2DM (n=4774), plasma TG in the highest quintile increased the risk (HR 1.47; 95%CI 1.11-1.96) for all vascular events compared plasma TG in the lowest quintile. In patients with T2DM (n=957), the risk for all vascular events was only slightly higher in quintile 5 compared to quintile 1 (HR 1.11; 95%CI 0.65-1.91), and the association between plasma TG and vascular events seemed slightly u-shaped. When analyzed continuously, the HR for all vascular events was similar for both groups (HR1.17; 95%CI 0.86-1.58 in T2DM vs. HR 1.23 ;95%CI 1.04-1.46) and p for interaction was 0.894. Plasma TG did increase the risk for all vascular events in patients without the metabolic syndrome (HR 1.29; 95%CI 1.00 – 1.66, p=0.047 for log-TG), but not in patients with the metabolic syndrome (HR 0.94; 95%CI 0.74-1.18, p=0.584 for log-TG); p for interaction by the metabolic syndrome was 0.051. HOMA-IR was only available in patients included from 2003 onwards and only few events occurred in this group. However, when additional adjustment was made for HOMA-IR in the non-diabetic patients, the hazard ratio for TG (log-transformed) was 1.28 (0.88 – 1.87). Although this result was not significant due to the small group size, the risk estimate is similar to the risk estimate in the entire group.

Plasma TG levels and risk of new vascular events according to the localization of vascular disease

In patients with coronary artery disease, increasing levels of TG were most clearly associated with increased risk of new vascular events (Table 3). Compared to quintile 1, the risk of new vascular events was increased with 45% in quintile 5 (HR 1.45; 95%CI 1.02-2.08). P for trend across quintiles was 0.027. After adjustment for HDL-C (model 3), higher TG levels the increase in risk did not reach statistical significance anymore (HR 1.34; 95%CI 0.92-1.96). In patients with cerebrovascular disease the risk in quintile 5 was slightly increased compared to quintile 1, although this was not statistically significant. Moreover, there was no clear trend across quintiles (p for trend 0.315). In peripheral artery disease/ AAA there was no relation between TG plasma levels and risk of future vascular events.

Plasma TG levels and risk of new vascular events according to baseline levels of LDL-C, nonHDL-C, HDL-C and TC/HDL-C ratio

As displayed in Table 4, higher plasma log-TG levels were still associated with increased risk of new vascular events when other lipids are at target level. Although the increase in risk with increasing plasma log-TG was statistically significant in some strata and not in others, the overall effect was not modified by either high or low stratum. When stratified for LDL-C, nonHDL-C, HDL-C and TC/HDL-C ratio, p for interaction was 0.472, 0.247,

Table 3. Risk of new vascular events in quintiles of plasma TG according to localization of vascular disease.

TG range (mmol/L)			Quintile 1 <0.97	Quintile 2 0.97-1.24	Quintile 3 1.25-1.60	Quintile 4 1.61-2.24	Quintile 5 >2.24
		Model					
Patients with coronary artery disease (n=3448)	# events / N		45/681	71/691	89/699	98/688	122/689
		I	Reference	1.30 (0.89-1.88)	1.36 (0.94-1.95)	1.55 (1.08-2.20)*	1.61 (1.14-2.28)**
		II	Reference	1.22 (0.84-1.78)	1.24 (0.86-1.79)	1.43 (0.99-2.05)	1.45 (1.02-2.08)*
Patients with cerebrovascular disease (n=1624)	# events / N		34/390	41/315	71/331	60/283	70/305
		I	Reference	1.19 (0.76-1.88)	1.47 (0.98-2.23)	1.45 (0.95-2.22)	1.38 (0.91-2.10)
		II	Reference	1.10 (0.70-1.76)	1.30 (0.85-1.99)	1.27 (0.81-1.97)	1.25 (0.81-1.92)
Patients with peripheral artery disease / AAA (n=1603)	# events / N		39/235	62/286	78/323	74/353	110/406
		I	Reference	0.96 (0.64-1.44)	1.02 (0.70-1.43)	0.97 (0.66-1.43)	1.13 (0.78-1.64)
		II	Reference	0.95 (0.63-1.42)	1.00 (0.68-1.47)	0.99 (0.66-1.47)	1.15 (0.79-1.69)

Hazard ratio (95% confidence interval), adjusted for:
Model I: age and gender + vascular disease at other localization
Model II: Model I + smoking, lipid-lowering medication, BMI and LDL-C
* $p < 0.05$
** $p < 0.01$
Vascular events: composite of myocardial infarction, ischemic stroke and vascular death
Patients could be classified in more than 1 vascular disease category
Abbreviations: AAA: aneurysm of the abdominal aorta. Further as in Table 1.

0.774 and 0.151 respectively, which implies that the risk conferred by TG is not different regardless of other lipid levels.

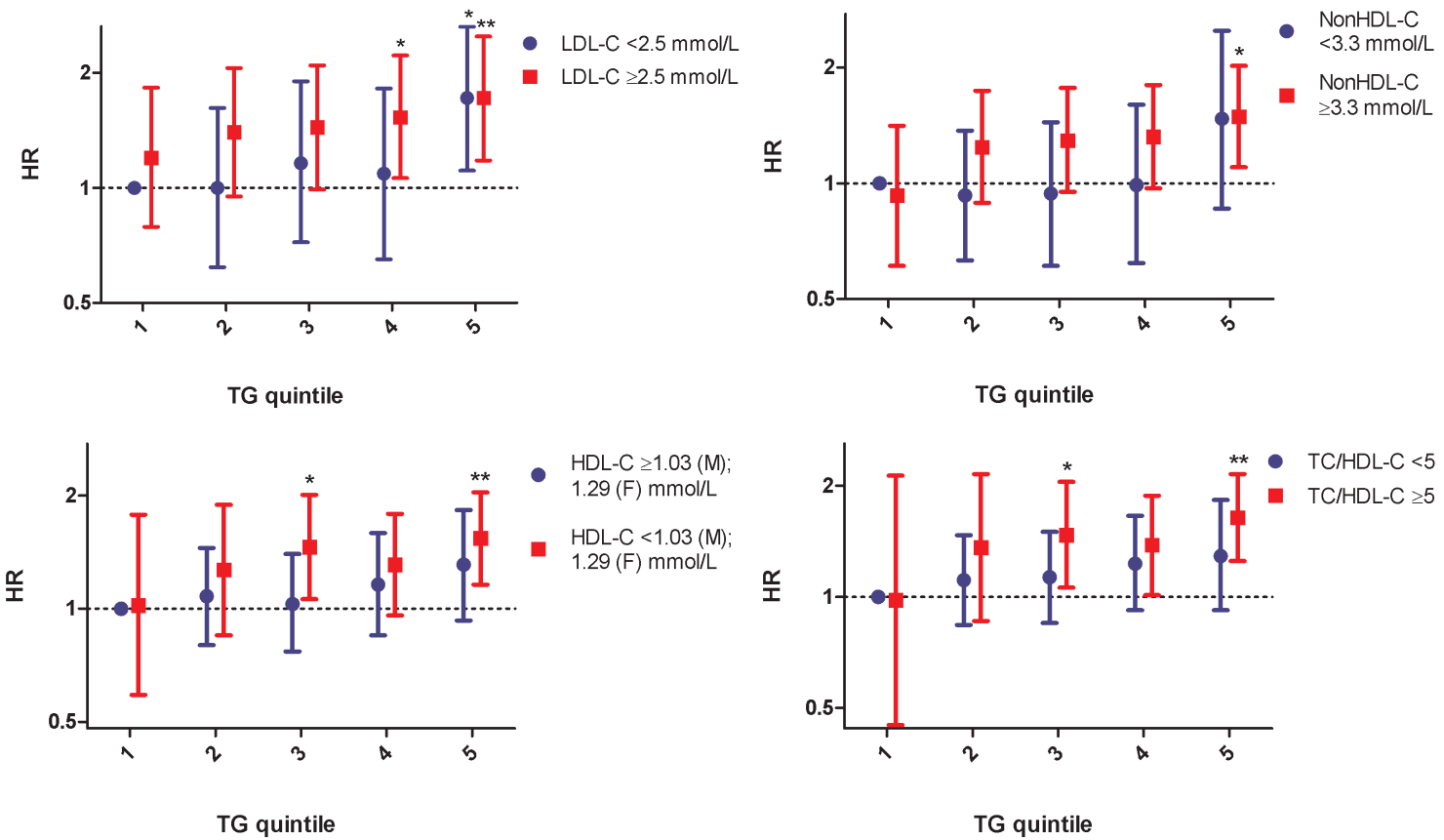
Figure 1 visualizes the risk of vascular events across plasma TG quintiles according to strata for LDL-C, nonHDL-C, HDL-C and TC/HDL-C ratio at baseline. The lower risk lipid stratum in the low TG quintile served as a reference category. This figure illustrates the results presented in Table 4, showing an increased risk with increasing plasma TG-levels irrespective of other lipid levels, and showing a contribution of plasma TG levels to increased vascular risk even when other lipids are at target level.

Discussion

In patients with clinically manifest vascular diseases, high plasma TG levels increase the risk of recurrent vascular events, particularly in patients with CAD. This risk is independent of the presence of T2DM and the use of lipid-lowering medication. Higher plasma TG levels increase risk in patients with either low (<2.5 mmol/L) or high (≥2.5 mmol/L) LDL-C and either low (<3.3 mmol/L) or high (≥3.3 mmol/L) nonHDL-C levels.

Previous meta-analyses showed that plasma TG is a risk factor for the development of cardiovascular diseases in healthy populations, independent of HDL-C(4;5). In patients with known CAD who were treated with statins, plasma TG levels have been shown a risk factor for recurrent vascular events, even at low LDL-C levels(6;7). The present study expands the results of previous trials to an unselected population of patients with CAD who are encountered in everyday clinical practice and treated intensively with lipid-lowering medication. However, plasma TG levels were not associated with an increased risk for recurrent vascular events in patients with CVD, PAD or AAA. Since the different vascular disease categories were not mutually exclusive, presence of interaction by location of vascular disease could not be statistically tested. The difference in vascular risk by plasma TG between these disease categories may be partially explained by the different metabolic profile of patients with CAD compared to patients with vascular disease at other locations. In our study, patients with coronary artery disease had a higher average BMI and waist circumference, but they had a lower blood pressure and were less likely to smoke. Therefore, vascular events in these patients may result from adipose tissue dysfunction and increase in plasma TG and may be 'fat-driven', more than in patients with vascular diseases at different locations. In short, these results show that in the current era of intensive treatment of vascular risk factors, plasma TG levels still contribute to residual risk, in particular in patients with CAD.

Figure 1. Risk for new vascular events for quintiles of TG stratified for other lipoprotein measures at baseline.



All HRs were adjusted for age, gender, smoking, lipid-lowering medication and BMI. In figure 1c, additional adjustment was made for LDL-C. * $p < 0.05$; ** $p < 0.01$

Table 4. Risk of new vascular events conferred by plasma log-triglyceride levels, stratified for other lipoprotein levels at baseline.

LDL-C Events / N	<2.5 mmol/L 169/2118	≥2.5 mmol/L 613/3613
HR Model I	1.30 (1.00-1.69)*	1.31 (1.10-1.55)**
HR Model II	1.27 (0.97-1.67)	1.29 (1.08-1.53)**

NonHDL-C Events / N	<3.3 mmol/L 178/2319	≥3.3 mmol/L 604/3412
HR Model I	1.21 (0.86-1.70)	1.22 (1.03-1.44)*
HR Model II	1.25 (0.88-1.78)	1.22 (1.03-1.45)*

HDL-C Events / N	<1.03 (M); 1.29 (F) mmol/L 360/2279	≥1.03 (M); 1.29 (F) mmol/L 422/3452
HR Model I	1.16 (0.94-1.44)	1.34 (1.08-1.65)**
HR Model II	1.14 (0.91-1.43)	1.22 (0.97-1.52)

TC/HDL-C ratio Events / N	<5 445/4098	≥5 337/1633
HR Model I	1.20 (0.97-1.50)	1.15 (0.91-1.44)
HR Model II	1.20 (0.96-1.51)	1.18 (0.93-1.49)

Hazard ratio (95% confidence interval), adjusted for:
Model I: age and gender
Model II: Model I + smoking, lipid-lowering medication, BMI and LDL-C (adjustment for LDL-C only when stratified for HDL-C)
* $p < 0.05$
** $p < 0.01$
Abbreviations: TC: total cholesterol. Further as in Table 1.

Higher plasma TG levels may be seen as a marker of insulin resistance, as is present in obesity, metabolic syndrome and T2DM, conditions known to be associated with an increased cardiovascular risk(14). Still, our results show that the risk associated with plasma TG is not due to its association with BMI, the metabolic syndrome or T2DM. Adjustment for BMI or stratification for T2DM did not essentially affect the vascular risk associated with high plasma TG, indicating that plasma TG is an independent risk factor

for vascular events in patients with clinically manifest vascular disease. Also adjustment for HOMA did not change the risk associated with plasma TG, making subclinical insulin resistance as an explanation unlikely. Only in patients with the metabolic syndrome, the risk associated with plasma TG seems to be absent. This may either indicate that plasma TG level on its own does not contribute risk in this metabolically unhealthy state; or that patients with metabolic syndrome but still low plasma TG levels have other comorbidities that decrease plasma TG.

Also after adjustment for LDL-C or nonHDL-C, high plasma TG levels were associated with higher risk of recurrent vascular events, in accordance with other studies in healthy populations and statin trials (7;15).Thus, these results may suggest a beneficial effect of lowering plasma TG in secondary prevention. However, drugs lowering plasma TG levels, such as fibrates, have not clearly proven to be effective in preventing vascular events(8;9). Therefore, triglyceride levels are not used as treatment targets in current guidelines (10;11). Instead, NCEP ATP III guidelines advise nonHDL-C as the treatment target in patients with TG >2.3 mmol/L (200 mg/dL). NonHDL-C includes TG-rich VLDL-particles in contrast to LDL-C (10). Data from the present study show that the risk associated with high TG levels is independent of nonHDL-C. Figure 1 shows an increased risk with TG even when the nonHDL-C target has been reached, although this increased risk pertains only to the highest TG quintile, which is a small group of the patients with nonHDL <3.3 mmol/L and therefore the confidence intervals are large and include 1. However, this may still imply that current lipid targets do not suffice in all patients with high TG and a search for other approaches to reduce plasma TG-associated residual risk may be worthwhile.

Apolipoprotein B (apoB) levels may explain the risk associated with plasma TG when nonHDL-C levels are low and have been proposed to estimate risk in hypertriglyceridemic patients. The Mercury II trial showed that for patients with TG ≥2.3 mmol/L (≥200 mg/dL) only 37% of the patients reaching the nonHDL-C goal of 3.37 mmol/L (130 mg/dL) during statin therapy also reached the apoB goal of 90 mg/dL (16). In the present study apoB was only available for 2075 patients, and consequently we could not verify this explanation. The Mercury II study also showed that on statin therapy a stricter nonHDL-C target (<2.6 mmol/L) is necessary to attain this apoB target (16). More studies are necessary to establish whether TG still confers additional risk at these low apoB/nonHDL-C goals.

In the present study, the risk associated with higher plasma TG levels disappeared after adjustment for HDL-C, which is tightly correlated with plasma TG. TG from TG-rich particles are transferred to HDL particles in exchange for cholesterol via cholesteryl ester transferase protein (CETP), after which the TG-enriched HDL particles are rapidly cleared (17). As HDL-C concentrations are more stable than TG concentrations, the relation of HDL-C with vascular events may be more clear. However, a part of the risk associated with

low HDL-C may actually reflect risk associated with increased levels of VLDL remnants and small dense LDL. The present results show a trend towards an increased risk associated with higher TG levels even if HDL-C is high.

A clinical implication of these results is the need for increased attention for plasma TG levels, in particular in patients with CAD. To reduce residual risk, lowering plasma LDL-C or even nonHDL-C to current targets may not be sufficient. As long as there are no TG-lowering drugs proven to be effective in reducing vascular events, focus should be on a strict nonHDL-C target. In patients with elevated plasma TG levels (≥2.3 mmol/L = ≥200mg/dL or even 1.7 mmol/L = ≥150 mg/dL) a strict nonHDL-C target (2.6 mmol/L =100 mg/dL) could be defined to lower the risk associated with plasma TG.

We acknowledge several limitations of the study. First, apoB levels were only available for a small proportion of patients, disabling the use in additional analyses. Furthermore, only baseline lipid levels were available. The use of lipid-lowering medication at baseline was 64%. It is likely that during follow-up lipid-lowering therapy was started in more patients, influencing plasma lipid levels. However, this will probably have resulted in only small changes in plasma TG levels. Moreover, the effect of starting lipid-lowering therapy will most likely result in a small underestimation of the effect of plasma TG, as the proportion of patients with lipid-lowering therapy at baseline was lowest in patients with high plasma TG.

In conclusion, high plasma TG levels confer an increased risk for recurrent vascular events in patients with clinically manifest vascular disease, especially patients with CAD. This relationship was independent of LDL-C and use of lipid-lowering medication and is present even if nonHDL-C is at target level.

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Chapter 6

Low HDL-cholesterol is not a risk factor for recurrent vascular events in patients with vascular disease on intensive lipid-lowering medication

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Abstract

Objectives To evaluate the vascular risk of low HDL-c in relation to use and intensity of lipid-lowering medication in patients with clinically manifest vascular diseases.

Background Low levels of high-density lipoprotein-cholesterol (HDL-c) are associated with increased risk for vascular diseases and may contribute to residual vascular risk in patients already treated for other risk factors. However, posthoc analyses from statin trials indicate that the vascular risk associated with low HDL-c may be low or even absent in patients using intensive statin therapy.

Methods Prospective cohort study of 6111 patients with manifest vascular disease. Cox proportional hazard models were used to evaluate the risk of HDL-c on vascular events in patients using no, usual dose or intensive lipid-lowering therapy.

Results New vascular events (myocardial infarction, stroke, vascular death) occurred in 874 subjects during a median follow-up of 5.4 years (interquartile range 2.9–8.6 years). In patients not using lipid-lowering medication at baseline (N=2153), 0.1 mmol/L increase in HDL-c was associated with a 5% reduced risk for all vascular events (HR 0.95; 95%CI 0.92-0.99). In patients on usual dose lipid-lowering medication (N=1910) there was a 6% reduced risk (HR 0.94, 95%CI 0.90-0.98). However, in patients using intensive lipid-lowering treatment (N=2046), HDL-c was not associated with recurrent vascular events (HR 1.02; 95%CI 0.98-1.07) irrespective of LDL-c level.

Conclusions In patients with clinically manifest vascular disease using no or usual dose lipid-lowering medication, low plasma HDL-c levels are related to increased vascular risk, in contrast to patients using intensive lipid-lowering medication, in whom HDL-c levels are not related to vascular risk.

