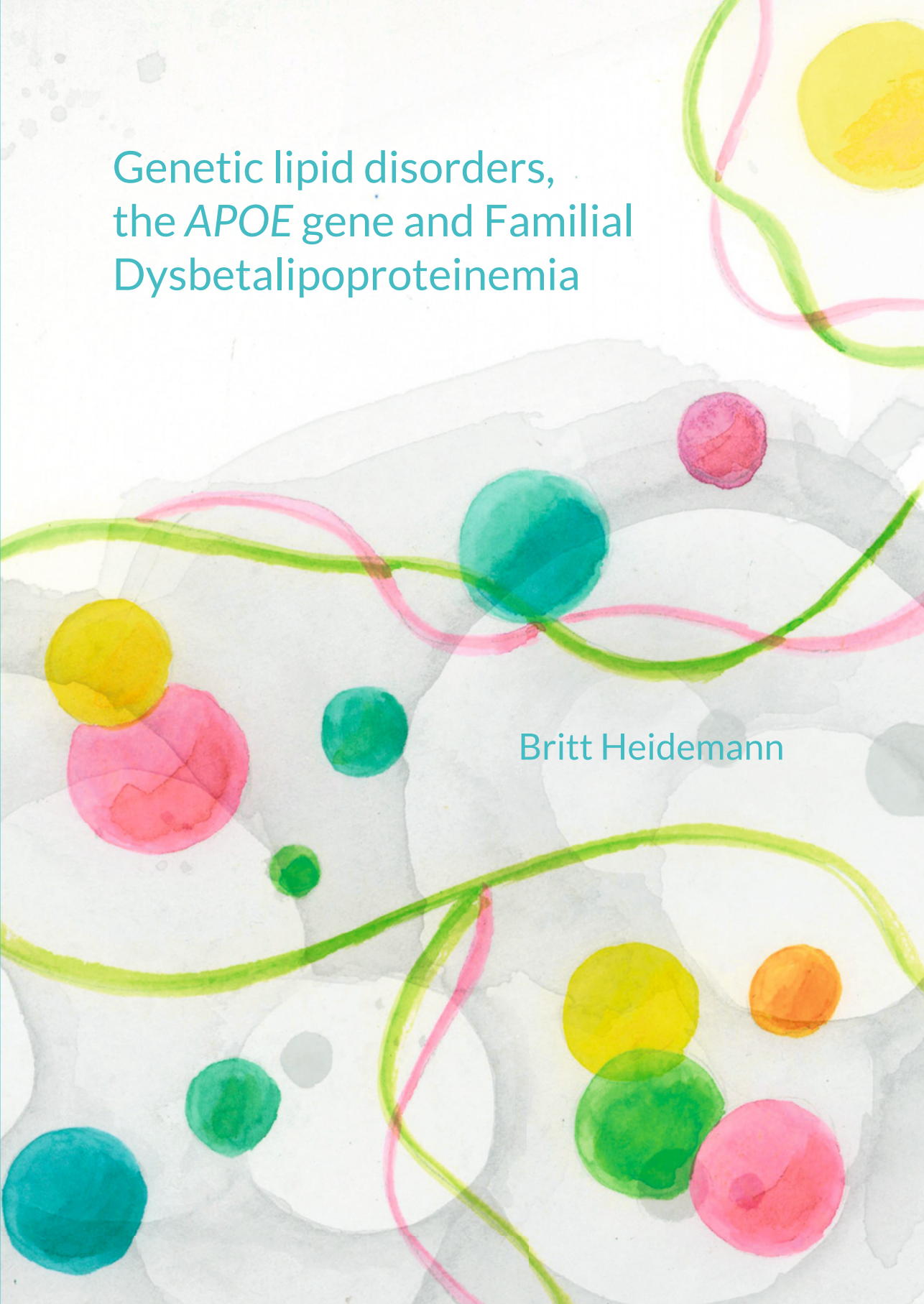
Genetic lipid disorders, the *APOE* gene and Familial Dysbetalipoproteinemia

Britt Heidemann



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Genetische lipidenstoornissen,
het *APOE* gen en Familiaire Dysbetalipoproteïnemie
(met een samenvatting in het Nederlands)