Introduction

Low levels of high-density lipoprotein-cholesterol (HDL-c) are recognized as an important risk factor for vascular disease, both in healthy populations (1-3) and in patients with known vascular disease (4-6). Although patients with clinically manifest vascular disease are usually intensively treated for risk factors such as high levels of low-density lipoprotein-cholesterol (LDL-c), these patients are still at a high residual risk for vascular events. Low HDL-c is a risk factor for vascular disease independent of LDL-c (7),even when LDL-c is at target level and this is still apparent in patients with vascular disease (4;6;8). However, in a recent analysis of the JUPITER (Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin) trial, the inverse association between HDL-c and vascular events was not observed in patients using intensive statin therapy in the primary prevention setting (9). Although these results have been disputed (10;11), statin trials in patients with coronary artery disease have seen a similar lack of the inverse relation between HDL-c and risk of vascular events in patients receiving (intensive) statin therapy (12;13). So far, findings concerning the absence of increased risk with low HDL-c levels mainly come from statin trials, in which patients are treated with a fixed statin dose, which is not a reflection of clinical practice as LDL-c is treated to target, according to current guidelines, with various dosages of different statins including combination therapy. However, as many patients with clinically manifest vascular disease are treated with statins, the residual risk by HDL-c in these patients may be smaller than initially thought. Currently, pharmacologic methods to increase HDL-c, such as cholesteryl ester transfer protein (CETP) inhibition, are investigated for their potential ability to further decrease vascular risk on top of intensive statin therapy in patients with vascular disease (14), but the results of CETP inhibitors in terms of reduction in vascular risk are disappointing as yet. The CETP inhibitor dalcetrapib did not show a beneficial effect on endothelial function(15) or vascular events(16) when added to statin therapy, despite a 30% increase in HDL-c. Also the AIM-HIGH (Atherothrombosis Intervention in Metabolic Syndrome with Low HDL/ High Triglycerides: Impact on Global Health Outcomes) study, in which extended release niacin was added to intensive statin therapy to increase HDL-c, did not show beneficial results, although the increase in HDL-c was only modest in this trial(17). The results of the HPS2 THRIVE (Heart Protection Study 2-Treatment of HDL to Reduce the Incidence of Vascular Events) trial, comparing extended release niacin / laropiprant and statin therapy(including ezetimibe if necessary) with statin (including ezetimibe if necessary) also showed no beneficial effects of niacin added to statin therapy(18;19).

In the present study, we evaluated the risk associated with plasma levels of HDL-c in patients with clinical manifestations of vascular disease with or without lipid-lowering

therapy. Furthermore, we evaluated the influence of the intensity of treatment on the relation between HDL-c and vascular events and whether this is influenced by achieved levels of LDL-c.

Methods

Patients

Data were used from patients enrolled in the SMART (Second Manifestation of ARTerial disease) cohort. This is a prospective, ongoing cohort study at the University Medical Center Utrecht, The Netherlands, designed to study the presence of concomitant arterial diseases and risk factors for atherosclerosis in a high risk population. Patients newly referred to our institution with clinically evident vascular disease or a vascular risk factor (hyperlipidemia, hypertension or diabetes) were asked to participate. Written informed consent was obtained from all patients. The Medical Ethics Committee of the University Medical Center Utrecht approved the study.

After inclusion, all patients underwent a vascular screening protocol including a health questionnaire, laboratory measurements and physical examination. A detailed description of the study design has been published previously (20).

For the present study, data were used from 6123 patients enrolled in the SMART study between September 1996 and March 2011, with either a history or a recent diagnosis of clinically manifest arterial disease: coronary artery disease, cerebrovascular disease, peripheral artery disease or aneurysm of the abdominal aorta. Patients could be classified into more than one disease category. Patients with HDL-c >3.0 mmol/L or <0.4 mmol/L were excluded (n=12) to exclude patients with monogenetic causes of low or high HDL-c, and hence the study population consisted of 6111 patients.

Single imputation methods were used to reduce missing covariate data for smoking (n=26; 0.4%), alcohol use (n=29; 0.5%), body mass index (BMI) (n=10; 0.2%), total cholesterol (n=1; 0.02%) and plasma triglycerides (TG) (n=2; 0.03%), since complete case analysis leads to loss of statistical power and to bias.

Laboratory assessment

Baseline lipid levels were obtained from fasting patients. Plasma total cholesterol and TG were measured using commercial enzymatic dry chemistry kits (Johnson and Johnson). HDL-c in plasma was determined using a commercial enzymatic kit (Boehringer-

Mannheim) after precipitation of LDL-c and VLDL-c with sodium phosphotungstate magnesium chloride. LDL-c was calculated using the Friedewald formula up to a plasma TG level of 9 mmol/L, which is in line with data showing that the Friedewald formula can be used up to this level (21), to avoid many missing values in the low HDL-c category. Calculated LDL-c levels <0.5 mmol/L were regarded as unreliable and not used for analyses. As a result, LDL-c levels could be calculated for 6085 of 6111 patients (99.6%).

Coding of lipid-lowering medication

Type and dose of lipid-lowering therapy was registered for all participants. To compare the intensity of different types of drugs, the theoretical percentage of LDL-c reduction per individual type and dose of lipid-lowering therapy was determined, based on (systematic) reviews and meta-analyses to the efficacy of statins and other lipid-lowering drugs (22-24). For the present study, intensive lipid-lowering medication was defined as lipid-lowering medication theoretically lowering LDL-c with ≥40%. This implies that for example pravastatin and fluvastatin will not fall in the intensive lipid-lowering group at any dose, whereas rosuvastatin will be defined as intensive lipid-lowering therapy in all doses. For atorvastatin and simvastatin, this will depend on the dose used, with ≥20 mg atorvastatin and ≥40 mg simvastatin being intensive lipid-lowering therapy. Furthermore, we accounted for the addition of other lipid-lowering drugs: e.g. combination therapy with ezetimibe 10 mg was also regarded as intensive therapy, since this was only used in combination with simvastatin and atorvastatin. Besides this, categories of lipid-lowering therapy were defined according to theoretical LDL-c reduction. This resulted in 4 categories, with reductions of 1-<30% (category 1), 30-<40% (category 2), 40-<45% (category 3), <45% (category 4). Detailed information about these categories is shown in Supplementary table 1.

Follow-up

All study participants received a questionnaire every 6 months during the follow-up period to obtain information about hospitalizations and outpatient clinic visits. All available relevant data from any reported possible event were collected. Death of a participant was reported by relatives, the general practitioner or the specialist who treated the participant. All events were classified independently by three members of the SMART Study Endpoint Committee, comprising physicians from different departments. The outcomes of interest for this study were a composite of vascular death, myocardial infarction or ischemic stroke (a definition of these outcomes is shown in Supplementary table 2). Follow-up duration

Table 1. Baseline characteristics according to sex-pooled quartiles of HDL-C.

	Quartile 1	Quartile 2	Quartile 3	Quartile 4	P
N	1560	1514	1523	1514	
HDL-c (mmol/l)	0.85 ± 0.12	1.08 ± 0.12	1.28 ± 0.14	1.68 ± 0.31	
HDL-c range - male	0.41-0.93	0.94-1.10	1.11-1.32	1.33-2.94	
- female	0.50-1.12	1.13-1.36	1.37-1.64	1.65-2.94	
Age (years)	58.4 ± 10.6	60.1 ± 10.2	60.2 ± 10.4	61.5 ± 10.2	<0.01
Male gender, n (%)	1169 (75)	1105 (73)	1134 (75)	1134 (75)	0.57
BMI (kg/m^2)	28.1 ± 4.3	27.1 ± 3.8	26.6 ± 3.8	25.5 ± 3.5	<0.01
Waist circumference* (cm)	99.3 ± 11.9	96.4 ± 11.6	95.1 ± 11.2	92.0 ± 11.6	<0.01
Metabolic syndrome, n (%)	1353 (87)	999 (66)	564 (37)	345 (23)	<0.01
Type 2 diabetes mellitus, n (%)	370 (24)	287 (19)	216 (14)	149 (10)	<0.01
Systolic blood pressure (mmHg)	139 ± 21	141 ± 21	141 ± 21	144 ± 21	<0.01
Diastolic blood pressure (mmHg)	81 ± 11	81 ± 11	81 ± 11	83 ± 11	<0.01
Total cholesterol (mmol/L)	4.8 ± 1.3	4.9 ± 1.2	4.9 ± 1.1	5.1 ± 1.1	<0.01
LDL-cholesterol (mmol/L)	3.0 ± 1.1	3.0 ± 1.1	2.9 ± 1.0	2.8 ± 1.0	<0.01
Triglycerides (mmol/L)	2.3 ± 1.4	1.7 ± 1.1	1.6 ± 1.3	1.2 ± 0.7	<0.01
eGFR (mL/min/1.73 m^2) †	75 ± 19	75 ± 18	76 ± 17	77 ± 18	0.04
Alcohol use during last year, n (%)	953 (61)	1000 (66)	1114 (73)	1250 (83)	<0.01
Current smoking, n (%)	628 (40)	512 (34)	453 (30)	425 (28)	<0.01
Lipid-lowering medication, n (%)	956 (61)	997 (66)	1026 (67)	979 (65)	<0.01
Intensive lipid-lowering medication, n (%)‡	484 (31)	520 (34)	544 (36)	498 (33)	0.04
Localization of vascular disease, n (%): Coronary artery disease	961 (62)	979 (65)	928 (61)	819 (54)	<0.01
Cerebrovascular disease	427 (27)	368 (24)	453 (30)	503 (33)	<0.01
Peripheral arterial disease	362 (23)	311 (21)	262 (17)	298 (20)	<0.01
Abdominal aortic aneurysm	157 (10)	138 (9)	123 (8)	124 (8)	0.18

* Available for patients included from 1999 onwards

† Glomerular Filtration Rate, estimated by the Modification of Diet in Renal Disease (MDRD) equation

‡ Defined as lipid-lowering medication lowering LDL-c with 40%

BMI = body mass index, eGFR = estimated Glomerular Filtration Rate, HDL-c = high-density lipoprotein-cholesterol , LDL-c = low-density lipoprotein-cholesterol

(years) was defined as the period between study inclusion and first cardiovascular event or death from any cause, date of loss to follow-up or the preselected date of 1 March 2011.

Data analysis

Baseline characteristics are presented according to quartiles of HDL-c. Males and females were divided separately into HDL-c quartiles and then combined (sex-pooled quartiles), to prevent overrepresentation of females in the highest HDL-c quartiles.

The effect of plasma HDL-c levels on the occurrence of vascular events was evaluated using Cox proportional hazard models. Hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated for HDL-c as a continuous variable, per 0.1 mmol/L increase in HDL-c. The HRs were adjusted for age and gender in model 1, with additional adjustment for Type 2 diabetes mellitus, body mass index (BMI) and plasma TG in model 2, all of which may confound the relationship between plasma HDL-c and vascular events. In model 3, model 2 was additionally adjusted for current smoking and use of alcohol. In an exploratory analysis, we adjusted for all baseline factors (model 4: model 3 + localization of vascular disease, systolic blood pressure, eGFR and LDL-c). Analyses were performed using the endpoints myocardial infarction, ischemic stroke, vascular death and a composite endpoint of all vascular events, comprising myocardial infarction, stroke and vascular death. Patients were censored if they were lost to follow-up (3.8%). In order to investigate whether the effect of HDL-c on vascular events was influenced by use of (intensive) lipid-lowering medication, these analyses were stratified into patients using no lipid-lowering medication, using non-intensive lipid-lowering medication or using intensive lipid-lowering medication.

To identify whether LDL-c level influenced the effect of HDL-c on vascular events, and whether a potential modification by lipid-lowering medication could be explained by the LDL-c lowering effect, additional analyses were performed with stratification for LDL-C level, at cut-off values of 2.0, 2.5, 3.0 and 4.0 mmol/L.

Additionally, the above analyses were visualized by calculating the HR for increasing quartiles of HDL-c, in strata of lipid-lowering medication or LDL-c level.

Finally, we divided the lipid-lowering therapy into 4 categories based on the amount of LDL-c reduction to estimate a HR for HDL-c in these categories (categories: lipid-lowering medication lowering LDL-c <30%, reducing LDL-c with 30-40%, reducing LDL-c with 40-45%, reducing LDL-c with ≥45%).

In order to test for interaction, i.e. whether the relation between plasma HDL-c levels and vascular events was modified by use of (intensive) lipid lowering medication or LDL-c level, we included an interaction term in the Cox model. If the p-value of the

Table 2. Risk of vascular events by HDL-c according to lipid-lowering treatment

		No lipid-lowering HR (95%CI)	Usual dose lipid-lowering HR (95%CI)	Intensive lipid-lowering HR (95%CI)
All vascular events				
	N (events)	2153 (454)	1910 (262)	2046 (158)
	Model 1	0.95 (0.92-0.97)	0.93 (0.89-0.97)	0.99 (0.95-1.04)
	Model 2	0.95 (0.92-0.98)	0.93 (0.89 – 0.97)	1.00 (0.96-1.05)
	Model 3	0.95 (0.92-0.99)	0.94 (0.90-0.98)	1.02 (0.98-1.07)
	Model 4	0.96 (0.93-1.00)	0.94 (0.90-0.98)	1.03 (0.98 – 1.08)
Myocardial infarction				
	N (events)	2153 (222)	1910 (157)	2046 (79)
	Model 1	0.92 (0.88-0.96)	0.90 (0.85 – 0.95)	0.98 (0.91-1.04)
	Model 2	0.93 (0.88-0.97)	0.90 (0.85 - 0.96)	0.98 (0.91-1.05)
	Model 3	0.93 (0.88-0.97)	0.91 (0.85 - 0.97)	0.99 (0.93-1.07)
	Model 4	0.93 (0.89-0.98)	0.91 (0.86-0.97)	1.00 (0.93-1.08)
Ischemic stroke				
	N (events)	2153 (108)	1910 (61)	2046 (49)
	Model 1	0.96 (0.90-1.01)	0.95 (0.87 – 1.03)	0.98 (0.90-1.06)
	Model 2	0.95 (0.89-1.02)	0.96 (0.88-1.05)	0.99 (0.91-1.07)
	Model 3	0.95 (0.89-1.01)	0.97 (0.88 – 1.06)	1.01 (0.93-1.10)
	Model 4	0.96 (0.90 – 1.03)	0.96 (0.88 – 1.06)	1.00 (0.91-1.09)
Vascular death				
	N (events)	2153 (312)	1910 (131)	2046 (69)
	Model 1	0.95 (0.92-0.99)	0.90 (0.85-0.96)	1.04 (0.98-1.11)
	Model 2	0.97 (0.93-1.01)	0.91 (0.85-0.97)	1.05 (0.98-1.13)
	Model 3	0.97 (0.93-1.01)	0.91 (0.85-0.98)	1.07 (1.00 – 1.15)
	Model 4	0.98 (0.94-1.02)	0.92 (0.86-0.98)	1.09 (1.01-1.17)

Hazard ratio (95% confidence interval) per 0.1 mmol/L increase in HDL-c, stratified for no lipid-lowering therapy, usual dose (e.g. pravastatin, atorvastatin 10 mg) or intensive lipid-lowering therapy (e.g. atorvastatin 20-80 mg, rosuvastatin)
Model 1: Adjusted for age and gender
Model 2: Model 1 + type 2 diabetes mellitus, body mass index and plasma triglyceride levels
Model 3: Model 2 + smoking and alcohol
Model 4: Model 3 + localization of vascular disease, systolic blood pressure, eGFR and LDL-c
CI = confidence interval, eGFR = estimated Glomerular Filtration Rate, HDL-c = high-density lipoprotein-cholesterol, HR = hazard ratio, LDL-c = low-density lipoprotein-cholesterol

interaction term was <0.05, effect-modification was considered to be present. Since the difference in baseline characteristics between the groups using no lipid-lowering therapy, usual dose lipid-lowering therapy and intensive lipid-lowering therapy could possibly explain a differential effect of HDL-c in the different strata, we constructed a propensity score to adjust for these between group differences. We constructed a propensity score for use of lipid-lowering therapy in general and a propensity score for use of intensive lipid-lowering therapy, using all baseline characteristics (including also use of antiplatelet agents, blood pressure lowering agents and time since inclusion) for this propensity score. The Cox model adjusted for age, gender, type 2 diabetes mellitus, body mass index, plasma triglycerides, smoking, alcohol usage, LDL-c level and the propensity score.

The proportional hazard assumption for the Cox model was tested using scaled Schoenfeld residuals, confirming proportional hazards. Only in patients using non-intensive lipid-lowering medication, the proportional hazard assumption did not hold overall, due to non-proportional hazards for age. In these patients, the HR should be interpreted as an average over time.

Analyses were performed using statistical package R 2.13 (R Core Team, Vienna, Austria). For all analyses, P <0.05 was considered significant.

Results

Baseline characteristics

With increasing quartiles of HDL-c, the mean BMI and waist circumference declined and the prevalence of the metabolic syndrome and type 2 diabetes mellitus was lower (Table 1). The proportion of patients using lipid lowering medication was approximately 65% and this did not differ across HDL-c quartiles. Almost two third of the patients had coronary artery disease, which was slightly lower in the highest quartile of HDL-c. About one third of the patients had cerebrovascular disease.

Plasma HDL-c and the risk of recurrent vascular events according to use of lipid-lowering medication

Median follow-up was 5.4 years (interquartile range 2.9-8.6 years). During this follow-up, 874 new vascular events occurred (myocardial infarction, stroke, and vascular death). Per 0.1 mmol/L increase in HDL-c the risk of vascular events decreased with 5% (HR 0.95, 95%CI 0.92-0.99) in patients not using lipid-lowering medication (Table 2). Additional

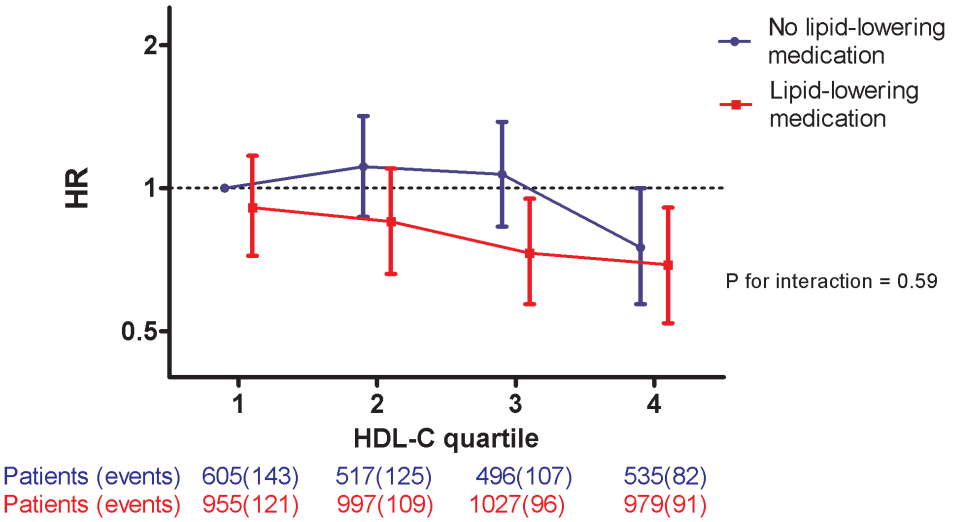
adjustment for baseline factors in model 4 did not affect this relation. Similar relations were seen between HDL-c and myocardial infarction (HR 0.93, 95%CI 0.88-0.97), ischemic stroke (HR 0.95, 95%CI 0.89-1.01) and vascular death (HR 0.97, 95%CI 0.93-1.01).

In patients on usual dose lipid-lowering therapy (n=1910) the risk for all vascular events per 0.1 mmol/L increase in HDL-c decreased with 6% (HR 0.94, 95%CI 0.90-0.98) (Table 2). However, in patients using intensive lipid-lowering medication HDL-c plasma levels were not associated with vascular events (HR 1.02, 95%CI 0.98-1.07). Furthermore, HDL-c was not associated with myocardial infarction (HR 0.99, 95%CI 0.93-1.07), ischemic stroke (HR 1.01, 95%CI 0.93-1.10) or vascular death (HR 1.07, 95%CI 1.00-1.16). The p for interaction in model 3, after additional adjustment for a possible differential effect of LDL-c level and propensity score, was 0.03, indicating modification of the effect of HDL-c by intensive lipid-lowering treatment, irrespective of LDL-c or baseline differences between the groups. In quartiles of higher HDL-c, the risk of vascular events was lower in patients on usual dose lipid-lowering therapy compared to the lowest HDL-c quartile (Figure 1A). For patients on intensive lipid-lowering therapy, the overall risk for vascular events is lower compared to patients using no or usual dose lipid-lowering, but there was no relation between HDL-c and vascular risk in that group (Figure 1B). An association between HDL-c and vascular events in patients on intensive lipid-lowering therapy was absent independent of the year of inclusion, and was absent both in patients with low levels of CRP (<2 mg/L) or high levels of CRP (≥2 mg/L). Analyses only based on statin treatment instead of lipid-lowering therapy in general did not change the results.

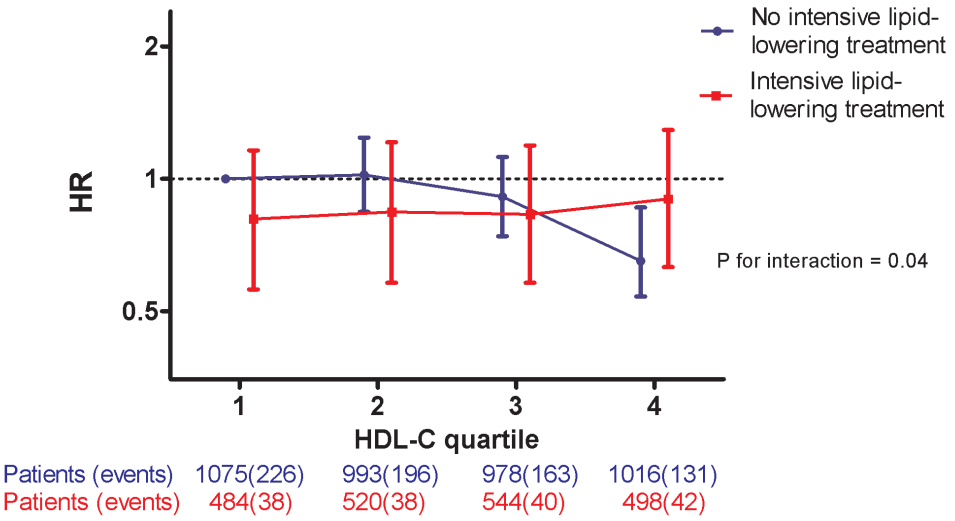
Plasma HDL-c and the risk of recurrent vascular events according to LDL-c level

The relation between HDL-c and risk for vascular events was not modified by LDL-c (p for interaction=0.3). Table 3 shows the HR per 0.1 mmol/L increase in HDL-c at different LDL-c levels, stratified for use of lipid-lowering medication. In patients using lipid-lowering medication, HDL-c was not related to risk of vascular events at LDL-c levels <2.0 mmol/L. Of these patients 70% used intensive lipid-lowering medication. In patients with LDL-c ≥2.0 mmol/L increasing HDL-c levels were related to a lower vascular risk. Adjustment for all baseline factors did not change the results (data not shown), although the number of events may not be sufficient in all strata to allow firm conclusions.

Figure 1.Risk for all vascular events across quartiles of HDL-c



A. Hazard ratio (95% confidence interval) by increasing quartiles of HDL-c, stratified for yes vs. no lipid-lowering therapy, adjusted for age, gender, body mass index, type 2 diabetes mellitus, plasma triglyceride level, alcohol use and smoking.



B. Hazard ratio (95% confidence interval) by increasing quartiles of HDL-c, stratified for yes vs. no intensive lipid-lowering therapy (defined as ≥40% LDL-c reduction) adjusted for age, gender, body mass index, type 2 diabetes mellitus, plasma triglyceride level, alcohol use and smoking. The reference group includes both patients using no lipid-lowering medication and patients using usual dose lipid-lowering medication.

Table 3. Risk of vascular events by HDL-c according to LDL-c level

LDL-c (mmol/L)		<2.0 HR (95%CI)	2.0-<2.5 HR (95%CI)	2.5-<3.0 HR (95%CI)	3.0-<4.0 HR (95%CI)	≥4.0 HR (95%CI)
Lipid-lowering therapy						
	N (events)	1027 (79)	997(84)	861 (88)	803 (123)	250 (42)
	Model 1	1.01(0.95-1.07)	0.96 (0.90-1.03)	0.97 (0.91-1.04)	0.90(0.84-0.96)	0.93(0.84-1.04)
	Model 2	1.00(0.94-1.07)	0.97 (0.90-1.04)	0.98 (0.91-1.06)	0.91(0.84-0.98)	0.94 (0.85-1.06)
	Model 3	1.02(0.95-1.09)	0.97 (0.90-1.05)	1.01 (0.94-1.09)	0.92(0.86-0.99)	0.96 (0.86-1.07)
No lipid-lowering therapy						
	N (events)		297 (43)	301 (46)	812 (180)	737 (183)
	Model 1		0.95 (0.87-1.03)	0.97 (0.90-1.05)	0.95(0.90-1.00)	0.94(0.89-0.99)
	Model 2		0.94 (0.85-1.03)	0.97 (0.90-1.06)	0.97(0.91-1.02)	0.93 (0.88-0.99)
	Model 3		0.94 (0.86-1.04)	0.98(0.90-1.07)	0.97(0.92-1.02)	0.94(0.89-1.00)

Hazard ratio (95% confidence interval) per 0.1 mmol/L increase in HDL-c, stratified for LDL-c level separately for use of lipid-lowering therapy.
For patients using no lipid-lowering therapy, few patients (n=124) had LDL-c levels <2.0 mmol/L, therefore these patients were added to the patients with LDL-c levels of 2.0-<2.5 mmol/L.
Model 1: Adjusted for age and gender
Model 2: Model 1+ type 2 diabetes mellitus, body mass index and plasma triglyceride levels
Model 3: Model 2 + smoking and alcohol
CI = confidence interval, HDL-c = high-density lipoprotein-cholesterol, HR = hazard ratio, LDL-c = low-density lipoprotein-cholesterol

Plasma HDL-c and the risk of recurrent vascular events according to intensity of lipid-lowering therapy

The intensity of lipid-lowering therapy modified the relation between HDL-c and vascular events (Table 4). In patients using lipid-lowering therapy that lowers LDL-c with <30%, the risk for vascular events was 5% lower (HR 0.95; 95%CI 0.88-1.02) for each 0.1 mmol/L increase in HDL-c. The inverse association between HDL-c and vascular events gradually changed with increasing intensity of lipid-lowering medication. In patients on lipid-lowering therapy that is considered to lower LDL-c with >45% there was no relation between HDL-c and vascular events (HR 1.06; 95%CI 0.99-1.15). The p for interaction by intensity of lipid-lowering therapy was 0.03. Adjustment for all baseline factors did not change the results (data not shown).

Table 4. Risk of vascular events by HDL-c in categories of increasingly potent lipid-lowering therapy

Category of lipid-lowering therapy	N (events)	LDL-c level (mmol/L)	Risk for vascular events per 0.1 mmol/L increase (HR 95%CI)		
			Model 1	Model 2	Model 3
1 (1 - <30%)	763 (114)	2.9 ± 0.8	0.92 (0.86-0.98)	0.93 (0.87-1.00)	0.95 (0.88-1.02)
2 (30 - <40%)	1144 (148)	2.7 ± 0.8	0.93 (0.88-0.99)	0.93 (0.88-0.99)	0.94 (0.88-1.00)
3 (40 - <45%)	1310 (92)	2.4 ± 0.8	0.98 (0.92-1.04)	0.99 (0.93-1.05)	1.01 (0.95-1.07)
4 (≥45%)	735 (65)	2.3 ± 0.9	1.02 (0.95-1.10)	1.04 (0.97-1.13)	1.06 (0.99-1.15)
P for interaction lipid-lowering therapy * HDL-c			0.03	0.03	0.03

Categories of lipid-lowering therapy were defined according to theoretical LDL-c reduction: 1-<30%, 30-<40%, 40-<45%, <45%
Model 1: adjusted for age and gender
Model 2: Model 1+ type 2 diabetes mellitus, body mass index and plasma triglyceride levels
Model 3: Model 2 + smoking and alcohol
CI = confidence interval, HDL-c = high-density lipoprotein-cholesterol, HR = hazard ratio, LDL-c = low-density lipoprotein-cholesterol

Discussion

In patients with clinically manifest vascular disease not treated with lipid-lowering medication or treated with usual dose lipid-lowering medication, low HDL-c was associated with an increased risk of recurrent vascular events, in contrast to patients on intensive dose lipid-lowering therapy in which HDL-c was not associated with vascular events. The plasma level of LDL-c did not modify the effect of HDL-c on vascular events.

Low plasma HDL-c is recognized as a risk factor for (recurrent) vascular events(1-4), and our results confirm these findings for patients using no or usual dose lipid-lowering therapy. However, several trials such as JUPITER, CARE and PROVE-IT TIMI-22, report absence of an association between low HDL-c and vascular events in patients with and without vascular disease on statin therapy (9;12;13). An important difference between these trials and clinical practice is the fixed statin dose used in trials instead of treating to a target as is advocated in European and American treatment guidelines(25;26). In a recent report from the Crusade registry, HDL-c was also not associated with recurrent myocardial infarction or death in patients with myocardial infarction on statin therapy, but type and dose of statin therapy was not specified (27). Our study is a real life situation in which patients are treated to an LDL-c target, as advocated in clinical guidelines, with information about different doses and types of lipid-lowering medication that were used, in contrast to a single fixed dose in clinical trials. We are able to evaluate a wide range of different lipid-lowering medication, instead of comparing two different doses or comparing a single dose with placebo. Therefore, we could demonstrate a gradual decrease of the inverse association between HDL-c and outcomes with increasing intensity of lipid-lowering treatment. This observation is consistent with a report from the TNT trial, in which HDL-c was associated with vascular events in the atorvastatin 10mg group, whereas it was not associated with vascular events in the atorvastatin 80mg group (28). Our results obtained in a cohort study indicate that not merely statin treatment, but also treatment intensity affects the relation between HDL-c plasma concentrations and vascular risk. In contrast to our findings, other cohort studies in patients with vascular disease treated with statins according to current guidelines have reported that HDL-c was still inversely associated with vascular events (8;29). However, intensity of lipid-lowering therapy was not reported, and the average LDL-c reduction of 28% (8) and/or the reached LDL-c level of approximately 130 mg/dL (3.4 mmol/L) (29) suggests that not all patients were on intensive statin therapy. In the present real-life study, all lipid-lowering therapy, mainly statins and cholesterol absorption inhibitors, were taken into account to estimate an expected proportion of LDL-c reduction as a measure of intensity of lipid-lowering treatment. Previous reports about lipid-lowering treatment modifying the relation

between HDL-c and vascular events specifically only considered statin therapy. The lipid-lowering treatment used in the present study was also mainly statin therapy and therefore these results can be compared with previous reports in statin-treated patients. In a sensitivity analysis, we performed analyses only based on statin treatment and that did not change the results.

In concordance with our results, studies in patients with and without vascular disease have shown that low HDL-c levels are related to increased vascular risk irrespective of plasma LDL-c level (4;7) even at LDL-c <1.8mmol/L (28;30). The present study confirms these findings, although only few patients reached very low LDL-c levels without receiving intensive statin therapy. The association between HDL-c and vascular events was not modified by LDL-c level. Only in patients with LDL-c <2 mmol/L on lipid-lowering therapy, the inverse association between HDL-c and vascular events was not present, consistent with the high prevalence of intensive lipid-lowering treatment in this group.

Although all patients in our study would qualify for treatment with lipid-lowering treatment, a part of these patients was not treated at baseline. These patients were in general included earlier in this study, which started in 1996, when the use of lipid-lowering therapy was lower. In addition, in patients with cerebrovascular disease, peripheral arterial disease or an abdominal aortic aneurysm the use of lipid-lowering therapy was generally lower at baseline compared to patients with coronary artery disease. However, the propensity score included also year of inclusion and localization of disease, and the p for interaction was still statistically significant after adjustment for a differential effect of the factors and the other baseline variables included in the propensity score.

Our results do not allow conclusions about the possible effect of HDL-c raising therapy in a population of patients with vascular disease. Randomized controlled trials are necessary to evaluate the effect of HDL-c raising therapy in these patients. Although a Mendelian randomization study indicates that HDL-c as such may not be a causal factor in vascular disease(31), and previous trials with CETP inhibitors (16;32) or niacin (17-19) to increase HDL-c on top of intensive statin treatment showed no reduction of cardiovascular events, the new CETP inhibitors anacetrapib and evacetrapib also reduce LDL-c and may therefore lower cardiovascular risk also due to LDL-c reduction. Besides, interventions targeted at increasing HDL-c that also affect HDL function could still be beneficial in patients with vascular disease. However, the results of the present study indicate that in patients using intensive statin therapy, HDL-c may not be a good secondary treatment target and these results could provide an alternative explanation for the failure of CETP inhibitors to show any benefit on top of intensive statin treatment. Explanations for the apparent absence of a relation between HDL-c and vascular events in patients treated with intensive lipid-lowering therapy can only be speculated upon. Although statins

marginally increase HDL-c depending on type and dose and beneficially influence several molecules in HDL metabolism such as CETP, lipoprotein lipase and paraoxonase-1 (33), the consequences of these effects on HDL function remain poorly understood. Whether the results of the present study can be explained by functional changes in HDL during intensive statin therapy, whether the relation between HDL function and plasma HDL-c level is lost in intensive statin therapy, or whether anti-inflammatory and anti-oxidant properties of HDL-c are less relevant as high-dose statin therapy already exerts anti-inflammatory and anti-oxidant actions is not known.

Study limitations

We acknowledge study limitations. Plasma lipid levels were measured only once at start of the study. Moreover, only data about baseline medication was available, which is likely to have changed during follow-up. Probably the number of patients receiving lipid-lowering therapy and the proportion of patients receiving intensive lipid-lowering treatment during follow-up has increased. Most patients receiving intensive statin therapy already at baseline are likely to continue use of intensive therapy during follow-up. Therefore, a 'drop-in' of lipid-lowering therapy in the patients not using lipid-lowering therapy at baseline would likely only decrease the difference between the groups and hence lead to an underestimation of the difference in effect of HDL-c in patients using or not using intensive lipid-lowering therapy. Secondly, stratification of our study population in different LDL-c categories resulted in a small number of events in some groups, attenuating the precision of the risk estimation. Drawing firm conclusions from the results in different LDL-c categories should therefore be done with caution, but these analyses serve to give an overall impression of the effect of HDL-c at increasing levels of LDL-c.

In conclusion, HDL-c levels are related to the risk of new cardiovascular events in patients with clinically manifest vascular disease treated with usual dose lipid-lowering therapy, but not in patients treated with intensive lipid-lowering medication, irrespective of LDL-c.

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Supplementary table 1. Coding of lipid-lowering medication into usual dose / intensive therapy or categories

Usual dose / intensive lipid-lowering therapy	Category of lipid-lowering therapy (theoretical LDL-c reduction)	Lipid-lowering medication in this category*
Usual dose	1 (1 - <30%)	pravastatin 10-40 mg, fluvastatin 20-40 mg, monotherapy with ezetimibe or bile acid sequestrants
	2 (30 - <40%)	simvastatin 10 and 20 mg, atorvastatin 10 mg
Intensive	3 (40 - <45%)	simvastatin 40 mg, atorvastatin 20 mg, rosuvastatin 5 mg; combination of simvastatin 10 mg and ezetimibe 10 mg
	4 (≥45%)	simvastatin 80 mg, atorvastatin 40-80 mg, rosuvastatin 10-40 mg; combination of simvastatin >10 mg and ezetimibe 10 mg, combination of atorvastatine and ezetimibe

* We accounted also for combination therapy with niacin and bile acid sequestrants, but this combination occurred only in 3 and 9 patients respectively.
LDL-c = low-density lipoprotein-cholesterol

Supplementary table 2. Endpoint definitions

Ischemic stroke	Relevant clinical features causing an increase in impairment of at least one grade on the modified Rankin scale, without signs of haemorrhage on repeat brain imaging
Myocardial infarction	At least two of the following criteria: (I) Chest pain for at least 20 min, not disappearing after administration of nitrates (II) ST-elevation >1 mm in two following leads or a left bundle branch block on the electrocardiogram (III) Troponin elevation above clinical cut-off values or creatinine kinase (CK) elevation of at least two times the normal value of CK and a myocardial band fraction >5% of the total CK
	Sudden death: unexpected cardiac death occurring within 1 h after onset of symptoms, or within 24 h given convincing circumstantial evidence
Vascular mortality	Death from stroke, myocardial infarction, congestive heart failure, or rupture of abdominal aortic aneurysm
	Vascular death from other causes
Composite vascular mortality	A composite of stroke, myocardial infarction and vascular mortality

Chapter 7

Statin therapy increases the risk of incident type 2 diabetes mellitus independent of known risk factors in patients with clinically manifest vascular disease; a cohort study

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Submitted

Abstract

Background Several trials and cohort studies have shown an increased incidence of type 2 diabetes mellitus (T2DM) in patients using statins. Whether this only applies to patients at already high risk for the development of T2DM or for all patients is still a matter of debate.

Methods Prospective cohort study of 4645 patients with established vascular disease without DM at baseline. 3057 patients used statins at baseline, of whom 1608 used intensive statin therapy, defined as statin therapy theoretically lowering LDL-c with ≥40%. Cox proportional hazard models were used to estimate the risk of incident T2DM with (intensive) statin therapy.

Results Statin therapy was associated with increased risk of incident T2DM (HR 1.63; 95%CI 1.15-2.32) when adjusted for age, sex, body mass index, plasma HDL-c and plasma triglyceride levels. Intensive statin therapy tended to be related to a higher risk of T2DM when compared to moderate statin therapy (HR 1.22; 95%CI 0.92-1.61, adjusted for age, sex, body mass index, plasma HDL-c and plasma triglyceride levels). The increase in risk was regardless of the number of metabolic syndrome characteristics or insulin resistance, but was particularly present in patients with low baseline glucose levels(<5.6 mmol/L; p for interaction 2.9×10^{-7}).

Conclusion Statin use increases the risk of incident T2DM in patients with clinically manifest vascular disease. The increase in risk was independent of the number of metabolic syndrome criteria, and was even more pronounced in patients with low baseline glucose levels.