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Chapter 1

General introduction

General introduction

Cardiovascular disease (CVD) is very common and still the number one cause of death worldwide. An estimated 17.9 million people die from CVD each year, representing 32% of global deaths.[1] The risk for CVD is driven by both genetic and lifestyle factors. The former is the random process of the inheritance of genes that is determined at conception. The latter includes smoking, harmful alcohol consumption, unhealthy diet, stress and lack of physical activity. Genetic and behavioral factors can lead to hypertension, hyperglycemia, dyslipidemia and obesity; all well-established risk factors for CVD.[1] With dyslipidemia the emphasis has historically been on low-density lipoprotein-cholesterol (LDL-C) as LDL-C is an established major causative factor for CVD.[2] Although LDL-C, but also high-density lipoprotein cholesterol (HDL-C), are well-known risk factors, and often referred to as the 'bad' and the 'good' cholesterol, dyslipidemia involves the metabolism of many more lipids and lipoproteins, and any imbalance in this metabolism can lead to an atherogenic lipid profile and CVD.

Lipoprotein metabolism

For the human body to function, three important lipids are required; cholesterol, triglycerides (TG) and phospholipids. Since lipids are water insoluble molecules, they are transported in lipoproteins. Lipoproteins are a combination of the words 'lipid' and 'protein', and make it possible to transport lipids through the body. Although lipoproteins form a continuum of different sizes and densities, their names are derived from their density measured by ultracentrifugation. With ultracentrifugation, lipoproteins are separated and named accordingly. The less dense particles are, the larger they are. The largest lipoproteins are chylomicrons (CM), then very-low-density lipoproteins (VLDL), followed by intermediate-density lipoproteins (IDL), low-density lipoprotein (LDL), and lastly high-density lipoproteins (HDL). All lipoproteins carry lipids in different quantities and several classes of apolipoproteins. Apolipoproteins have a number of functions, including functioning as coenzymes for receptors. CM, VLDL, IDL and LDL all carry one molecule of apolipoprotein B (apoB). HDL carries apolipoprotein A (apoA) and CM, VLDL, IDL and some subspecies of HDL carry apolipoprotein E (apoE).[3]

TGs are mainly obtained through diet. After TGs are absorbed by the intestines they are incorporated in CMs, resulting in very large particles. Subsequently, TG in the CM undergo lipolysis by lipoprotein lipase (LPL), leading to the hydrolysis of one glycerol and three fatty acids which can be used as an energy source by the human body. When they are not used, TGs are stored in adipose tissue. At the same time, after eating a meal, the liver produces large VLDLs in response to increased supply of TG by the portal circulation. VLDLs are rich in TGs and after release into the circulation their TGs are also hydrolyzed by LPL.

Through lipolysis, VLDLs become smaller and relatively cholesterol-rich, resulting in IDL or 'remnants'. CM, VLDL and their lipolytic remnants are collectively called triglyceride rich lipoproteins (TRLs).[4] Remnants are then transported to the liver, where some are cleared directly and some are further remodeled and hydrolyzed with help of hepatic lipase into the final product, LDL. HDL is produced by the liver and interacts directly with the other lipoproteins, mainly through the cholesteryl ester transfer protein (CETP) enzyme, leading to the exchange of cholesterol from HDL to TRLs and HDL receiving TG from apoB containing TRLs. When TRLs are not properly cleared, this process of cholesterol transfer leads to cholesterol enrichment of TRLs, which increases their atherogenicity.

Lipids and cardiovascular risk

Cholesterol plays a crucial role in human metabolism, but it also plays a role in the development of atherosclerosis. Atherosclerosis, initiated by the retention of circulating LDL and smaller TRLs in arterial walls, leads to a multifactorial, complex and inflammatory process that causes foam cell accumulation and the formation of plaques. When these plaques become larger and unstable, they may rupture, leading to thrombosis and eventually result in symptomatic CVD.[5,6] The cholesterol content in LDL, reflected as LDL-C, is an established risk factor for CVD.[2] For many years, guidelines emphasize the importance of LDL-C as a treatment goal as well as a component in cardiovascular risk prediction models.[7,8] LDL-C is causally related to CVD and there is abundant evidence that targeting LDL-C levels reduces the risk of CVD.[2] The relative risk of major cardiovascular events is reduced by approximately 20% for every 1 mmol/L reduction in LDL-C.[9] Statins, ezetimibe and Proprotein Convertase Subtilin Kexin (PCSK) 9 monoclonal antibodies lower LDL-C levels efficiently, thereby lowering CVD risk.[10] However, even among patients with optimal treatment and low LDL-C levels, residual risk of CVD remains.[11] Therefore, in recent years, increasing attention has been paid to lipid pathways leading to CVD beyond LDL-C. Genetic and epidemiologic studies provide strong support for a causal relation between TRLs and CVD,[4,12,13] and there is evidence that residual CVD risk is caused by TRLs.[14] This raised the question whether TRLs might be a risk factor for recurrent vascular disease and whether this effect is independent from LDL-C and lipid-lowering therapy.