Introduction

Statins are a safe and effective therapy to reduce the risk of first or subsequent vascular events and their use is recommended for all patients at high risk of vascular events (1-3). Recently it was reported that statins confer an increased risk for the development of type 2 diabetes mellitus (T2DM). In the JUPITER trial, statin treatment was associated with a 25% increased risk of T2DM (4). Subsequent meta-analyses indicated that statins increase the risk of T2DM and this risk is higher in patients using more intensive statin therapy, with odds ratios around 1.1 for statin therapy and also 1.1 for intensive versus moderate statin therapy (5;6). Several cohort studies and registries have substantiated the evidence for statin-induced T2DM in the general population and patients with coronary artery disease (7-11). Furthermore, the increase in risk of diabetes with statin therapy may be more pronounced in women and elderly (5;12;13). There are various potential mechanisms by which statins could lead to development of T2DM, among others dysfunction of voltage-gated calcium channel of the pancreatic beta cells leading to decreased insulin secretion(14), mitochondrial dysfunction in myocytes and pancreatic beta cells causing peripheral insulin resistance and decreased pancreatic beta cell function(12;15), and decreased expression of the insulin induced glucose receptor causing peripheral insulin resistance(16).

Whether only patients who are already at high risk to develop T2DM are prone to acquire T2DM due to statin therapy, or whether statin therapy also conveys T2DM risk in patients who are considered to be at low risk to develop T2DM remains a matter of debate. Results of the TNT and IDEAL studies indicate that only patients at already elevated risk for T2DM according to previously determined risk factors in their study (resembling metabolic syndrome criteria) are at increased risk to develop T2DM by statin therapy (11), but in the JUPITER study the risk of T2DM with statin therapy was independent of baseline glucose levels (17). For clinical practice, identification of risk factors to develop T2DM with statin therapy are of importance to guide individual therapy. This is particularly relevant for patients with established arterial disease, who are in need of LDL-cholesterol lowering therapy, but who are also at elevated risk of developing statin-induced T2DM.

In the present study, we investigated the relation between statin therapy and intensity of statin therapy and incident T2DM in a cohort of patients with clinically manifest vascular disease. Furthermore, we investigated whether the effect of statin therapy on incident T2DM was dependent on the presence of risk factors for T2DM.

Methods

Patients

Data were used from patients enrolled in the SMART (Second Manifestation of ARTerial disease) cohort. This is a prospective, ongoing cohort study at the University Medical Center Utrecht, The Netherlands, designed to study the presence of concomitant arterial diseases and risk factors for atherosclerosis in a high risk population. Patients newly referred to our institution with clinically evident vascular disease or a vascular risk factor (hyperlipidemia, hypertension or diabetes) were asked to participate. Written informed consent was obtained from all patients. The Medical Ethics Committee of the University Medical Center Utrecht approved the study.

After inclusion, all patients underwent a vascular screening protocol including a health questionnaire, laboratory measurements and physical examination. A detailed description of the study design has been published previously (18).

For the present study, data were used from 4645 patients enrolled in the SMART study between September 1996 and March 2011 who did not have DM at baseline, with either a history or a recent diagnosis of clinically manifest arterial disease: coronary artery disease, cerebrovascular disease, peripheral artery disease or aneurysm of the abdominal aorta. Patients could be classified into more than one disease category. Patients who had died (n=383) or were lost to follow-up (n=67) before the assessment of T2DM in 2006 were excluded.

Single imputation methods were used to reduce missing covariate data for body mass index (BMI) (n=8, 0.2%), plasma glucose levels (n=30, 0.6%), systolic blood pressure (n=21, 0.4%), total cholesterol (n=24, 0.5%), HDL-cholesterol (n=35, 0.7%), triglycerides (n=32, 0.7%), eGFR (n=22, 0.5%) and smoking (n=21, 0.5%), since complete case analysis leads to loss of statistical power and bias.

Classification of lipid-lowering medication

Type and dose of lipid-lowering therapy was registered for all participants at baseline. To compare the intensity of different types of statins, the percentage of LDL-c reduction per individual type and dose of statin therapy was determined, based on (systematic) reviews and meta-analyses to the efficacy of statins and other lipid-lowering drugs(19;20). For the present study, intensive statin therapy was defined as statin therapy lowering LDL-c with ≥40%. This implies that for example pravastatin and fluvastatin are not considered intensive statin therapy at any dose, whereas all doses of rosuvastatin are considered

intensive statin therapy. For atorvastatin and simvastatin, this will depend on the dose used, with ≥20 mg atorvastatin and ≥40 mg simvastatin being intensive statin therapy.

Statin use during follow-up was known for 1093 patients. We used a logistic regression model to estimate statin use during follow-up for all participants for whom no follow-up information about statin use was present. Patients who started or stopped treatment during follow-up were assumed to have used statin treatment for half of the follow-up time. For subjects without follow-up information, we used the estimates from the logistic regression model. This resulted in a continuous scale for statin therapy, 0 meaning that no statin therapy was used at any time during the study and 1 meaning that statin therapy was used at all time during the study.

Follow-up

All study participants received a questionnaire every 6 months during the follow-up period to obtain information about hospitalizations and outpatient clinic visits. All available relevant data from any reported possible event were collected. In order to assess the incidence of diabetes, all patients that had been included until June 2006 without diabetes at baseline received a questionnaire in the period between June and December 2006 to assess the incidence of T2DM after study inclusion. After 2006, information about diabetes was biannually collected in the questionnaire. Patients reporting diabetes received a supplementary questionnaire regarding date of diagnosis, initial and current treatment (oral medication or insulin) and family history of diabetes. Patients and/or their general practitioners were contacted by telephone for further information if the answers were incomplete or unclear and also non-responders were contacted. All diabetes cases were audited and classified by two independent physicians. Cross validation with the hospital diagnosis registry revealed that none of the patients who reported not to have diabetes had a physician's diagnosis of diabetes. Follow-up duration (years) was defined as the period between study inclusion and the date of incident T2DM, death from any cause, date of loss to follow-up or the preselected date of 1 March 2012. From 1996 until 1 March 2012, 120 out of the 4645 patients (2.6%) were lost to follow-up.

Data analysis

The effect of statin use on incident T2DM was evaluated using Cox proportional hazard models. In model 1, adjustment was made for age and gender. In model 2, additional adjustment for BMI, plasma triglyceride level and plasma HDL-cholesterol level was made. Furthermore, a propensity score was constructed, since patients using statins differ from

patients not using statins and these differences themselves may influence the risk to develop DM. In order to make this propensity score, the probability to use statin therapy was estimated using a logistic regression model, including baseline characteristics (except current LDL-c levels). In model 3, we adjusted for this propensity score.

To compare the effect of different types and different intensities of statin therapy, we stratified these analyses by type and intensity. Pravastatin or fluvastatin and atorvastatin or rosuvastatin were grouped together, since the group sized were too small, and these types of statin therapy have shown similar effects on T2DM risk (9;21). Type and dose of statin treatment were not available during follow-up, therefore, these analyses only compare baseline differences. Furthermore, we stratified by number of metabolic syndrome criteria present as an estimate of T2DM risk, to evaluate whether statin therapy increases T2DM risk independent of baseline T2DM risk. To further investigate this, we also stratified for quartiles of each of the metabolic syndrome criteria. We used the TyG index (Ln[fasting triglycerides(in mg/dL) x fasting glucose (in mg/dL)/2]) as an estimate of insulin resistance (22), since HOMA-IR could only be calculated for patients included from July 2003 onwards due to absence of insulin measurements before July 2003.

In order to test for interaction, i.e. whether the relation between statin therapy and incident T2DM was modified by the metabolic syndrome, insulin resistance or by any of the individual metabolic syndrome criteria, we included an interaction term in the Cox model. Furthermore, we also tested for interaction with age and sex.

The proportional hazard assumption for the Cox model was tested using scaled Schoenfeld residuals, confirming proportional hazards.

The statistical package R 2.13 was used for all analysis. A p-value of <0.05 was considered statistically significant.

Results

Baseline characteristics

Baseline characteristics according to baseline statin use are shown in Table 1. The mostly used statins in the patients using moderate statin therapy were pravastatin (36%) and (low dose) simvastatin (43%). In patients using intensive statin therapy, mostly used statins were (high dose) simvastatin (51%) and atorvastatin (34%). Patients using no statin therapy were in general included earlier in the study (mean time since inclusion 7.7 ± 3.9 years) than patients using moderate statin therapy (6.3 ± 3.5 years) or intensive statin therapy (3.8 ± 2.9 years). Furthermore, patients with cerebrovascular disease or peripheral

Table 1. Baseline characteristics according to statin treatment

N	No statin 1588	Moderate statin 1449	Intensive statin 1608
Age (years)	58.4 ± 11.3	59.7 ± 10.2	59.0 ± 9.7
Male sex, n (%)	1092 (69)	1092 (75)	1231 (77)
BMI (kg/m^2)	26.1 ± 3.9	26.8 ± 3.7	27.0 ± 3.7
Plasma glucose (mmol/L)	5.7 ± 0.8	5.8 ± 0.9	5.8 ± 0.8
Systolic blood pressure (mmHg)	141 ± 22	139 ± 21	139 ± 21
Total cholesterol (mmol/L)	5.6 ± 1.2	4.8 ± 1.0	4.3 ± 1.0
LDL-cholesterol (mmol/L)	3.6 ± 1.0	2.8 ± 0.8	2.4 ± 0.8
HDL-cholesterol (mmol/L)	1.26 ± 0.40	1.24 ± 0.35	1.25 ± 0.37
Triglycerides (mmol/L)	1.4 (1.0-2.1)	1.4 (1.0-2.0)	1.2 (0.9-1.8)
eGFR (mL/min/1.73 m^2)*	77 ± 18	76 ± 16	77 ± 17
HsCRP (mg/L)**	3.3 ± 3.0	2.6 ± 2.6	2.4 ± 2.5
Current smoking, n (%)	638 (40)	408 (28)	504 (31)
Metabolic syndrome, n (%)	710 (45)	746 (52)	770 (48)
TyG index †	8.8 (8.4-9.2)	8.8 (8.4-9.1)	8.6 (8.3-9.0)
Use of antiplatelet agents, n (%)	935 (59)	1184 (82)	1436(89)
Use of blood pressure-lowering agents	842 (53)	1225 (85)	1303 (81)
Type of statin:			
Pravastatin	0 (0)	514 (36)	0 (0)
Fluvastatin	0 (0)	68 (5)	0 (0)
Cerivastatin	0 (0)	7 (1)	0 (0)
Simvastatin	0 (0)	628 (43)	813 (51)
Atorvastatin	0 (0)	232 (16)	547 (34)
Rosuvastatin	0 (0)	0 (0)	248 (15)
Localisation of vascular disease, n (%):			
Coronary artery disease	609 (38)	1076 (74)	1123 (70)
Cerebrovascular disease	555 (35)	286 (20)	469 (29)
Peripheral arterial disease	446 (28)	196 (14)	187 (12)
Abdominal aortic aneurysm	168 (11)	96 (7)	80 (5)
Mean time since inclusion in the cohort (years)	7.7 ± 3.9	6.3 ± 3.5	3.8 ± 2.9

* Glomerular Filtration Rate, estimated by the Modification of Diet in Renal Disease (MDRD) equation

** Only if hsCRP < 15 mg/L

† TyG index calculated as Ln[fasting triglycerides(in mg/dL) x fasting glucose (in mg/dL)/2]

artery disease were less likely to use statin therapy at baseline, with 58% and 46% using statin therapy at baseline compared to 78% in patients with coronary artery disease.

Effect of statin therapy on the risk of incident T2DM

During follow-up, 353 patients developed T2DM, the overall incidence of T2DM was 1.29 per 100 patient years. Statin therapy increased the risk of incident T2DM with 63% (HR 1.63, 95%CI 1.15-2.32) when adjusted for age, sex, body mass index, plasma HDL-c and plasma triglyceride levels (Table 2). This risk persisted when adjusted for a propensity score (HR1.66, 95%CI 1.14-2.42).

Baseline use of intensive statin therapy as compared to usual dose statin therapy tended to be related to an increased risk of T2DM, although this finding was not statistically significant (HR 1.21, 95%CI 0.90-1.63 in the fully adjusted model). When types of baseline therapy used at baseline were compared, simvastatin and especially atorvastatin or rosuvastatin confer a higher risk of developing T2DM than pravastatin or fluvastatin. However, this trend was only weak for baseline use of simvastatin and of

Table 2. Risk of incident T2DM with statin treatment

	Events/N	Incidence per 100 person year	HR Model 1	HR Model 2	HR Model 3
Statin use vs. no statin use	353/4645	1.29	1.71 (1.22-2.41)	1.63 (1.15-2.32)	1.66 (1.14-2.42)
Intensive statin vs. usual dose	212/3057	1.37	1.18 (0.90-1.57)	1.22 (0.92-1.61)	1.21 (0.90-1.63)
Simvastatin vs fluvastatin/ pravastatin	137/2023	1.31	1.20 (0.84-1.73)	1.26 (0.88-1.82)	1.25 (0.86-1.81)
Atorvastatin / rosuvastatin vs fluvastatin/ pravastatin	117/1609	1.38	1.38 (0.94-2.03)	1.32 (0.90-1.93)	1.41 (0.95-2.13)

Hazard ratio (HR) and 95% confidence interval for incident T2DM, adjusted for:
Model 1: Age and sex
Model 2: Model 1 + BMI, HDL-cholesterol, triglycerides
Model 3: Age, gender and propensity score-adjusted (propensity score with age, sex, localisation of vascular disease, BMI, HDL-cholesterol, plasma triglycerides, systolic blood pressure, plasma glucose, platelet inhibitors, blood pressure lowering medication, smoking, eGFR and time since inclusion)

borderline significance for baseline use of atorvastatin or rosuvastatin (HR 1.41, 95%CI 0.95-2.13, p=0.09 in the fully adjusted model).

Since incident T2DM was initially not registered in this cohort, we performed sensitivity analyses by excluding patients who were included >7 year ago or by adjusting for years since inclusion in the cohort. This led to similar results.

Effect of risk factors for T2DM on the relation between statin use and incidence of T2DM

The increased risk of T2DM with statin therapy was independent of the number of metabolic syndrome criteria present (Table 3). The p for interaction by number of metabolic syndrome criteria was 0.91. When stratified according to TyG index, a measure of insulin resistance, the risk of T2DM with statin therapy was elevated in the upper TyG index quartile. However, the p for interaction by TyG index was not statistically significant (p=0.62).

Table 3. Risk of incident T2DM with statin therapy stratified by number of metabolic syndrome criteria and quartile of TyG index*

Parameter	Events / N	Incidence per 100 person year	HR Model 1	HR Model 2
Number of metabolic syndrome criteria				
0-2	72/2416	0.50	1.84 (0.88-3.83)	1.65 (0.79-3.47)
3	92/1188	1.33	1.51 (0.76-2.99)	1.33 (0.67-2.65)
4	121/762	2.77	1.58 (0.86-2.93)	1.57 (0.85-2.92)
5	68/276	4.59	1.28 (0.55-2.94)	1.62 (0.67-3.95)
TyG index				
Quartile 1 (<8.37)	12/1147	0.22	1.28 (0.23-7.29)	1.23 (0.21-7.42)
Quartile 2 (8.37-8.72)	48/1145	0.70	1.67 (0.68-4.11)	1.11 (0.49-2.80)
Quartile 3 (8.72-9.10)	84/1171	1.15	1.45 (0.72-2.91)	1.34 (0.67-2.67)
Quartile 4 (≥9.10)	209/1181	2.79	2.16 (1.35-3.45)	2.37 (1.46-3.83)

Hazard ratio (HR) and 95% confidence interval for incident T2DM, adusted for:
Model 1: Age and sex
Model 2: Model 1 + BMI, HDL-cholesterol, triglycerides
* TyG index calculated as Ln[fasting triglycerides(in mg/dL) x fasting glucose (in mg/dL)/2]

Table 4. Risk of incident T2DM with statin therapy stratified by levels of metabolic parameters

Parameter	Events / N	Incidence per 100 person year	HR Model 1	HR Model 2	P for inter-action
Plasma glucose level					$2.8*10^{-7}$
Quartile 1 (<5.3 mmol/L)	17/1073	0.24	3.97 (0.83-18.90)	5.11 (0.98-26.73)	
Quartile 2 (5.3-5.6 mmol/L)	19/909	0.34	4.95 (0.95-25.81)	3.87 (0.72-20.72)	
Quartile 3 (5.6-6.1 mmol/L)	70/1425	0.85	1.86 (0.86-4.04)	1.58 (0.72-3.51)	
Quartile 4 (≥6.1 mmol/L)	247/1238	4.00	1.09 (0.72-1.65)	1.11 (0.73-1.69)	
Body mass index					0.18
Quartile 1 (<24.1 kg/m^2)	36/1146	0.50	1.08 (0.42-2.77)	1.06 (0.41-2.76)	
Quartile 2 (24.1-26.3 kg/m^2)	63/1195	0.87	2.25 (0.97-5.24)	2.37 (1.01-5.57)	
Quartile 3 (26.3-28.7 kg/m^2)	100/1138	1.51	1.07 (0.57-2.04)	1.13 (0.59-2.18)	
Quartile 4 (≥28.7 kg/m^2)	154/1166	2.47	1.65 (0.95-2.89)	1.95 (1.10-3.43)	
Plasma triglyceride level					0.44
Quartile 1 (<1.0 mmol/L)	21/1130	0.39	1.41 (0.36-5.47)	1.34 (0.34-5.29)	
Quartile 2 (1.0-1.4 mmol/L)	61/1178	0.91	1.62 (0.74-3.57)	1.22 (0.53-2.76)	
Quartile 3 (1.4-1.9 mmol/L)	94/1170	1.29	1.56 (0.79-3.06)	1.35 (0.68-2.68)	
Quartile 4 (1.9-10 mmol/L)	175/1155	2.25	2.27 (1.37-3.74)	2.61 (1.55-4.39)	
Plasma HDL-c level					0.23
Quartile 1 (<1.0 mmol/L)	126/1109	1.86	1.80 (1.00-3.26)	1.84 (1.00-3.39)	
Quartile 2 (1.0-1.2 mmol/L)	103/1161	1.56	1.35 (0.72-2.54)	1.29 (0.68-2.44)	
Quartile 3 (1.2-1.4 mmol/L)	77/1197	1.10	1.82 (0.88-3.74)	1.60 (0.76-3.37)	
Quartile 4 (≥ 1.4 mmol/L)	47/1175	0.69	2.52 (0.98-6.48)	2.20 (0.83-5.81)	
Systolic blood pressure					0.71
Quartile 1 (<125 mmHg)	64/1147	0.94	1.64 (0.74-3.62)	1.71 (0.75-3.91)	
Quartile 2 (125-137 mmHg)	81/1161	1.21	2.49 (1.20-5.19)	2.27 (1.06-4.88)	
Quartile 3 (137-151 mmHg)	92/1124	1.39	1.88 (0.96-3.69)	1.79 (0.90-3.56)	
Quartile 4 (≥ 151 mmHg)	116/1213	1.63	1.29 (0.72-2.33)	1.26 (0.69-2.30)	

Hazard ratio (HR) and 95% confidence interval for incident T2DM, adusted for:
Model 1: Age and sex
Model 2: Model 1 + BMI, HDL-cholesterol, triglycerides
P for interaction adjusted for the model 2 variables.

When the variables included in the metabolic syndrome were considered separately, the effect of statin therapy was similar in increasing quartiles of each of the variables. The only exception was glucose level, with a larger increase in the risk of T2DM with statin use for patients with low baseline glucose levels compared to patients with higher baseline glucose levels. The p for interaction by baseline glucose level was 2.9 x 10^{-7}, adjusted for age, gender, BMI, plasma triglycerides and HDL-c. When adjusted for age, gender and a propensity score interaction term, this interaction remained statistically significant (p=0.006). In an exploratory analysis to explain this finding, the increased risk to develop T2DM with statin treatment seemed confined to patients with low baseline glucose levels but a high TyG index.

No effect modification by age or gender was present (p for interaction 0.35 and 0.93 respectively, adjusted for age, gender, BMI, plasma triglycerides and HDL-c).

Discussion

Statin use increases the risk of incident T2DM in patients with clinically manifest vascular disease, with a higher risk for patients using intensive statin therapy. The increase in risk of T2DM with statin therapy was independent of the number of metabolic syndrome criteria or insulin resistance.

Previous studies have convincingly shown an increased risk of incident T2DM associated with statin therapy in the general population and in patients with coronary artery disease (5-9;11). The present study confirms these findings in a cohort of patients with different manifestations of arterial disease. Our results show that increasing intensity of statin therapy is likely to be associated with increased risk of developing T2DM, which is in accordance with other studies (6;11). Similarly, our results are in accordance with the notion that pravastatin may have limited effect on the risk of T2DM, whereas rosuvastatin and atorvastatin may in particular increase the risk of T2DM (9;21). However, the benefits of statin therapy in the prevention of (recurrent) vascular events and mortality have been shown convincingly in clinical trials and meta-analyses (1) and outweigh the vascular risk of T2DM development in patients with established vascular disease (23). Intensive statin therapy has shown favorable effects on recurrent vascular events and mortality compared to moderate statin therapy (1;11) and again this benefit outweighs the increased risk of T2DM in these patients at high risk for both recurrent vascular events and T2DM. Therefore, not treating patients with established vascular disease with a statin, because of the increased risk of T2DM during statin treatment, would be the wrong choice. Treating physicians and patients should however be aware of the elevated risk of developing T2DM

during statin therapy. In the end it is the task of the treating physician to weigh these risks and identify those patients who benefit most from intensive statin treatment(24), and to identify the patients who are at the highest risk for developing T2DM during treatment and who should be monitored accordingly.

To identify these patients who are at increased risk to develop T2DM with statin therapy, results from the TNT and IDEAL studies suggested that baseline risk factors for T2DM could be used(11). In their analyses, the risk associated with statin therapy increased with increasing number of T2DM risk factors (resembling metabolic syndrome criteria)(11). In the present study, statin therapy increases the risk of T2DM in patients independent of the number of metabolic syndrome criteria. This may be due to the smaller sample size of the present study with fewer incident T2DM cases than in the TNT and IDEAL studies, since in the TNT and IDEAL studies the effect modification was weak with a p for interaction by number of T2DM risk factors of 0.07(11). Since the metabolic syndrome can be regarded as mainly a measure of insulin resistance (25), a continuous measure of insulin resistance might be used to define patients at high risk to develop T2DM with statin treatment. In our analyses, the increased risk to develop T2DM with statin treatment was indeed only statistically significant in the highest quartile of the TyG index (Ln[fasting triglycerides(in mg/dL) x fasting glucose (in mg/dL)/2])(22) as a measure of insulin resistance, but there was no formal effect modification. Therefore, determining insulin resistance alone may not be appropriate to reliably identify patients at high risk to develop T2DM with statin treatment. Furthermore, the present study suggests that the relative risk of developing T2DM due to statin therapy is higher in patients with low glucose levels at baseline. This finding contrasts the results of the JUPITER study where it was demonstrated that there was no interaction with baseline glucose level (17). Although there were few patients in the present study with low glucose levels who developed T2DM, which means that only a few additional T2DM cases can lead to high relative risks, the decrease in relative risk of T2DM due to statin treatment with increasing glucose levels was highly statistically significant, making this result less likely to be a chance finding. In our cohort study patients were already at statin treatment at baseline, therefore in theory a possible effect on plasma glucose level could already be incorporated in the plasma glucose level at baseline in patients in the highest glucose quartile. This would especially be the case for patients treated already for a longer period of time. We did not have information on initiation dates of statin therapy, hence we could not adjust for duration of statin use before inclusion in the cohort. Still, the issue then remains that patients with baseline glucose levels lower than 5.3 mmol/L have a high relative risk to develop T2DM with statin. In an explorative analysis to explain these findings, the patients developing T2DM with statin therapy despite low baseline fasting glucose levels seemed to be patients with

a high TyG index. These patients who are insulin resistant with normal fasting glucose, but probably impaired glucose tolerance, may therefore be in particular vulnerable for the increased risk of T2DM with statin therapy. Although impaired glucose tolerance and impaired fasting glucose are both risk factors for T2DM, they are considered two different entities of insulin resistance(26), which could explain a possible different susceptibility for statin-induced T2DM. However, since these exploratory analyses were performed in small groups of patients, with limited numbers of T2DM cases especially in the patients with low fasting glucose or low TyG index, they need confirmation in other studies.

Strengths of this study are the observational cohort, reflecting clinical practice and the good ascertainment of incident T2DM cases by two independent physicians. We acknowledge study limitations. Statin use was not continuously monitored during follow up and the use was estimated on baseline information, and follow-up information in a subset of patients. However, few patients stopped statin treatment during follow-up, since all patients included had an indication for statin therapy. Patients starting treatment were estimated to have used statin therapy during 50% from the time to the follow-up visit where statin therapy was registered. This will probably underestimate the treatment time and thus lead to underestimation of the effect of statin therapy, since these patients received a therapy advice at the start of the study period. Another limitation is the registration of type and dose of statin therapy which occurred only at baseline. This will probably underestimate the difference between the patients using moderate dose statin therapy and patients using intensive statin therapy, since part of the patients using moderate statin therapy is likely to have progressed to intensive statin therapy during follow-up. Finally, statin use was not randomly assigned and a different distribution of diabetes risk factors could (partially) explain our results. After adjustment for confounders or adjustment for a propensity score the effects of statin therapy on incident T2DM remained similar, although we cannot rule out some residual confounding.

In conclusion, statin use increases the risk of incident T2DM in patients with clinically manifest vascular disease. This increase in risk was independent of metabolic syndrome characteristics or insulin resistance. Intensive statin therapy was related to a higher risk of incident T2DM than low or moderate statin treatment.

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Chapter 8

The SMART prediction rule for estimating lifetime risk of recurrent vascular events in patients with clinically manifest vascular disease

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Submitted

Abstract

Background Estimates of 10-year risk of vascular events are strongly dependent on the age of the patient. Lifetime risk estimates may therefore add valuable information, particularly in young patients. The present study aims to estimate lifetime risk of recurrent vascular events in patients with clinically manifest vascular disease and to identify patients in whom estimating lifetime risk may be useful.

Methods Prospective cohort study of 6090 patients with clinically manifest vascular disease. A modified Fine & Gray proportional subdistribution hazard model was used to develop a prediction model for risk of all vascular events (myocardial infarction, stroke, vascular death) up to 85 year, using prespecified clinical and laboratory parameters as used in the SMART risk score. The performance of the model was internally validated at 10 years of follow-up.

Results During a median follow-up of 5.5 year (interquartile range 2.7-8.7), 928 vascular events occurred. All prespecified predictors remained in the model. The optimism-corrected C-statistic of the model was 0.693 and calibration was satisfactory for the 10-year estimates. The average predicted lifetime risk was 45±14% for patients aged 45-54; 40±15% for patients aged 55-64, and 35±16% for patients aged 65-74, whereas the average predicted 10-year risk was 14±7%, 19±10% and 32±17% in these age groups. Defining a 'very high risk' group as being the upper quartile of predicted risk for patients aged 45-75 year leads to a reclassification of 30% of the patients of 45-54 year old to 'very high risk' when using a lifetime risk estimate instead of a 10-year risk estimate. In patients aged >65 year, a lifetime risk model did not identify additional patients at high cardiovascular risk when compared to a 10-year risk model.

Conclusion The lifetime risk model identified young patients at high and at relatively low cardiovascular risk and could be used to discriminate between these groups of patients with clinically manifest vascular disease. The lifetime risk model did not add clinically relevant information in patients >65 year with clinically manifest vascular disease.

Introduction

Patients with symptomatic vascular disease are generally regarded to be at high 10-year risk of recurrent vascular events (1;2). However, similar to the primary prevention setting, also in the secondary prevention setting there is a large variation in 10-year risk of vascular events(3). A major determinant of 10-year risk of vascular events is age. Therefore, at young age the 10-year risk is generally low, also in patients with established arterial disease. However, the treating physician *and* the patients themselves intuitively realize that the risk to encounter a new vascular event during the remaining lifetime may be very high for patients who developed arterial disease already at a young age, but models to estimate this total risk are not available. Another remaining question is whether a lifetime risk model will change clinical practice, since all patients deserve treatment according to current guidelines(1;2) . A prediction model to estimate lifetime risk could potentially direct treatment decisions, provided that patients are reclassified from low 10-year risk to high lifetime risk or from high 10-year risk to low lifetime risk. At a younger age, there may be still more to gain and therefore firm treatment of risk factors at young age may lead to large reductions in the lifetime risk of recurrent vascular events. Expensive lifestyle improvement programs or costly new drugs may sort the largest effect in young patients, in whom a lifetime risk model estimates a very high risk of recurrent disease.

Thus far, no lifetime risk prediction model for recurrent vascular events is available. In the present study, we developed and validated a model for prediction of lifetime risk of recurrent vascular events in individual patients with various types of arterial disease. The focus will be on identification of patient groups in whom lifetime risk prediction provides additional information on vascular risk.

Methods

Study population

Data were used from patients enrolled in the SMART (Second Manifestation of ARTerial disease) cohort. This is a prospective, ongoing cohort study at the University Medical Center Utrecht, The Netherlands, designed to study the presence of concomitant arterial diseases and risk factors for atherosclerosis in a high risk population. Patients newly referred to our institution with any clinical manifestation of vascular disease or a vascular risk factor (hyperlipidemia, hypertension or diabetes) were asked to participate. Patients with a terminal malignancy, patients not sufficiently fluent in Dutch, patients not independent in daily activities or patients referred back to the referring specialist immediately after one

visit were excluded. Written informed consent was obtained from all patients. The Medical Ethics Committee of the University Medical Center Utrecht approved the study.

After inclusion, all patients underwent a vascular screening protocol including a health questionnaire, laboratory measurements and physical examination. The health questionnaire included among others medical history, smoking status and current medication use. Based on the questionnaire, the age at first diagnosis of vascular disease was determined. A detailed description of the study design has been published previously (4).

For the present study, data were used from 6090 patients aged 45 year and older, enrolled in the SMART study between September 1996 and March 2012, with either a history or a recent diagnosis of arterial disease: coronary artery disease, cerebrovascular disease, peripheral artery disease or aneurysm of the abdominal aorta. Patients could be classified into more than one disease category.

Follow-up

All study participants received a questionnaire every 6 months during the follow-up period to obtain information about hospitalizations and outpatient clinic visits. All available relevant data from any reported possible event were collected. Death of a participant was reported by relatives, the general practitioner or the specialist who treated the participant. All events were classified independently by three members of the SMART Study Endpoint Committee, comprising physicians from different departments. The outcomes of interest for this study were a composite of vascular death, myocardial infarction or ischemic stroke.

Model derivation

Single imputation methods were used to reduce missing covariate data for total cholesterol (n=28, 0.5%), HDL-cholesterol (n=44, 0.7%), systolic blood pressure (n=44, 0.7 %), hs-CRP (n=69, 1.1%), eGFR(n=25, 0.4%) and age at first event (n=18, 0.3%). Continuous predictors were truncated at the 1st and 99th percentile to limit the effect of outliers.

To account for competing risk in the lifetime risk setting, we used a subdistribution hazard model according to methods described earlier (5). In short, in this method patients with an earlier competing event remain in the risk set and receive with a weight depending on the censoring and truncation distributions. This method allows regression on the subdistribution hazard as in the Fine and Gray model (6), thus accounting for competing risk, while also allowing delayed entry. We estimated the lifetime risk up to an age of 85 years, since few patients in the cohort were alive beyond this age. A Cox proportional

hazard model for all-cause mortality using the same predictors as the main model was developed to estimate average survival beyond this age.

Candidate predictors were the predictors of the 10-year SMART risk score (age, sex, presence of diabetes mellitus, current smoking, systolic blood pressure, total cholesterol, high-density lipoprotein cholesterol, hs-CRP, years since first cardiovascular event, history of coronary artery disease, cerebrovascular disease, peripheral artery disease or aneurysm of the abdominal aorta and eGFR). Since age was used as the underlying time function in the present analysis, age and time since first cardiovascular event were not included in the present analysis. The age at study entry was used to define the delayed entry time in the study, enabling to study age-specific hazards. Continuous predictors were transformed if a transformation was used in the original SMART risk score for estimation of 10-year cardiovascular risk(3;7). The optimism in the predictions was determined by fitting a prediction model in 1000 bootstrap samples. To prevent overfitting, the coefficients were shrunk by a linear shrinkage factor to obtain optimism-corrected coefficients(7).

The proportional hazard assumption was assessed by testing the correlations between scaled Schoenfeld residuals for the various predictors and time.

Model validation

Since no 'lifetime' follow-up was available, the performance of the model was validated at 10 year of follow-up. We used the lifetime risk model to calculate 10-year risk predictions for all study participants. The discriminatory ability of the model was expressed by the concordance statistic for regression models with competing risk (8). The optimism of this concordance statistic was determined by fitting a prediction model in 1000 bootstrap samples and calculating the difference in concordance statistic of these models in the bootstrap sample and the original data. The optimism was subsequently subtracted from the apparent concordance statistic to obtain the optimism corrected concordance statistic. The model calibration was demonstrated by calibration plots, again accounting for competing risk when calculating the observed risk.

In a sensitivity analysis to investigate whether the short follow-up time of a part of the patients influences the results, we excluded patients who were included <5 year March 2012.

Comparison with 10-year risk

We compared the estimates of lifetime risk of vascular events with the 10-year risk estimates of the SMART risk score(3). Comparisons were made for patients <55 year of

age, for patients ≥55 and <65 year of age, and for patients for patients ≥65 and <75 year of age. For patients ≥75 year of age, the estimated lifetime risk up to an age of 85 year will not add anything to the 10-year risk prediction, since the 10-year risk prediction estimates risk for a longer period of time in these patients. We compared the risk reclassification of patients when using the upper two quartiles of predicted lifetime risk to identify high risk and extremely high risk patients instead of using the upper two quartiles of predicted 10-year risk. In addition, we calculated the 10-year and lifetime risk of vascular events for some example patients.

All analysis were performed in the statistical package R 2.13.2. The add-on packages mstate, rms, Hmisc, cmprsk, survival, pec and xlxs were used.

Results

Baseline characteristics according to age categories are shown in Table 1. With increasing age, systolic blood pressure and prevalence of diabetes mellitus increased, whereas the eGFR and prevalence of current smoking decreased. During a median follow-up of 5.5 year (interquartile range 2.7-8.7) and a total follow-up of 36,445 patient-years, 928 vascular events occurred and 1065 patients died (all-cause mortality).

Derivation and validation of the lifetime cardiovascular risk model

All prespecified predictors contributed significantly to the overall model. Testing the proportional hazard assumption showed proportional hazards for the overall model and for all of the individual predictors. The optimism was 4.25%, hence all coefficients were shrunk by multiplying the individual coefficients with 0.9575. The computational formulas for the lifetime risk estimates of patients aged 45 year old are presented in Table 2.

The optimism-corrected concordance statistic was 0.693. The calibration plot of the lifetime risk model estimates at 10 years of follow-up versus observed risk is shown in Figure 1. Exclusion of patients included <5 year before March 2012 did hardly change model coefficients and lifetime risk estimates.

Comparison of SMART 10-years risk estimates and lifetime risk estimates

In young patients (≥45 and <55 year of age), the average estimated 10-year risk was 14%(standard deviation(SD) 7%). The average lifetime risk up to 85 year was 45±14% in

Table 1. Baseline characteristics according to age

Age category (years)	**45-54**	**55-64**	**65-74**	**75-84**
N	1471	2217	1917	485
Age (years)	50.3 (2.8)	59.6 (2.8)	69.1 (2.8)	76.6 (1.3)
Age at first manifestation of CVD (years)	48.8 (5.4)	56.6 (6.8)	64.9 (8.3)	70.6 (10.3)
Male gender	1103 (75)	1692 (76)	1419 (74)	350 (72)
Body mass index (kg/m^2)	27.2 (4.4)	27.0 (3.9)	26.6 (3.6)	26.0 (3.3)
Diabetes mellitus	194 (13)	416 (19)	389 (20)	105 (22)
Systolic blood pressure (mmHg)	135 (19)	140 (21)	146 (22)	150 (22)
Total cholesterol (mmol/L)	5.0 (1.2)	4.9 (1.2)	4.8 (1.2)	4.8 (1.2)
LDL-cholesterol (mmol/L)	3.0 (1.1)	2.9 (1.0)	2.9 (1.0)	2.9 (1.1)
HDL-cholesterol (mmol/L)	1.2 (0.4)	1.2 (0.4)	1.3 (0.4)	1.3 (0.4)
Triglycerides (mmol/L)	1.5 (1.0-2.2)	1.4 (1.0-2.0)	1.3 (1.0-1.9)	1.3 (0.9-1.8)
eGFR (ml/min/1.73 m^2)	83 (17)	77 (16)	69 (17)	65 (17)
Glucose (mmol/L)	6.1 (1.8)	6.3 (1.8)	6.3 (1.6)	6.4 (1.9)
Hs-CRP (mg/L)	1.8 (0.9-4.0)	2.0 (0.9-4.3)	2.2 (1.1-4.6)	2.6 (1.3-5.9)
Use of lipid-lowering medication	976 (66)	1550 (70)	1233 (64)	263 (54)
Current smoking	707 (48)	713 (32)	403 (21)	71 (15)
Metabolic syndrome	783 (54)	1185 (54)	1030 (54)	243 (50)
Localization of vascular disease:				
Coronary artery disease	843 (57)	1405 (63)	1191 (62)	281 (58)
Cerebrovascular disease	409 (28)	602 (27)	558 (29)	170 (35)
Peripheral artery disease	330 (22)	409 (18)	352 (18)	105 (22)
Aneurysm of the abdominal aorta	53 (4)	170 (8)	256 (13)	86 (18)

All data are displayed as mean (SD), median (interquartile range) or n (%).
Abbreviations: eGFR: glomerular filtration rate, estimated by the Modification of Diet in Renal Disease (MDRD) equation; HDL-cholesterol: high-density lipoprotein cholesterol; Hs-CRP: high-sensitivity C-reactive protein; LDL-cholesterol: low-density lipoprotein cholesterol.