Genes, lipids and cardiovascular risk

Plasma concentrations of lipids and lipoproteins are influenced by genes and lifestyle factors. Hundreds of genetic variants influencing lipid levels have been identified with each having their own small effect.[15] The cumulative effect of the small contribution of many variations in genes has been shown to affect lipids, resulting in a large effect on life time risk of CVD in patients from the general population.[16] The contribution of (poly)genetic variation in lipid-genes on clinical outcomes in patients with prevalent vascular disease is unknown.

In addition to the small cumulative effects of many genes on lipids, specific variants in selected genes closely related to lipoprotein metabolism can have major effects on plasma lipid concentrations, lipoprotein composition and consequently on CVD risk. These monogenic lipid disorders include several diseases; such as Familial Hypercholesterolemia (FH), involving the *LDLR, APOB* or *PCSK9* gene, and monogenic chylomicronemia involving the *LPL, APOA5, APOC2, LMF1* or *GPIHBP1* gene.[17] Monogenic chylomicronemia is a very rare disease, and not much is known about the different ways this disease can present in clinical practice.

Another gene in lipid metabolism is the *APOE* gene, which accounts for a significant proportion of lipid variability in the general population.[18] The *APOE* gene encodes for the apolipoprotein E (apoE) protein which is located on all lipoproteins, except LDL and some subspecies of HDL. ApoE plays an important role in the hepatic clearance of TRLs by binding to the LDL-receptor (LDL-R) and heparan sulphate proteoglycans (HSPG).[19] The *APOE* gene has three main variants (ε2, ε3 and ε4) encoding three isoforms (apoE2, apoE3 and apoE4), with an estimated allele frequency in the general population of 7%, 82% and 11% respectively.[18] Compared to the wild type ε3 allele, the ε4 allele is associated with increased levels of LDL-C and increased risk of CVD. The ε4 allele is also known for its strong association with Alzheimers' disease.[20] The ε2 allele is associated with generally lower LDL-C levels and a lower risk of CVD relative to subjects carrying the ε3 variant.[18] In addition to the common isoforms of the apoE protein, some rare variants in the *APOE* gene can lead to a dysfunctional ApoE protein, which (under certain circumstances), can lead to a variety of diseases, including several types of dyslipidemias, but also non-lipid related diseases.[21,22]

Approximately 1% of the general population is homozygous for the ε2 allele, which leads to a significantly reduced binding of TRLs to the LDL-receptor (LDL-R) compared to patients with the wild-type apoE3 protein,[23,24] leading to upregulation of the LDL-R and consequently lower LDL-C levels. However, approximately 10-15% of these healthy ε2ε2 subjects transform to the very atherogenic lipid disorder Familial Dysbetalipoproteinemia (FD).[25]

Familial Dysbetalipoproteinemia

FD, also known as hyperlipoproteinemia type III or remnant removal disease, is the second most common monogenic lipid disorder after FH, with an estimated prevalence of 1 in 1000 to 1 in 2500 subjects in the general population.[25] FD is characterized by the accumulation of atherogenic cholesterol-enriched TRLs, particularly pronounced during the postprandial phase, and is associated with a very high risk of CVD.[4,26]

Etiology

In FD, the protective ε2 lipoprotein profile transforms to a highly atherogenic lipoprotein profile during the course of life. This 'switch' from the favorable hypolipidemic to the dysbetalipoproteinemic state is most likely caused by metabolic stress, which usually is adiposity and insulin resistance but can also happen during pregnancy.[27-32] Since evidence for the development of FD is limited to cross-sectional studies, the direction of the association between metabolic stress and the development of FD is unsure. The prospective association between risk factors and the development of FD in healthy ε2ε2 subjects remains to be determined.

Although the underlying pathophysiological mechanism for the development of FD is unclear, it is hypothesized that the HSPG system plays an important role.[33] Individuals with an ε2ε2 genotype cannot clear remnants efficiently by the LDL-R, but in most patients this is of little consequence for lipid metabolism, probably because the second remnant clearing system, the HSPG system, functions normally in these subjects. A study in mice identified that dysregulation of the heparan sulfate glucosamine-6-O-endosulfatase-2 (*SULF2*) gene disrupts HSPG structure.[34] This gene encodes the sulf2 enzyme that decreases the sulphation grade of the HSPG, thereby impairing the binding and consequently the clearance of TRLs from the circulation. Another study demonstrated that inhibition of the sulf2 enzyme completely normalized binding of TRLs in mice with type 2 diabetes (T2DM).[35] In addition, it was shown that a specific variant (rs2281279) in the *SULF2* gene was associated with postprandial dyslipidemia and insulin resistance in healthy and obese T2DM individuals, but this evidence was limited to small studies showing conflicting results of its effect on metabolic parameters and CVD.[36-39] Why some ε2ε2 subjects develop FD while others do not, and the role of *SULF2* in this process, is not completely understood and warrants further research.

Diagnostic challenges

The diagnosis of FD requires a specific lipoprotein phenotype in combination with a specific *APOE* genotype. Determining both the phenotype and genotype in FD is essential for proper diagnosis of FD, but both face some difficulties in clinical practice.

Lipoprotein phenotype

Although the dysbetalipoproteinemic lipoprotein profile is abnormal and highly specific, with the presence of cholesterol-enriched remnants,[40] it is not possible to determine this profile with standard lab measurements. With standard lab measurements, the lipid phenotype of FD is often seen as a (non-specific) mixed hyperlipidemia with increased cholesterol and TG, at an approximate 2:1 molar ratio,[25] but this can vary greatly. The reference standard for diagnosis of FD is ultracentrifugation. With ultracentrifugation,

an FD lipoprotein phenotype is defined as an increased VLDL-C/VLDL-TG ratio or increased VLDL-C/total TG ratio.[40,41] Ultracentrifugation is however not straightforward and currently only used in specialized lipid laboratories. Therefore, in clinical practice, alternatives based on standard laboratory lipid measurements have been developed, including several algorithms incorporating apoB levels.[42-45]

When a patient is diagnosed with FD, based on genetics and lipid phenotype, lipid levels should be monitored. In clinical practice, healthcare professionals tend to focus on lowering LDL-C levels, but in FD, LDL-C levels are low or even absent.[46] In addition, the most commonly used method to estimate LDL-C levels, the Friedewald formula, is not applicable in patients with FD.[47] According to the Friedewald formula, LDL-C is calculated as follows: TC minus HDL-C – TG/2.2 (in mmol/L). This formula assumes a fixed ratio of VLDL-C to VLDL-TG, which is not valid in FD because of the presence of cholesterol-enriched VLDL and remnant lipoproteins. Alternatively, several homogeneous assays for the direct measurement of LDL-C have been developed.[48] In clinical practice, these are often used to determine LDL-C levels in FD, but it is not known how well they perform in this context. In addition to these direct assays, other options for determining LDL-C levels are the Martin-Hopkins formula (using an adjustable VLDL-C/VLDL-TG ratio)[49] and polyacrylamide gradient gel electrophoresis (PGGE) that separates lipoproteins based on size and stains neutral lipid.[46] These methods may be more appropriate for determining LDL-C levels in patients with FD.

Relationship FD and new variants in the *APOE* gene

In 90% of the cases, FD is recessively inherited with homozygosity for the ε2 allele (ε2ε2 genotype). The remaining 10% involves other variants in the *APOE* gene.[33] These are rare and often inherited in a dominant manner. Next Generation Sequencing (NGS) is becoming more widely available and can reveal variants in the *APOE* gene for which the relationship with FD is unknown or uncertain. The American College of Medical Genetics and Genomics (AMCG) guideline[50] could be used to determine pathogenicity of a new variant. However, it is often not feasible to perform thorough laboratory tests for every variant currently found, so they are often considered a variant of uncertain significance (class 3) leaving healthcare provider and patient in uncertainty. Also, as previously mentioned, the *APOE* gene is a heterogeneous gene with variants associated not only with FD, but also with FH, hypertriglyceridemia or lipoprotein glomerulopathy.[21,22] Therefore, demonstrating pathogenicity is not the same as demonstrating a causal relationship with FD. Guidance for determining the relationship of variants in the *APOE* gene and FD in clinical practice is needed.