Table 2. Equation to calculate lifetime risk of recurrent vascular disease (up to 85 year of age) at 45 and 55 year of age

Estimated lifetime risk of vascular events at 45 year of age(%) = (1-0.3886543^exp(A)) x 100%
Estimated lifetime risk of vascular events at 55 year of age (%) = (1-0.4680942^exp(A)) x 100%
A = -0.229 [if female] +0.255 [if current smoker] + 0.00517 x systolic blood pressure in mmHg + 0.307 [if diabetic] + 0.404 [if history of coronary artery disease] + 0.507 [if history of cerebrovascular disease] + 0.617 [if abdominal aortic aneurysm] + 0.250 [if peripheral artery disease] -0.226 x HDL-cholesterol in mmol/L + 0.102 x total cholesterol in mmol/L -0.0342 x eGFR in mL/min/1.63m^2 +0.000155 x(eGFR in mL/min/1.63m^2)^2+0.168 x log(hs-CRP in mg/L)

Abbreviations: eGFR: glomerular filtration rate, estimated by the Modification of Diet in Renal Disease (MDRD) equation; HDL-cholesterol: high-density lipoprotein cholesterol; Hs-CRP: high-sensitivity C-reactive protein.

Figure 1. Calibration plot of predicted and observed risk of vascular events at 10 year.

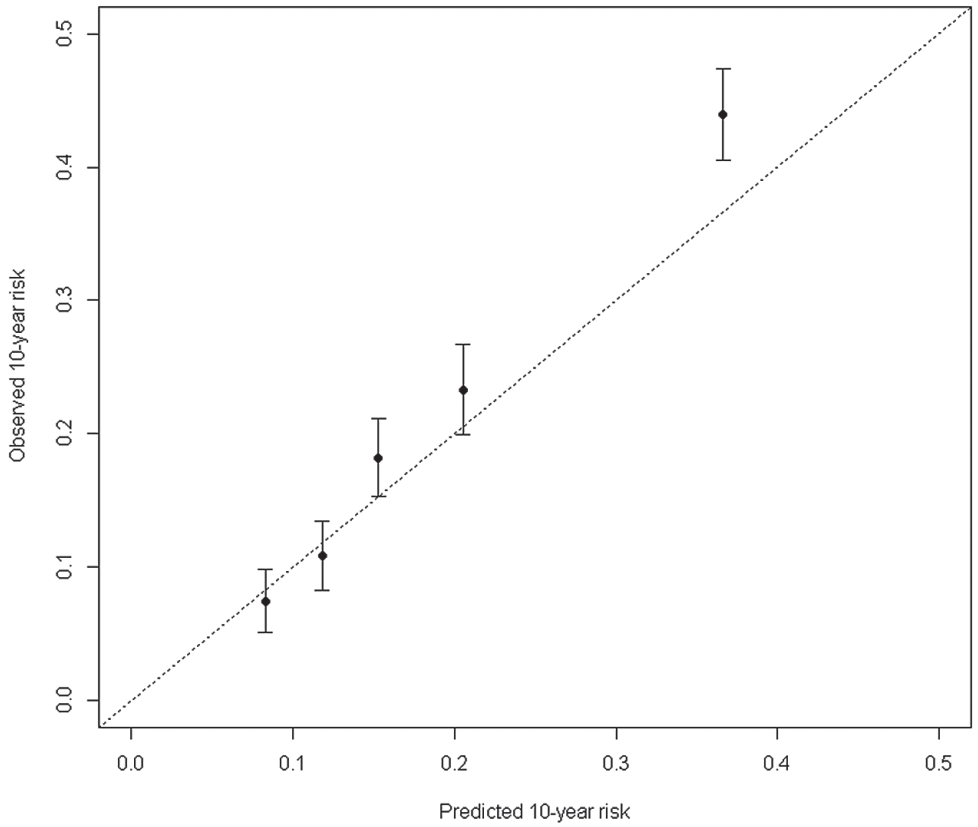
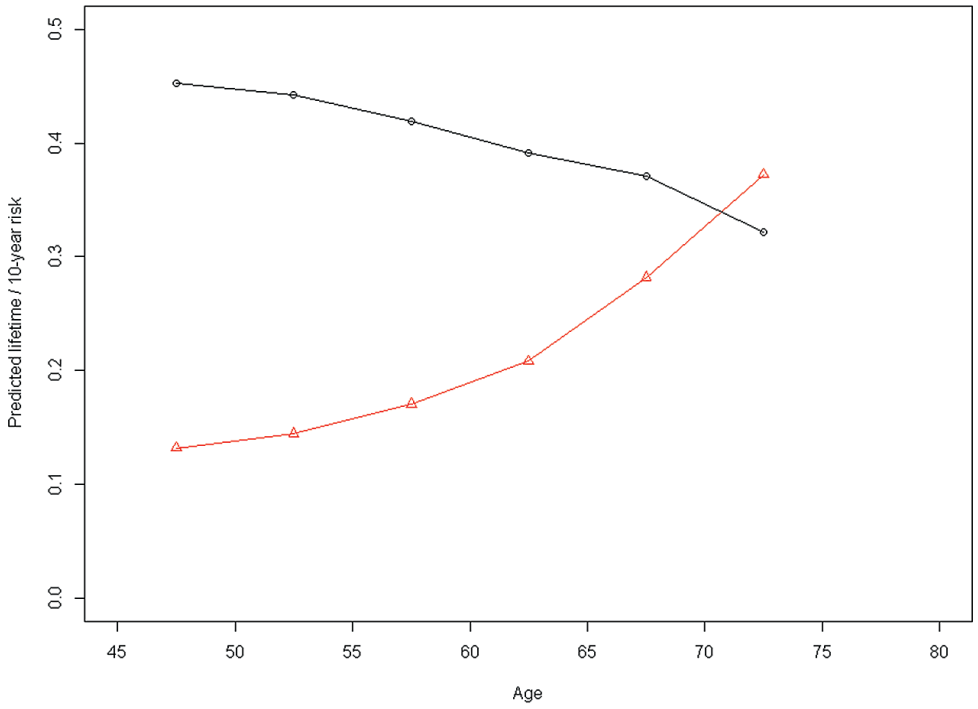


Figure 2. Average predicted lifetime risk and average predicted 10-year risk by age



Black: lifetime risk estimates; red: 10-year estimates

these patients. With a Cox model for all-cause mortality it was estimated that 39% of the patients aged 45-55 year would survive beyond 85 years of age. In patients ≥55 and <65 year of age, the average estimated lifetime risk was 40±15% and the average 10-year risk was 19±10%. In older patients (≥65 up to 85 year), the lifetime risk up to 85 year estimates the risk for a shorter period than the 10-year risk for a part of the patients. The average lifetime risk in these patients was 35±16% and the average 10-year risk was 32±17%.The difference between average lifetime risk and average 10-year risk is illustrated in Figure 2.

Defining the upper quartile of predicted risk for patients aged ≥45 and <85 year as 'very high risk', the threshold of very high risk would be 31% for 10-year risk and 47% for lifetime risk up to 85 year. Table 3 shows that using lifetime risk instead of 10-year risk to identify very high risk patients will lead to reclassification to a higher risk category for 66% of the patients aged 45-54. In contrast, it will lead to reclassification to a lower risk category for 52% of the patients aged 65-74 and reclassification to a higher risk category for only 1% of the patients. For patient aged 75-84, the 10-year risk will estimate the risk

Table 3. Reclassification of patients into quartiles of risk when using lifetime risk instead of 10-year risk of recurrent vascular events

		Lifetime risk			
Age (years)		Quartile 1-2	Quartile 3	Quartile 4	Total (any lifetime risk)
45-54	10-year risk quartile 1-2	436 (30)	480 (33)	295 (20)	1211 (83)
	10-year risk quartile 3	0 (0)	18 (1)	191 (13)	209 (14)
	10-year risk quartile 4	0 (0)	0 (0)	46 (3)	46 (3)
	Total (any 10-year risk)	436 (30)	498 (34)	532 (36)	1466
55-64	10-year risk quartile 1-2	918 (42)	389 (18)	78 (4)	1385 (63)
	10-year risk quartile 3	88 (4)	222 (10)	278 (13)	588 (27)
	10-year risk quartile 4	0(0)	23 (1)	215 (10)	238 (11)
	Total (any 10-year risk)	1006 (45)	634 (29)	571 (26)	2211
65-74	10-year risk quartile 1-2	421(22)	12 (1)	0(0)	433 (23)
	10-year risk quartile 3	515 (27)	114 (6)	18 (1)	647 (34)
	10-year risk quartile 4	275(14)	204 (11)	351 (18)	830 (43)
	Total (any 10-year risk)	1210 (63)	330 (27)	369 (19)	1910
75-84	10-year risk quartile 1-2	6 (1)	0 (0)	0 (0)	6 (1)
	10-year risk quartile 3	74 (15)	0 (0)	0 (0)	74 (15)
	10-year risk quartile 4	308 (64)	55 (11)	42 (9)	405 (84)
	Total (any 10-year risk)	388 (80)	55 (11)	42 (9)	485

Data shown as n (%)
Quartiles of 10-year risk: quartile 1-2: ≤19.1%; quartile 3: 19.2% - ≤ 30.8%; quartile 4: >30.8%
Quartiles of lifetime risk: quartile 1-2: ≤ 36.0%; quartile 3: 36.1 - ≤ 47.3%; quartile 4: >47.3%

for a longer period of time than the lifetime risk up to 85 year, hence lifetime risk estimates will lead to lower risk estimates.

Table 4. Comparison of lifetime risk up to 85 year of age and 10-year risk estimates of recurrent vascular events for example patients

	10-year risk	Lifetime risk up to 85 year
Patient 1		
- 45 year	6%	26%
- 55 year	8%	22%
Patient 2		
- 45 year	9%	49%
- 55 year	11%	41%
Patient 3		
- 45 year	16%	74%
- 55 year	19%	67%
Patient 4		
- 45 year	30%	92%
- 55 year	35%	87%

Patient 1 (low risk factor level): female, coronary artery disease, no DM, non-smoking, systolic blood pressure 130 mmHg, total cholesterol 4.0 mmol/L, HDL-cholesterol 1.4 mmol/L, hs-CRP 0.6 mg/L, eGFR 80 mL/min/1.73 m^2.
Patient 2 (average risk factor level):male, coronary artery disease, no DM, smoking, systolic blood pressure 140 mmHg, total cholesterol 4.5 mmol/L, HDL-cholesterol 1.1 mmol/L, hs-CRP 1.2 mg/L, eGFR 80 mL/min/1.73 m^2 .
Patient 3 (high risk factor level):male, coronary artery disease, DM, non-smoking, systolic blood pressure 160 mmHg, total cholesterol 7.0 mmol/L, HDL-cholesterol 1.0 mmol/L, hs-CRP 1.5 mg/L, eGFR 60 mL/min/1.73 m^2 .
Patient 4 (very high risk factor level) male, coronary artery disease and peripheral artery disease, DM, non-smoking, systolic blood pressure 160 mmHg, total cholesterol 6.0 mmol/L, HDL-cholesterol 1.0 mmol/L, hs-CRP 2.5 mg/L, eGFR 40 mL/min/1.73 m^2.

Patient examples

We estimated lifetime risk for 4 example patients at different ages and compared this with the estimated 10-year risk (Table 4). For a patient with coronary artery disease disease and a low risk factor level, 10-year risk of recurrent vascular events is low (6% at 45 year of age), but the estimated lifetime risk of recurrent vascular events up to 85 year of age is also low (26% at 45 year of age). For a patient coronary artery disease and peripheral artery disease

with a very high risk factor level, the 10-year risk is already very high (30% at 45 year of age), hence the lifetime risk will also be very high (92% at 45 year of age). For patients with clinically manifest vascular disease and average or high risk factor levels, the estimated 10-year risk of vascular events is relatively low (9-16%), whereas the estimated lifetime risk up to 85 year of age is still very high (49-74%). If possible, additional medical and lifestyle changes could be of benefit to reduce lifetime risk in these patients. For example, in the 45-year old patient 3 an additional 2 mmol/L reduction in total cholesterol, 0.2 increase in HDL cholesterol and a 10 mmHg decrease in systolic blood pressure would decrease the lifetime risk with 10%, and the age at which this patient reaches the 50% of cardiovascular risk (the expected age at which a vascular event will occur) will be postponed from 69 to 76. A similar intervention if patient 3 would be 55 year old would postpone the expected age at which a vascular event will occur from 77 to 82.

Discussion

In the present study, we developed and validated a model to predict lifetime risk of vascular events in patients with arterial disease. The model showed satisfactory discrimination and calibration at 10 year of follow-up. For patients at young age (<55 year), average lifetime risk was 45±14% whereas the average 10-year risk was 14±7% . The average lifetime risk was lower for patients at older age (35±16% for patients 65-74 year), whereas the average 10-year risk was higher (32±17% for patients 65-74 year). Defining the upper quartile of predicted risk for patients aged 45-75 year as 'very high risk' leads to a reclassification of 30% of the patients to 'very high risk' when using a lifetime risk estimate instead of a 10-year risk estimate.

Previous studies have estimated lifetime risk of vascular disease in subjects without vascular disease at baseline (9-17). The present study extends the concept of lifetime risk to the secondary prevention setting. Compared with healthy subjects, lifetime risk in this population is obviously higher. However, lifetime risk estimates from the Cardiovascular Lifetime Risk Pooling Project in historical US data give even higher estimates for subjects in the general population than we found in the present study (9;11), with lifetime risk estimates up to 60% for men en 55% for women. In contrast to these studies, which estimated the lifetime risk up to 95 year of age, we estimated the risk of vascular events up to an age of 85 year since there were few patients in the present study beyond an age of 85 year. Therefore, the actual 'lifetime' risk is higher, since 39% of the patients between 45 and 55 year of age will survive beyond 85 year of age. Moreover, the lifetime risk model in the contemporary QRISK showed considerably lower lifetime risk estimates than the lifetime risk estimates in the historical US data, consistent with the overestimation of

vascular risk by historical US algorithms in the current European setting (14). Our results in a contemporary European cohort show higher lifetime risk estimates up to 85 year of age than the QRISK lifetime risk estimates up to 95 year of age, which estimated a median lifetime risk of 35-40% in males and 25-30% in females aged 45-55 year old. Compared with the QRISK estimates, our results show a higher lifetime risk of vascular events for patients with established vascular disease(14).

In the primary prevention setting, lifetime risk estimates can be used to identify patients who are at high risk to develop vascular disease despite low 10-year risk, to start preventive measures in an early stage, although it remains a matter of debate whether medical therapy should be used in these patients(18). However, in secondary prevention, patients are already treated to reduce the risk of recurrent events and also lifestyle changes are advised if applicable(1;2). Guidelines do define a 'very high risk' group(1;2), but almost all patients with clinically manifest vascular disease will qualify for this group and no formal risk calculation is involved in this definition. Therefore, currently lifetime risk estimates are unlikely to change medical treatment in patients with established vascular disease, although they could be used to define young 'very high risk' patients who could benefit from e.g. more intensive statin therapy instead of the usual statin therapy necessary to attain the treatment target. Lifetime risk estimates could also be used to identify patients at highest lifetime risk who could benefit most from novel treatment or expensive lifestyle improvement programs, which may be of more value in young patients. Moreover, a patient who has encountered a vascular event at a young age is interested in long-term prognosis, and not only in the 10-year risk. The results of the present study show that the present lifetime risk model can discriminate between patients with low lifetime risk and high lifetime risk of recurrent vascular disease.

The benefit of using a lifetime risk model to estimate risk of recurrent vascular events may in particular apply for young patients. In these patients, the average lifetime risk is highest and average 10-year risk is lowest, hence a considerable part of the patients will be reclassified into the highest quartile of risk when using a lifetime risk estimate instead of a 10-year risk estimate. At a higher age, the difference in timespan between lifetime risk up to 85 year of age and 10-year risk is smaller, decreasing the difference in risk estimates. Up to 65 year of age, a considerable part of the patients is reclassified into a higher risk group, although this obviously depends on the threshold chosen. For patients older than 65 year of age, no additional high risk patients are identified with a lifetime risk model and using a lifetime risk model in these patients may not be helpful in clinical practice. The patient examples given illustrate the possible benefit of lifetime risk estimates in young patients, specifically in patients with average levels of risk factors. For patients with very high levels of risk factors, 10-year risk is already very high. The

very high 10-year risk in these young patients will prompt the clinicians to aggressively treat every risk factor, and providing a lifetime risk estimate would not change that. For patients with very low levels of risk factors, lifetime risk estimates will also be low, 26% in the example, which would probably not change the current treatment. It should be noted that low levels of risk factors in a young patient with a vascular event is very unlikely. In contrast, for a young patient with average or high risk factor levels, the lifetime risk up to 85 year of age is high, despite a relatively low 10-year risk. For these patients, lifetime risk estimates could be of added clinical value, to guide lifestyle changes and to consider intensification of risk factor treatment if possible. In the patient example 3, it is shown that an additional improvement leading to 2 mmol/L reduction in total cholesterol, 0.2 mmol/L increase in HDL-cholesterol and 10 mmHg decrease in systolic blood pressure would decrease lifetime risk with 10%. Although intensive treatment for patients with high 10-year risk may seem more cost-effective that intensive treatment for patients with high lifetime risk, this example also shows that additional treatment in this patient would postpone a recurrent vascular event on average with 8 year, whereas it would only postpone a recurrent event with 5 year if this patient would have been 55 year of age. Despite a relatively low 10-year risk for this patient at 45 year of age, this treatment could therefore still be cost-effective. In addition to a possible relevance for treatment decisions, the lifetime risk estimate is obviously relevant for all patients with regard to informing a patient about the long-term prognosis, and merely this reason may already favor use of lifetime risk estimates for recurrent events in clinical practice.

Study limitations need to be considered. Since patients were followed for a median of 5.5 year (interquartile range 2.7-8.7, maximum 15.5 years), the presented lifetime risks are not based on actual observed lifetime. However, since the treatment for patients with established vascular disease has changed drastically during the last decades, patients followed for a lifetime, starting decades ago, would not be representative anymore for the lifetime risk of contemporary patients. Still, the patients in the present study who developed arterial disease at a young age will be different from patients who develop arterial disease at an older age, and a patient who developed arterial disease at 50 years of age may have a higher risk at the age of 70 year than a patient who developed arterial disease 65 year of age. This may cause some underestimation of risk in young patients. In addition, we did not account for natural increase in risk factor burden over time, but assumed that risk factor levels remained constant throughout lifetime. Although older patients were less likely to smoke, the blood pressure increased with age, as well as presence of diabetes mellitus, and the eGFR decreased with age. This natural course of risk factor burden may result in increased risk of vascular events, therefore our approach may underestimate the lifetime risk. Furthermore, this lifetime risk prediction score was

not externally validated, therefore, the performance in other datasets is likely to be lower. However, to prevent overfitting we used prespecified predictors in the model and we applied uniform shrinkage to the model coefficients.