Treatment

Due to the presence of atherogenic cholesterol-enriched TRLs in the circulation, and with increased and prolonged postprandial TRL concentrations, patients with FD have a high risk of premature CVD.[26,51] However, the exact level of CVD risk is unknown because longitudinal cohort studies in FD patients are lacking. In a large cross-sectional study including 305 FD patients (mean age 60.9 ± 14.4 years) the prevalence of CVD was 29%.[52] Because LDL-C levels in patients with FD are low and do not reflect actual CVD risk, treatment goals for patients with FD are based on non-HDL-C levels. Non-HDL-C treatment goals are <3.4 mmol/L in patients without CVD or T2DM and <2.6 mmol/L in patients with pre-existent CVD or T2DM.[7] Current options to achieve non-HDL-C goals in FD consist of statins, and fibrates which reduce fasting and postprandial lipids and lipoproteins.[53] However, in clinical practice 60% of FD patients do not achieve non-HDL-C treatment goals with current lipid-lowering medication,[52] indicating the need for more intensive lipid-lowering treatment.

PCSK9 monoclonal antibodies neutralize circulating PCSK9 and thereby reduce degradation of the LDL-R. PCSK9 monoclonal antibodies proved to lower LDL-C by 50-60%[54,55] and reduce CVD risk with 20% in high-risk patients.[56,57] Also, in patients with T2DM, PCSK9 monoclonal antibodies have been shown to lower postprandial TRLs by about 30-40%.[58-61] The effect of the PCSK9 monoclonal antibody evolocumab was recently evaluated in a non-randomized study in three FD patients who were intolerant or resistant to statins and fibrates. This study showed that evolocumab reduced fasting non-HDL-C by 42% and TG by 36%.[62] However, data on the effects of PCSK9 monoclonal antibodies in FD patients are limited to this study and therefore the effects of PCSK9 lowering on fasting and post fat load lipids and lipoproteins in FD are largely unknown. Furthermore, the effects of PCSK9 monoclonal antibodies on protein and (apo)lipoprotein concentration, distribution and composition in patients with FD are not known.

Objectives of this thesis

The general objectives of this thesis are:

- To evaluate the relation between genes involved in lipoprotein metabolism and cardiovascular disease in patients at high risk of CVD.
- To evaluate etiologic pathways, diagnostic criteria and new therapeutic options for Familial Dysbetalipoproteinemia.

Outline of this thesis

The first part of the thesis focuses on genetic lipid disorders and cardiovascular disease. **Chapter 2** describes two case reports related to genetic lipidology. The first case report presents cases from three families with monogenic chylomicronemia. In three families, different variants in different genes are involved, leading to different clinical presentations, demonstrating the large clinical heterogeneity in monogenic chylomicronemia. The second case describes a patient with a heterozygous variant in her *LIPC* gene that leads to apparently very high HDL-C levels. In **Chapter 3**, the relationship between VLDL-C and risk of cardiovascular events is examined in patients with manifest cardiovascular disease. In **Chapter 4**, the relationship between genetic variants associated with LDL-C and systolic blood pressure and the risk of recurrent cardiovascular disease is assessed in patients with established vascular disease. **Chapter 5** investigates the association between a genetic variant in the *SULF2* gene, metabolic parameters, and vascular disease and T2DM in patients at high cardiovascular risk.

The second part of this thesis focuses on the *APOE* gene and Familial Dysbetalipoproteinemia. **Chapter 6** longitudinally evaluates the relationship between adiposity and the development of dyslipidemia in subjects with an *APOE* ε2ε2 genotype. **Chapter 7** establishes the relation between Familial Dysbetalipoproteinemia and genetic variants of unknown significance in the *APOE* gene and provides two different approaches to ascertain the relationship of a variant in the *APOE* gene with FD. **Chapter 8** investigates different methods of determining LDL-C in patients with FD. It is known that the Friedewald formula to estimate LDL-C is not applicable in FD, but other methods such the Martin-Hopkins formula, direct measurement of LDL-C with a homogeneous assay or PGGE might perform better. **Chapter 9** reports the effect of the PCSK9 monoclonal antibody evolocumab on fasting and post fat load lipids and lipoproteins in 28 patients with FD, examined in a multicenter, randomized, double-blind, placebo-controlled, crossover trial. **Chapter 10** further investigates the effect of evolocumab in FD and presents the effects on protein and lipoprotein distribution and composition in patients with FD. The main findings of this thesis are discussed in **Chapter 11** and summarized in **Chapter 12**.