In conclusion, the present study shows the development and validation of a lifetime risk model for recurrent vascular events in patients with clinically manifest vascular disease. Comparison of the estimates of lifetime risk with the estimates of 10-year risk showed that the difference between these two risk estimates may be most relevant for young patients with vascular disease and with average risk factor levels. In patients with clinically manifest vascular disease older than 65 year of age, lifetime risk estimates are unlikely to add clinically relevant information.

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Chapter 9

General discussion

In this thesis, we studied several aspects of plasma lipids and the (vascular) risks conferred by these aspects. Although the field of lipids and genetics has been studied for decades, still many questions remain unanswered. The present thesis sheds new light on some of these questions and their provisional answers.

Should we genotype lipid-associated SNPs in patients with arterial disease?

Recent GWAS have identified numerous lipid-associated SNPs(1-3) and a part of these SNPs is associated with vascular events(4;5). Although the identified loci may lead to the discovery of new mechanisms involved in lipid metabolism and Mendelian randomization studies may give new insights in the causal pathway of lipids in atherosclerotic disease, direct applications of these SNPs have not entered clinical practice yet. Since the differences in plasma lipid levels caused by these SNPs are only small, measuring the plasma lipids provides more information about the level of atherogenic particles and thus is likely to provide more information about vascular risk as well. However, unlike measured plasma levels, genes carry information about lifetime exposure to plasma lipid levels, and a lifetime exposure to a minor increase in plasma lipid levels could still have a major effect on the risk of vascular events. This is exemplified by genetic variants in the PCSK9 gene: although leading to a reduction in low-density lipoprotein cholesterol (LDL-c) of only 15%, the risk of vascular events is reduced with 47% (6). This could raise the question whether lipid-associated SNPs should be genotyped in clinical practice.

In **chapter 4**, we studied the association between four LDL-c related SNPs and LDL-c in patients with clinically manifest vascular disease, and we investigated the relation between these SNPs and recurrent vascular events. We showed that even in patients treated with (intensive) lipid-lowering medication, the SNPs were still related with LDL-c. Furthermore, a score comprising these four SNPs was related with the risk of myocardial infarction in patients treated with lipid-lowering medication. In addition, we investigated the relations between a SNP in the APOA5-A4-C3-A1 gene complex and plasma triglycerides (TG), the metabolic syndrome and vascular events in **chapter 3**. This SNP was strongly associated with plasma TG levels, also in a population of patients with clinically manifest vascular disease and therefore in general a worse metabolic profile than the general population. The SNP was also related with metabolic syndrome in these patients, however, it was not associated with vascular events.

Since many genes are involved in the regulation of plasma lipid levels and many small changes in these genes can influence plasma lipid level (1;7), these results serve as an example for the regulation of plasma lipid levels in patients with established vascular disease. In patients with different clinical manifestations of vascular disease,

plasma LDL-c levels still depend on these small variances in genetic information, despite LDL-c lowering therapy. Moreover, also the risk of recurrent vascular events seems still dependent on genetic factors associated with plasma LDL-c. However, as yet it is not possible to fully determine the genetic profile which determines plasma LDL-c or other plasma lipid levels, and these four studied SNPs in LDL-c only explain a very small part of plasma LDL-c level and vascular risk. Even all known lipid-associated SNPs do explain only a part (approximately 10%) of the total variation in plasma lipid levels(1;3), which implies that genotyping all known variants would be costly while providing only incomplete information. Although genotyping these variants would provide information about lifetime exposure, as yet there is no evidence supporting the notion that this information would improve current risk prediction models. Furthermore, the phenotype resulting from the genotype may also depend on metabolic or other patient characteristics, as was shown in **chapter 3**. Information about these genotype-phenotype interactions is still scarce. As a result of these interactions, a change in e.g. diet, weight or medication could have a different phenotypic effect dependent on genotype, and conversely the difference between patients with a different genotype may only be expressed with a certain diet, weight or medication. The actual plasma lipid levels are not simply the addition of 'lipid genes' and 'secondary factors', but the result of an intricate network of processes involving these genes and secondary factors(8). This implies that the networks determining plasma lipid levels should be clear before the effects of genotype on plasma lipid levels can be fully understood, and that understanding these networks could even lead to identification of new genes involved in regulation of plasma lipid levels(9). Moreover, understanding these networks would enable development of personalized treatments targeted at the mechanism that will sort the largest effect and would enable personalized lifestyle and diet advice. This 'network medicine' is a field of study currently under development (9), but the complexity of the networks involved will imply that clinical applications as described above are rather a matter of decades than of years. For the time being, genotyping the known lipid-associated SNPs will not alter clinical practice(10). From a scientific standpoint it is useful to identify new lipid-related genes and to study the effect of genes on plasma lipid levels in order to better understand lipid metabolism and to find new drug targets. Measuring SNPs should not routinely be performed in clinical practice in patients with or without clinical manifestations of vascular disease.

Is plasma TG a better treatment target in addition to LDL-c than HDL-c?

LDL-c has demonstrated to be an appropriate treatment target to reduce the risk of vascular events(11-13). However, residual risk remains, and since high-density lipoprotein

cholesterol (HDL-c) is known for its robust inverse association with vascular events, HDL-c has been identified as a potential treatment target already long ago (14). However, the search for a drug significantly increasing HDL-c level whilst reducing the risk of vascular events has proven to be difficult. The older drug niacin increases HDL-c levels in addition to reducing LDL-c level, and is effective in reducing vascular events, although this may not result from the increase in HDL-c level (15). Only more recently, niacin has been studied as add-on therapy to statin treatment, showing disappointing results (16;17). A new class of drugs to increase HDL-c is currently under study. These drugs inhibit cholesteryl esterase transfer protein (CETP), a protein that facilitates the exchange of TG and cholesterol between apoB-containing lipoproteins and apoA-containing lipoproteins. The net result of CETP action is a transfer of TG from TG-rich lipoproteins to HDL particles, after which these HDL particles are rapidly cleared, decreasing plasma HDL-c levels (18). CETP-inhibitors increase plasma HDL-c levels and the more potent CETP inhibitors also have a LDL-c lowering effect. However, the development of two CETP inhibitors (torcetrapib and dalcetrapib) has been halted, because of detrimental effects (torcetrapib, which increased blood pressure and mortality rate) or futility (dalcetrapib) (19;20). Both CETP inhibitors were studied as an add-on to statin therapy in a population of (mostly) patients with established vascular disease. However, in these patients, evidence for a beneficial effect of HDL-c on vascular events is equivocal.

In **chapter 6**, we show that HDL-c is associated with vascular events in patients with clinically manifest vascular disease who are not treated with lipid-lowering medication or who are treated with usual dose lipid-lowering medication. However, in patients treated with intensive lipid-lowering medication, HDL-c was not associated with vascular events. There was a gradual decrease of the beneficial effect of HDL-c with increasing intensity of lipid-lowering medication. These results were in accordance with results from some statin trials reporting no effect of HDL-c on vascular events in the patients using statin therapy (21-23). In contrast, a very recent meta-analysis of eight statin trials provides evidence that on statin HDL-c is still associated with vascular events(24). Yet, several cohorts of patients with CAD (patients generally using potent statin therapy) report absence of an association between HDL-c and vascular events, indicating the uncertainty of the association between HDL-c and vascular events among patients with established vascular disease treated with statins(25;26). Moreover, the aforementioned meta-analysis showed that the (small) increase in HDL-c with statin therapy was not associated with a reduction in vascular events(24). This may indicate that HDL-c raising therapy is not helpful to prevent vascular events in patients using intensive statin therapy or even in general. Furthermore, also another line of evidence does not support a beneficial effect of HDL-c increasing therapy on vascular events. In Mendelian randomization studies,

genetic variability in HDL-c levels is not related with vascular events(27;28). Although a variant in CETP was associated with a decreased risk of vascular events, this variant also lower LDL-c, which could also explain the effect on vascular events(27). Since two CETP inhibitors currently under study, (anacetrapib and evacetrapib) are more powerful than dalcetrapib, thus reducing also LDL-c next to increasing HDL-c, and because they have a better safety profile than torcetrapib, these drugs may still be successful in decreasing the risk of vascular events(29;30). However, the effect of an isolated HDL-c increase will likely be disappointing.

Yet, HDL particles can have a beneficial effect on the arterial wall and the prevention of vascular disease via several well established mechanisms. In addition to reverse cholesterol transport, HDL particles have anti-inflammatory and anti-oxidant properties, and stimulates endothelial nitric oxide production (31;32). These functions are not likely to depend on the cholesterol content of HDL particles, but are likely to depend on the number of HDL particles. In general, HDL-c is correlated with the number of HDL particles, which could explain why HDL-c is inversely associated with vascular events(33). An analysis in the JUPITER trial showed that after adjustment for HDL particle number, HDL-c was not associated with vascular events, also in the patients allocated to placebo(34). Methods to increase HDL particle number are under study. Infusion of apoA1 or nascent HDL particles is an option, but due to its complexity not suitable for standard care(35). Oral drugs stimulating apoA1 production or acting as mimetic apoA1 are still in a very early stage of development and results need to be awaited whether these therapies are more effective than HDL-c increasing therapies(35).

In addition to HDL particle number, plasma TG levels may also explain some of the vascular risk associated with low HDL-c levels. Since CETP shuttles TG from TG-rich lipoproteins to HDL particles, high plasma TG levels result in more TG shuttling to HDL particles(18). After the (rapid) clearance of these HDL particles, the net result is a lower HDL-c(18). This mechanism explains that high TG is a key mechanism determining HDL-c level. Therefore, plasma TG may be one of the underlying mechanisms causing atherosclerosis, although the more stable HDL-c reflects average TG level better and are hence the association between HDL-c and vascular events may be stronger. This was also reviewed in **chapter 2**. In **chapter 5** we studied the effect of plasma TG on recurrent events. After adjustment for age, sex, smoking, lipid-lowering medication, body mass index and LDL-c, plasma TG was shown to be associated with vascular events. This association was also present in patients with low nonHDL-c, the advocated treatment target in patients with elevated TG levels. After adjustment for HDL-c, the association between plasma TG and vascular events disappeared, but as was explained above, this does not rule out a causal association between plasma TG and vascular events. Also pathophysiologic and

genetic evidence points toward direct effects of plasma TG on atherosclerosis. In **chapter 2**, we discuss evidence showing that triglyceride-rich remnant particles are taken up in the arterial wall, resulting in formation of atherosclerotic plaques. Genetic studies have shown that variants associated with plasma TG levels increase the risk of vascular events, also independent of HDL-c(36;37).

This evidence of a causal role of plasma TG on vascular events makes plasma TG a potential treatment target next to HDL-c or instead of HDL-c. Although the attention for plasma TG has increased and specific guidelines have been developed(38;39), pharmacological therapies to lower plasma TG level produce at best modest reductions in vascular event risk. Niacin reduces plasma TG level next to increasing HDL-c level, but as discussed before fails to lower the risk of vascular events when added to statin therapy(17;40). Fibrates have been shown to reduce the risk of vascular events when added to statins, but only in a selected subgroup of patients with low HDL-c (<0.88 mmol/L) and high TG levels (>2.3 mmol/L)(41;42). Omega-3 fatty acids are also effective in reducing plasma TG level, but have not shown beneficial effects in trials assessing vascular outcomes(43;44). Other drugs to reduce plasma TG level are currently under development, such as dual PPAR-α/δ agonists and diacylglycerol O-acyltransferase 1 inhibitors, which have been shown to reduce plasma TG levels in human studies, but their effects on vascular events is not known yet(45).

To summarize, current pharmacological options to reduce plasma TG while effectively reducing the number of vascular events are limited. The same is true for current options to increase plasma HDL-c, whereas HDL-c may not be the appropriate treatment target. Considering the disappointing effect on vascular events of the existing drugs reducing plasma TG or increasing plasma HDL-c, both plasma TG levels and plasma HDL-c levels may currently not be appropriate treatment targets to reduce residual risk of vascular events, although they are useful risk markers. The only exception is for patients with high TG and low HDL-c, for whom therapy (mainly fibrates) can be advised. Obviously, the results of research into new drugs may change views on treatment targets for plasma TG or HDL-c. And perhaps therapies to improve HDL function or increase HDL particle number have the potential to be more effective than a treatment targeted at one of these traditional lipid parameters.

Should high-dose statin be avoided in patients at high risk of developing type 2 diabetes mellitus?

During the last two decades, statins have obtained a prominent place in the prevention of vascular events(13;46). Administration of statins combines a robust decrease in the

risk of vascular events with a favorable safety profile(12). The recent demonstration of an increased incidence of type 2 diabetes mellitus (T2DM) with statin therapy shows that also statin therapy has its adverse effects(47;48). How to use this knowledge of increased incidence of T2DM in clinical practice is still a matter of debate.

In **chapter 7**, we confirm the increased incidence of T2DM in statin treated patients in a cohort of patient with established vascular disease. More intensive statin therapy increases the risk to develop T2DM compared to less intensive statin therapy, with fluvastatin and pravastatin being more favorable than simvastatin and especially atorvastatin or rosuvastatin. Also other studies have demonstrated that more potent statins or a higher dose of any statin confers a higher risk to develop T2DM than less potent or lower dose statins(48-50). If patients with a high risk to develop T2DM due to statin treatment could be identified, avoidance of high dose atorvastatin and rosuvastatin might be an option to prevent T2DM in these patients. Results from the TNT and IDEAL studies suggested that risk factors for T2DM also increase the relative risk to develop T2DM with statin treatment(51). In patients with few of these risk factors (resembling metabolic syndrome criteria), statin treatment did not increase the risk to develop T2DM(51). This would imply that for patients at low risk to develop T2DM, intensive statin therapy could be prescribed without fear to induce T2DM. In patients with several baseline risk factors for T2DM, statin treatment did increase the risk to develop T2DM(51). Hence, the reaction to this finding could be to prescribe a statin with a small effect on the incidence of T2DM, such as pravastatin, if necessary with addition of other (non-statin) LDL-c lowering drugs. However, the TNT and IDEAL studies also showed that for patients at the highest risk of T2DM, intensive statin is of most benefit to reduce the risk of recurrent vascular events(51). Therefore, the increased risk of vascular events when prescribing less potent statin therapy should be carefully weighed against the decreased risk to develop T2DM. Moreover, there is few additional evidence suggesting that the increased risk to develop T2DM with statin therapy related to baseline risk factors for T2DM. Our results in **chapter 7** do not support the findings in the TNT and IDEAL studies. The increase in risk was similar across patients with different numbers of metabolic syndrome criteria, as a proxy of risk of T2DM development and across patients with different levels of insulin resistance. Although the increase in risk of T2DM with statin therapy was only statistically significant in patients in the highest quartile of insulin resistance, a possible effect of insulin resistance or other baseline risk factors for T2DM on the increased risk of T2DM with statin therapy is likely to be small. Also in patients at high risk of T2DM, atorvastatin and rosuvastatin should be used to decrease the risk of vascular events, instead of less potent pravastatin. In these patients, the risk of vascular events will also be higher since risk factors for T2DM are also risk factors for vascular disease(52), and therefore the reduction in absolute risk

of vascular events will be higher than in patients with few risk factors for T2DM. In the TNT and IDEAL trials, intensive statin therapy as compared to moderate statin therapy for an average follow-up of 4.8-4.9 years overall resulted in a reduction of 17 major vascular events while inducing 9 cases of T2DM per 1000 patients (12). Both numbers will increase in patients at high risk for T2DM, but the current evidence is insufficient to state that the balance will be changed. More studies are necessary to understand the mechanism by which statins increase the risk of T2DM and to whom this risk applies. For the time being, also intensive statin therapy should be administered if needed according to guidelines, without regarding baseline risk factors for T2DM. The treating clinician should be aware of this increased risk, and because of the higher absolute risk in patients with baseline risk factors, he should focus primarily on patients at high baseline risk for T2DM. However, identifying specific patients at risk to develop T2DM with statin therapy is not possible.

Is there any added value of calculating lifetime risk of recurrent vascular events in clinical practice?

Conventional vascular risk prediction models estimate 10-year risk of vascular events(53-55). In the primary prevention, the estimated 10-year risk is used to decide about starting treatment to prevent vascular events(46). Although this practice to use 10-year risk estimates is useful to identify patients for whom treatment will be beneficial and cost-effective, it has its shortcomings. By definition, 10-year risk is low in young patients, whereas in these patients there may be much to gain as disease processes leading to damage to the vessel wall are still reversible at younger age. Therefore, the concept of lifetime risk is now increasingly studied in the primary prevention setting(56;57). These lifetime risk models estimate the cumulative risk of vascular disease in general up to an age of 95 year to provide a more complete picture of vascular risk. In patients with a low 10-year risk but a high lifetime risk, lifestyle changes should be advised and start of medical therapy may be considered despite the low 10-year risk, because in general this high lifetime risk indicates a high risk factor burden(46). By the time the 10-year risk passes the threshold of high risk, this high risk factor burden will already have translated into considerable damage to the vascular wall. Treating risk factors at a younger age will prevent this damage and will lead to a larger gain in healthy life years than treating only when 10-year risk passes the arbitrary threshold of high risk(56).

In the secondary prevention setting, so far no model is available to estimate lifetime risk of recurrent events. In **chapter 8** we developed and validated a model to estimate the lifetime risk of recurrent events using clinical characteristics and laboratory parameters. In particular for young patients, there was considerable difference between

the 10-year risk estimate and the lifetime risk estimate of recurrent events. In patients between 45 and 55 year of age, the average 10-year risk was 14%, whereas the average lifetime risk was 45%. However, in secondary prevention, all patients are regarded at high 10-year risk according to guidelines. Although the above results and other studies have shown that a part of the patients and especially young patients are not at the assumed 10-year risk of >20%, guidelines do not advocate differentiation in treatment according to estimated risk. Therefore, the clinical consequences of these lifetime risk estimations are less evident than in the primary prevention setting and the question could raise whether estimating lifetime risk will have any consequences in the secondary prevention setting. However, also in the secondary prevention setting risk estimation is becoming increasingly important. More intensive statin treatment than necessary to attain the LDL-c target, expensive new therapies that may become available such as immunomodulants or biologicals(58;59) and expensive lifestyle improvement programs may not be cost-effective in all patients or may cause more harm than benefit in some patients. Selecting patients for these treatments may be performed with 10-year risk estimation models, but our results in **chapter 8** show that a lifetime risk model can identify a large group of patients <65 years who are at high lifetime risk despite having a low 10-year risk. In these patients, the 10-year risks may be low, but the lifetime risk is high and may even be higher than the lifetime risk of older patients at high 10-year risk. Although treating these patients with expensive therapy will be costly since the treatment time will be longer, the benefits could also be larger. Especially for these patients, who encountered a vascular event at young age and are at high risk for a recurrent event during the remaining lifespan, aggressive treatment to reduce residual risk seems appropriate.

Furthermore, the benefits of lifetime risk estimates may extend beyond a more accurate identification of patients in need of additional therapy. For young patients, a 10-year horizon is very short. For a 45-year old patient, the risk of vascular events up to 55 year of age is useful, but would be more useful if put into perspective by an estimate of the risk up to 85 year of age. Adding a lifetime risk estimate to a 10-year risk estimate will provide more information about the risk of recurrent events for this patient. In addition, providing information about long term risk has been shown to improve patient adherence and realization of lipid targets in a study in primary prevention setting in which 'cardiovascular age' was calculated, an alternative method to communicate lifetime risk (60). In this study, 'cardiovascular age' could not be calculated for patients with clinically manifest vascular disease(60), but these results show that informing the patient about long-term risk may also improve the realization of treatment targets.

A future step in the clinical application of lifetime risk estimates may be estimation of lifetime treatment effects. Although the model we derived in **chapter 8** is formally

not developed for this type of predictions, we showed that small changes in risk factor levels can have large effects on lifetime risks, especially in young patients. To estimate the treatment effect on lifetime risk, the hazard ratios derived in trials could be used (61), or the methods to estimate individualized treatment effect in trials could be adapted to estimate lifetime treatment effects(62). However, these methods will still estimate the lifetime effects based on observed treatment times of in approximately 5 year, whereas genetic studies show that for example lifetime low LDL-c levels will lead to much larger decreases in risk than a comparable reduction attained in statin trials(6). Since trials with lifetime treatment are unlikely to be performed, estimation of lifetime treatment effects will continue to be affected by this problem. While taking this into account, lifetime risk estimation of treatment effects will further help to find the best treatment for every young patients to further decrease the risk of (recurrent) vascular events during the remaining lifespan. In current clinical practice, the presented lifetime risk model in **chapter 8** can be applied immediately to inform patients with clinically manifest vascular disease about their long-term prognosis and to guide more aggressive therapy if available.

In conclusion, the studies presented in this thesis showed that:

- SNPs associated with plasma LDL-c and TG levels and with risk of coronary artery disease in the general population are still associated with plasma LDL-c and TG levels in patients with various clinical manifestations of vascular disease, and LDL-c related SNPs seem to be related with recurrent myocardial infarction.
- The association between rs964184 in the APOA5-A4-C3-A1 gene complex and plasma TG level is modulated by BMI
- Plasma TG levels are associated with recurrent vascular events independent of LDL-c or nonHDL-c levels.
- Plasma HDL-c levels are inversely associated with recurrent vascular events in patients not treated with lipid-lowering medication or treated with moderate dose lipid-lowering medication, but are not associated with recurrent vascular events in patients treated with intensive lipid-lowering medication.
- Treatment with statins increases the risk of T2DM in a dose dependent manner, independent of the number of metabolic syndrome criteria present.
- A model to lifetime risk of recurrent vascular events is useful to identify additional high-risk patients in patients aged <65 years compared to a 10-year risk model.

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Chapter 10

Summary
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Summary

The plasma lipid levels measured in clinical practice are plasma triglyceride (TG) and cholesterol levels. These lipids are transported in lipoproteins to facilitate solubility. Triglycerides are mainly transported in chylomicrons, produced in the intestines, and very low density lipoprotein (VLDL) particles. After hydrolysis of the triglycerides in VLDL, intermediate density lipoprotein (IDL) and ultimately low density lipoprotein (LDL) is left. The cholesterol content of the plasma is mainly located in LDL and high-density lipoprotein (HDL) particles. Uptake of LDL particles provides cholesterol for peripheral tissues. HDL particles take up cholesterol mainly in the peripheral circulation, and transport cholesterol to the liver.

Plasma lipid levels are strongly associated with the risk of vascular events. Moreover, drugs lowering plasma LDL-cholesterol (mainly statins) are very effective in reducing the risk of vascular events. However, residual risk remains, and plasma levels of TG and HDL-cholesterol may explain a part of this risk after reduction of LDL-cholesterol with statin therapy.

Adiposity and plasma lipid levels

In **chapter 2** we review the literature regarding the effect of adiposity on plasma lipid levels and the consequences of this effect for the risk of vascular events. Overweight and obesity are an increasing problem in the Western society with impact also on plasma lipid levels, which are determined by genetic factors and secondary factors. Excessive storage of triglycerides in adipocytes and the consequent hypertrophy and proliferation of adipocytes results in a low grade inflammation of adipose tissue. The inflammatory changes lead to an altered adipose tissue function with change in the profile of the adipokines released, and insulin resistance will be the consequence. The inflammation, insulin resistance and altered adipokine profile together result in an increase in TG production and a decrease in TG catabolic rate an hence increased plasma TG levels. Increased plasma TG levels have impact on other lipid levels as well: via cholesteryl ester transfer protein (CETP), TG is shuttled for cholesterol, and high TG plasma levels directly lead to low HDL-cholesterol levels and formation of small dense LDL particles. High plasma TG levels are associated with atherosclerosis via several mechanisms, via direct effect on endothelial inflammation and uptake of VLDL remnants in the arterial wall, but also via small dense LDL, which is more prone to uptake in the arterial wall and via low HDL-cholesterol, which is associated with atherosclerosis.

Genotype and phenotype

Since both genotype and secondary factors influence plasma lipid levels, the association between plasma lipid levels and vascular events could possibly be explained by secondary factors. Several studies have however shown that genetic determinants of plasma lipid levels are still associated with vascular events, this is most clearly shown for LDL-cholesterol. The contribution of both genetic determinants of plasma lipid levels as well as measured plasma lipid levels to vascular risk is less clear in patients with clinically manifest vascular disease.

In **chapter 3** and **chapter 4,** we study whether single nucleotide polymorphisms (SNPs) that are associated with LDL-cholesterol or plasma triglyceride level in the general population are still related with LDL-cholesterol or triglyceride level in patients with arterial disease, of whom a large part uses lipid-lowering medication. We show that these SNPs are still related with the respective lipid levels and that they are related with the probability to be at the lipid treatment target. Despite lipid-lowering medication, these SNPs still influence plasma lipid levels. Furthermore, our results suggested that the LDL-cholesterol related SNPs were associated with vascular events in patients using lipid-lowering medication.

In **chapter 5**, we study the effect of plasma TG levels on vascular events in patients with various clinical manifestations of vascular disease. We show that plasma TG levels increase the risk of vascular events, after adjustment for confounding factors and even after adjustment for nonHDL-cholesterol. After adjustment for HDL-cholesterol, the association was considerably weakened, but plasma TG levels were also a risk factor in patients with high levels of HDL-cholesterol. Since it is argued that plasma TG levels are only a risk factor for vascular events because of the association with insulin resistance of type 2 diabetes mellitus, we adjusted also for insulin resistance and derived estimates in patients with or without type 2 diabetes mellitus separately. The results show that plasma TG levels are also a risk factor for patients without type 2 diabetes mellitus. The increased risk of vascular events with plasma triglyceride levels was particularly present in patients with coronary artery disease.

HDL-cholesterol is known for its robust inverse association with vascular events. However, some studies suggest that this inverse association is not present in patients treated intensively with LDL-cholesterol lowering medication. In **chapter 6** we therefore investigate the association between HDL-cholesterol and vascular events in a population of patients with established arterial disease, of whom a large part is treated with (intensive) LDL-cholesterol reducing therapy. Since the treatment was not randomly allocated, we adjusted for a propensity score comprising all baseline characteristics. Our

results show that the inverse association between HDL-cholesterol and vascular events gradually weakens with increasing intensity of LDL-cholesterol reducing therapy. This finding seemed independent of the attained plasma LDL-cholesterol levels.

Adverse effects of lipid-lowering therapy

Every treatment will also convey some risk of adverse events, also lipid-lowering therapies. Statins are generally known as well-tolerated with a favorable adverse effect profile. Recent reports have indicated that statin treatment may increase the risk of type 2 diabetes mellitus. Although it was suggested that only patients already at high risk to develop type 2 diabetes mellitus are affected by this increased risk, it is not fully clear which patients are in particular at risk to develop type 2 diabetes mellitus.

In **chapter 7,** we quantify the effect of statin therapy on incident type 2 diabetes mellitus in patients with various clinical manifestation of vascular disease. We confirm the increased risk of type 2 diabetes mellitus with statin therapy. Intensive statin therapy is associated with a higher risk to develop type 2 diabetes mellitus. The increase in risk was independent of the number of metabolic syndrome criteria or insulin resistance, although the increase was statistically significant only in the upper quartile of insulin resistance. The increase in risk of type 2 diabetes mellitus with statin therapy seems in particular present in patients with low baseline glucose, which might be due to insulin resistance in these patients despite a low fasting glucose.

Long-term risk estimation in patients with clinically manifest vascular disease

Estimation of vascular risk is important to guide (lipid-lowering) therapy. Although all patients with known arterial disease are considered to be at high risk of recurrent events and treated according to guidelines, risk estimation could be used to guide new expensive or hazardous therapy. A 10-year risk estimation could be used for this purpose, but these risk estimates by definition provide low risk estimates in young patients. Therefore, a long-term or lifetime risk model may provide additional information in these patients.

In **chapter 8**, we therefore develop and validate a model to estimate the risk of vascular events up to an age of 85 year in patients with various clinical manifestations of vascular disease. We used a pre-specified model with predictors from the SMART risk score. The model could reasonably discriminate between patients at high risk and patients at low risk of vascular events. Although the average 10-year risk in patients aged 45 up to 54 year of age was only 14%, the lifetime risk up to 85 year was 45% in these

patients. With increasing age, the difference between 10-year risk and lifetime risk up to 85 year obviously decreased. For patients up to 65 year of age, using the lifetime risk model resulted in classification of patients into higher risk categories. Our results show that the lifetime risk model could be used to provide additional information about the risk of vascular events for patients up to 65 year of age.

Nederlandse samenvatting

In het bloed worden verschillende soorten vetten (lipiden) vervoerd. Deze lipiden worden getransporteerd door eiwitten. De combinatie van de lipiden met deze eiwitten worden lipoproteïnen genoemd. De belangrijkste lipidenwaarden voor de arts zijn vooral het triglyceridengehalte en het cholesterol. Het cholesterol wordt onderscheiden in het low density lipoprotein cholesterol (LDL-cholesterol, 'het slechte cholesterol') en high density lipoprotein cholesterol (HDL-cholesterol, 'het goede cholesterol). Zoals de naam laat zien, wordt dit onderscheid gemaakt op basis van de soort lipoproteïne waarin het vervoerd wordt.

De lipidenwaarden in het bloed hangen sterk samen met het risico op hart- en vaatziekten. Medicijnen om het LDL-cholesterol te verlagen, zoals statines, zijn erg effectief in het verlagen van het risico op hart- en vaatziekten. Ze voorkomen echter niet alle hart- en vaatziekten. De (andere) lipidenwaarden in het bloed zouden een deel van dit overgebleven risico kunnen verklaren.

Overgewicht en de lipidenwaarden in het bloed

Overgewicht is in de huidige westerse maatschappij een gezondheidsprobleem geworden, met ook invloed op de lipidenwaarden in het bloed. Lipidenwaarden worden namelijk bepaald door genetische factoren, maar ook door andere factoren zoals overgewicht en eetpatroon. In **hoofdstuk 2** geven we een overzicht van wat er bekend is over het effect van overgewicht op lipidenwaarden. We kijken vooral naar de triglyceridewaarden en gaan ook na welk effecten ze hebben op het risico op hart- en vaatziekten. Overgewicht leidt tot veranderingen in het vetweefsel en geeft een chronische lichte ontsteking van het vetweefsel. De balans van de signaalstoffen die het vetweefsel produceert (zogenaamde adipokines) verandert en de gevoeligheid voor insuline vermindert. Deze veranderingen zorgen voor een verhoogde productie van triglyeriden en een verminderde afbraak van triglyceriden, dus een verhoging van de triglyceridenwaarden in het bloed. Deze verhoging van de triglyceridenwaarden beïnvloedt ook andere lipidenwaarden: het leidt tot een lagere waarde van het 'goede cholesterol', het HDL-cholesterol en kleiner worden van de 'slechte' LDL deeltjes. De hogere triglyceridenwaarden verhogen ook het risico op hart- en vaatziekten. De triglyceriden-rijke deeltjes hebben een direct effect op ontsteking van de vaatwand. Daarnaast kunnen de restanten van triglyceriden-rijke deeltjes opgenomen worden in de vaatwand en daar schade aanbrengen. Het effect op hart- en vaatziekten gaat ook via de kleinere LDL deeltjes, omdat deze eerder opgenomen

worden in de vaatwand, en via een verlaging van het HDL-cholesterol wat samenhangt met hart- en vaatziekten.

Genetische informatie en gemeten lipidenwaarden

Zowel genetische factoren alsook andere factoren bepalen de lipidenwaarden in het bloed. Het effect van lipidenwaarden op hart- en vaatziekten zou daarom mogelijk verklaard kunnen worden door factoren die tegelijk de lipidenwaarden beïnvloeden en ook het risico op hart- en vaatziekten vergroten, zonder dat de lipidenwaarden de oorzaak zijn van de hart- en vaatziekten. Genetische studies hebben echter laten zien dat genetische factoren die de lipidenwaarden beïnvloeden ook samenhangen met hart- en vaatziekten. Deze studies tonen dus een oorzakelijk verband aan tussen lipidenwaarden en hart- en vaatziekten. In mensen die al hart- en vaatziekten hebben, is de bijdrage van genetische factoren en de gemeten lipidenwaarden in het bloed minder duidelijk.

In **hoofdstuk 3** en **hoofdstuk 4** onderzoeken we of kleine genetische veranderingen (zogenaamde SNPs) die samenhangen met het LDL-cholesterol of met de triglyceridenwaarden in de algemene bevolking nog steeds samenhangen met LDL-cholesterol of triglyceridenwaarden in patiënten met hart- en vaatziekten, waarvan er velen cholesterolverlagers gebruiken. We laten zien dat deze genetische veranderingen nog steeds samenhangen met de lipidenwaarden, ondanks behandeling. Ook tonen we aan dat deze genetische veranderingen samenhangen met de kans om het behandeldoel voor de lipidenwaarden te bereiken.

In **hoofdstuk 5** onderzoeken we het verband tussen het triglcyeridengehalte in het bloed en nieuwe hart- en vaatziekten in patiënten die al hart- en vaatziekten hebben. We laten zien dat de triglyceridenwaarden samenhangen met nieuwe hart- en vaatziekten. Het verhoogde risico door verhoogde triglyceridenwaarden kon niet alleen verklaard worden door een verminderde insulinegevoeligheid. Het lijkt echter weinig informatie toe te voegen naast het HDL-cholesterol.

HDL-cholesterol staat bekend als het 'goede cholesterol' en een laag HDL-cholesterol hangt samen met hart- en vaatziekten. Sommige studies suggereren echter dat dit niet zo is bij patiënten die behandeld worden met sterke cholesterolverlagers (gericht tegen LDL-cholesterol, het 'slechte cholesterol'). In **hoofdstuk 6** onderzoeken we daarom het verband tussen HDL-cholesterol en nieuwe hart- en vaatziekten en patiënten die al hart- en vaatziekten hebben en waarvan een groot deel behandeld wordt met cholesterolverlagers. Het blijkt uit onze resultaten dat het verband tussen een laag HDL-cholesterol en hart- en vaatziekten steeds minder sterk wordt bij sterker wordende

cholesterolverlagers. In patiënten die krachtige cholesterolverlagers gebruiken, leek het HDL-cholesterol niet meer samen te hangen met nieuwe hart- en vaatziekten.

Bijwerkingen van cholesterolverlagers

Alle behandelingen hebben ook bijwerkingen, zo ook cholesterolverlagers. De meest gebruikte cholesterolverlagers, statines, staan over het algemeen bekend als medicijnen met weinig (ernstige) bijwerkingen. Recent bleek uit studies dat statines wel het risico op type 2 diabetes mellitus ('ouderdomssuikerziekte') verhogen. Het is niet helemaal duidelijk voor welke patiënten het risico op type 2 diabetes mellitus verhoogd wordt door statine therapie. Zijn dit alleen mensen die toch al bijna type 2 diabetes mellitus hebben, of ook andere patiënten?

In **hoofdstuk 7** onderzoeken we het effect van statinebehandeling op het ontwikkelen van type 2 diabetes mellitus in patiënten met hart- en vaatziekten. We vinden inderdaad een verhoogd risico op type 2 diabetes door statine behandeling. Hoe sterker de behandeling, des te hoger ook het risico om type 2 diabetes te ontwikkelen. Dit risico leek niet af te hangen van de risicofactoren voor type 2 diabetes mellitus die deze patiënten al hadden.

Lange termijn risicoschatting in patiënten met hart- en vaatziekten

Het schatten van het risico op hart- en vaatziekten is belangrijk om te bepalen welke mensen er wel of niet met cholesterolverlagers of andere medicijnen behandeld moeten worden. Patiënten met hart- en vaatziekten worden automatisch gezien als een risicogroep voor nieuwe hart- en vaatziekten en worden daarom ook intensief behandeld. Toch zou een risicoschatting gebruikt kunnen worden om alleen mensen met heel hoog risico te behandelen met bepaalde dure medicijnen of medicijnen met veel bijwerkingen. Een 10-jaars risicoschatting kan hiervoor gebruikt worden, maar dit levert per definitie lage geschatte risico's op in jonge patiënten, omdat leeftijd erg sterk doorweegt in deze risicoschatting. Voor deze mensen zou een lange termijn risicoschatting extra informatie kunnen geven over hun risico op hart- en vaatziekten.

In **hoofdstuk 8** ontwikkelen we daarom een model om het risico op nieuwe hart- en vaatziekten te schatten tot de leeftijd van 85 jaar bij patiënten die al hart- en vaatziekten hebben. Dit model kan gebruikt worden om onderscheid te maken tussen patiënten met een hoog risico en een laag risico. Het 10-jaarsrisico op nieuwe hart- en vaatziekten was 14% voor patiënten van 45-54 jaar oud, maar hun totale risico tot een leeftijd van 85 jaar was 45%. Hoe ouder de patiënten, hoe kleiner het verschil tussen de

risicoschatting tot 85 jaar en de schatting van het 10-jaars risico. Voor patiënten tot 65 jaar schat dit lange termijn model vaker in dat de betreffende patiënt een hoog risico heeft dan wanneer het 10-jaars risico model gebruikt wordt.

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Soli Deo Gloria

Curriculum Vitae

Anton van de Woestijne was born on the 29th of July, 1986 in Mariekerke, the Netherlands. After graduating from secondary school in 2004, he obtained a Bachelor of Science degree at University College Roosevelt Academy in Middelburg in 2007. During this time, he performed research at the Department of Cardiology in the hospital in Goes. After obtaining his Bachelor's degree, he was accepted to the Selective Utrecht Medical Master (SUMMA). His scientific internship was performed at the department of Vascular Medicine of the University Medical Center Utrecht, under supervision of prof. dr. F.L.J. Visseren. A part of the work described in this thesis was performed during this scientific internship. He continued the work on this thesis under supervision of prof. dr. F.L.J. Visseren and prof. dr. Y. van der Graaf after obtaining his Medical Degree in 2011. He combined his research with a Master's study in Clinical Epidemiology, from which he graduated in 2013. As of November 2013, he is working at the Emergency Department of the Streekziekenhuis Koningin Beatrix in Winterswijk.

Anton is married to Hanneke van de Woestijne-Bisschop and together they have one child (Matthias, 2013).

Appendix

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