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Part I

Genetic lipid disorders, triglyceride-rich lipoproteins and cardiovascular disease

Chapter 2

Case reports in genetic lipidology

Chapter 2.1

Clinical heterogeneity in monogenic chylomicronemia

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In revision

Abstract

Chylomicronemia accompanies severe hypertriglyceridemia that is usually the result of a polygenic predisposition in combination with secondary risk factors. Monogenic chylomicronemia represents a small subgroup of patients with hypertriglyceridemia. This article describes three patients and illustrates the large heterogeneity in the clinical presentation of monogenic chylomicronemia. The first case is a male with mild hypertriglyceridemia based on two compound heterozygote variants in the *LMF1* gene, without relevant medical history. The second case is a woman who is a double heterozygote of variants in the *LPL* and *APOA5* genes. She experienced severe pancreatitis. The third case is a male, with recurrent pancreatitis attributed to severe hypertriglyceridemia and is homozygous for a variant in the *APOC2* gene. This article highlights that in patients with hypertriglyceridemia, the absence of pancreatitis or the presence of mild hypertriglyceridemia does not exclude monogenic chylomicronemia. Genetic screening should be considered in patients with unexplained or severe hypertriglyceridemia, to determine appropriate treatment and follow-up.

Introduction

Triglycerides are transported through the blood in lipoproteins for distribution to muscle and adipose tissue. Hypertriglyceridemia is defined as fasting plasma triglycerides (TG) ≥2.0 mmol/L and severe hypertriglyceridemia as fasting TGs ≥10 mmol/L.[1] TGs from the diet are incorporated in chylomicrons and the liver secretes TG in very-low density lipoproteins (VLDL). These lipoproteins and their lipolytic remnants that vary in size and density, are collectively called triglyceride-rich lipoproteins (TRLs). Chylomicrons are the largest lipoproteins with the highest TG content, but are cleared from the circulation rapidly because TGs are efficiently removed by lipolysis that processes chylomicrons and VLDL to remnants lipoproteins. Severe hypertriglyceridemia is usually caused by the pathological presence of chylomicrons in the fasting state.[2] Both mild to moderately (2.0–9.9 mmol/L) and severely increased TGs are in most cases caused by a polygenic predisposition in combination with common secondary causes of increased TG such as insulin resistance, uncontrolled diabetes mellitus, hypothyroidism, alcohol use and nephrotic syndrome.[1,3] Although the vast majority of patients with severe hypertriglyceridemia have a polygenic background, a monogenic cause resulting in a deficiency in lipolysis explains about 1-2% of cases. Lipolysis is performed by lipoprotein lipase (LPL) and related factors. Monogenic chylomicronemia (formerly known as hyperlipoproteinemia type I or familial chylomicronemia syndrome) is caused by homozygous or compound heterozygous loss-of-function variants in each of genes coding for proteins involved in the lipolysis of TGs.

Case presentations

Case 1

A male in his 50s was referred to the Vascular Medicine outpatient clinic for evaluation of hypertriglyceridemia. The patient contacted his general practitioner because of concern about his cardiovascular risk profile, as his maternal grandfather and uncle had died of myocardial infarction in their 6th and 5th decades respectively. His medical history was unremarkable, he had no symptoms and took no medication. He had been smoking for 8 years and consumed 5 alcoholic beverages per week. Physical examination revealed no abnormalities, except for a BMI of 28.6 kg/m^2 and mild hypertension. There were no eruptive xanthomata. The patient's fasting lipid profile was: TG 10.2 mmol/L, total cholesterol (TC) 7.6 mmol/L, LDL-C 4.5 mmol/L and HDL-C 1.0 mmol/L.

Case 2

The case of a female in her 40s was published previously.[4] In short, she had no remarkable medical history, had a BMI of 29.7 kg/m^2 and used an oral contraceptive pill (ethinyloestradiol/drospirenone 20 mcg/3mg) and ezetimibe 10 mg once daily. She did not consume alcohol. She presented to the emergency unit with pancreatitis. Because of hemodynamic instability she was admitted to the Intensive Care Unit (ICU), where she developed pneumonia and epiglottitis. A biliary cause of pancreatitis was excluded by abdominal ultrasound. Her maximum TG level was 28 mmol/L.

Case 3

A male in his 20s, known to have visual impairment and consanguinity (his parents are cousins) was hospitalized six times with recurrent pancreatitis during a period of 2 years, two of which led to admission to the ICU. He was not using any medication and never consumed alcohol. His BMI was 25.8 kg/m^2. On ultrasound, his bile duct system was normal. Imaging during the second episode showed a severe necrotizing pancreatitis with disruption of the pancreatic duct, which most likely also contributed to the recurrent episodes. On first admission, his lipid profile revealed TG levels of 15 mmol/L, which increased during follow-up to a maximum of 66 mmol/L. The patient reported that his father and mother had type 2 diabetes mellitus (T2DM), but no increased TG levels. The patient had five sisters and two brothers. One brother died of sudden cardiac arrest in his 40s. No other family members had pancreatitis and their TG levels were unknown.

Investigations

Case 1

Secondary factors of hypertriglyceridemia were excluded, including T2DM (glucose 5.8 mmol/L and HbA1c 34 mmol/mol), hypothyroidism (TSH level 0.58 mU/L) and nephrotic syndrome (no proteinuria). Familial Dysbetalipoproteinemia (FD) was evaluated by a non-HDL-C/ApoB ratio of 4.23 mmol/g (>3.69 is suggestive of FD)[5], but FD was ruled out by sequencing his *APOE* gene, which revealed an ε2ε3 genotype without any other pathogenic variants in his *APOE* gene. Next Generation Sequencing (NGS) showed two missense variants in the *LMF1* gene; (c.1351C>T; p.Arg451Trp) and (c.41C>G; p.Ser14Trp), confirming the diagnosis of monogenic chylomicronemia. A post-heparin test for the evaluation of LPL activity showed a TG reduction of 22% (*Figure 1*). The patient underwent preventive cardiovascular screening. Computer Tomography (CT) imaging revealed no coronary calcifications (Agatston score 0). Sonography of the carotid arteries and abdomen as well as an electrocardiogram was normal.

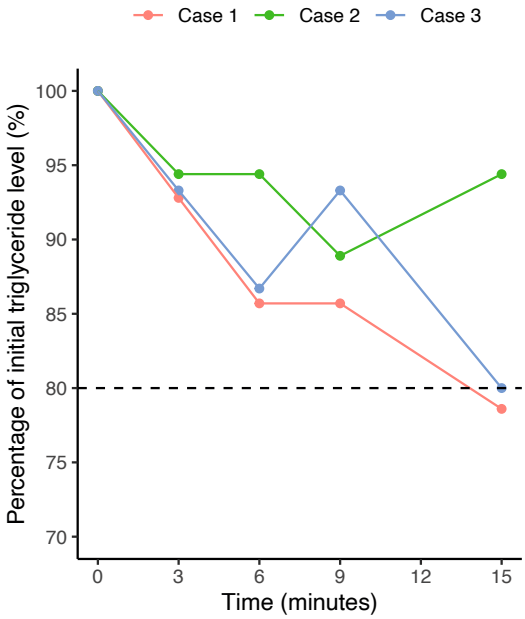

Figure 1. Post-heparin lipase tests of 3 cases

The intravenous injection of heparin leads to the release of LPL from the endothelium. In the normal situation, all LPL molecules become highly active and lead to increased lipolysis and consequently a reduction in TG. In a healthy situation, the TG level should decrease by at least 20% within the first 15 minutes compared to the patient's TG level at the start of the test (dotted line is 80% of the initial TG value).

The test from case 1 was performed without use of medication and shows a post-heparin TG reduction of 22% (red line).

The test from case 2 was performed during use of gemfibrozil 600 mg twice daily and shows a post-heparin TG reduction after 15 minutes of 6% (green line).

The test from case 3 was performed during use of bezafibrate 400 mg once daily and rosuvastatin 20 mg once daily and shows a TG reduction of 20% (blue line).

Case 2

Besides being overweight and taking the oral contraceptive pill, there were no other relevant potential secondary causes of hypertriglyceridemia. She had an ε2ε3 genotype, ruling out FD. Genetic testing showed a pathogenic heterozygous variant in her *LPL* gene (c.173C>G, p.Pro58Arg) and a pathogenic heterozygous variant in her *APOA5* gene (c.161+5G>C). A post-heparin LPL test showed a reduction in TG of only 6%[4] (normal >20% reduction).

Case 3
There were no secondary causes of hypertriglyceridemia: TSH was normal (1.7 mU/L), there was no proteinuria, the non-fasting glucose concentration was 7.4 mmol/L but insulin resistance was unlikely (HbA1c levels 38 mmol/mol). A genetic cause was suspected because secondary causes were absent. NGS showed a homozygous variant in his *APOC2* gene (c.245T>G, p.Met82Arg). A post-heparin LPL test while on lipid-lowering medication including fibrate showed a reasonable reduction in TG of 20% (normal >20% reduction) (*Figure 2*).

Differential diagnosis

Typically, hypertriglyceridemia is caused by a polygenic background in combination with secondary factors including T2DM, metabolic syndrome, abdominal obesity, polycystic ovary syndrome, nephrotic syndrome, end-stage kidney disease or hemodialysis, alcohol use, pregnancy, hypothyroidism, or specific medication such as steroids (estrogens and glucocorticoids), antipsychotic medication, or antiretroviral medication. A very fatty diet may also unmask impaired lipolysis. Hypertriglyceridemia may also result from genetic disorders, including Familial Dysbetalipoproteinemia and monogenic chylomicronemia, which is defined by the presence of variants in genes related to TG lipolysis.

Treatment

Case 1
At first rosuvastatin 10 mg once daily was started, but because of side effects (muscle complaints) the dose was lowered to 5 mg once daily. The patient stopped smoking and a low-fat diet was advised by a dietitian.

Case 2
A fat-free diet was advised by a dietician and gemfibrozil 600 mg twice daily was prescribed. Also, oral contraceptives were discontinued.

Case 3
A strict fat-free diet, bezafibrate 400 mg once daily and rosuvastatin 20 mg once daily were prescribed to keep TGs below 8.0 mmol/L.

Outcome and follow-up

Case 1

One year later, still on treatment with 5mg of rosuvastatin, his lipid profile was: TG 4.5 mmol/L, TC 5.3 mmol/L, LDL-C 1.8 mmol/L, HDL-C 1.47 mmol/L, ApoB 1.12 g/L, Lp(a) <30 mg/L. His BMI remained around 29 kg/m^2. Cascade screening of his father without cardiovascular disease (CVD) until he was in his 80s, showed a normal lipid profile (TG 0.91 mmol/L, LDL-C 1.69 mmol/L, HDL-C 1.82 mmol/L, ApoB 0.7 g/l, Lp(a) 171 mg/L). Genetic testing revealed the p.Ser14Trp variant in the father's *LMF1* gene. Approximately one year after the genetic screening the father died due to complications of Parkinson's disease. The mother of the patient had died from a brain tumor in her 60s, but had no CVD. Her lipid values were unknown. Due to heterozygosity for the variant in the *LMF1* gene in the father, the mother was most likely a carrier of the p.Arg451Trp variant. Genetic testing in the patient's brother, who had no history of CVD or dyslipidemia (TG 1.7 mmol/L, TC 6.0 mmol/L, LDL-C 3.5 mmol/L, HDL-C 1.68 mmol/L) showed none of the variants in the *LMF1* gene. The index patient had two young children who will be genetically tested when they are older. Two siblings of the mother had CVD, one in his 40s and the other at an unknown age. Lipid values were unknown in both. The pedigree of the family is shown in *Figure 2*.

Case 2

Gemfibrozil was discontinued due to hair loss (a known side effect of fibrates). With a strict diet alone, her triglycerides were stable around 4.0–5.0 mmol/L. The mother of the index patient was referred to the Vascular Department for assessment as she had dyslipidemia for about 25 years, for which she received several statins and gemfibrozil of which all caused severe muscle complaints. She had no other relevant medical history and took barnidipine 10 mg once daily for hypertension. Her BMI was 30.9 kg/m^2 and no clinical stigmata of dyslipidemia were found on physical examination. Her lipid profile showed a mixed hyperlipidemia: TG 9.4 mmol/L, TC 9.9 mmol/L, HDL-C 0.7 mmol/L, non-HDL-C 9.2 mmol/L and directly measured LDL-C of 3.2 mmol/L. Genetic analysis found both variants in her *LPL* and *APOA5* genes. She was already following a low-fat diet. Ezetimibe 10 mg once daily was initiated, she started using fish oil (over the counter) and continued her low-fat diet with help of a dietician. After this, her lipid levels were TG 4.2 mmol/L, TC 5.7 mmol/L, HDL-C 0.8 mmol/L, non-HDL-C 4.9 mmol/L and LDL-C 3.0 mmol/L.

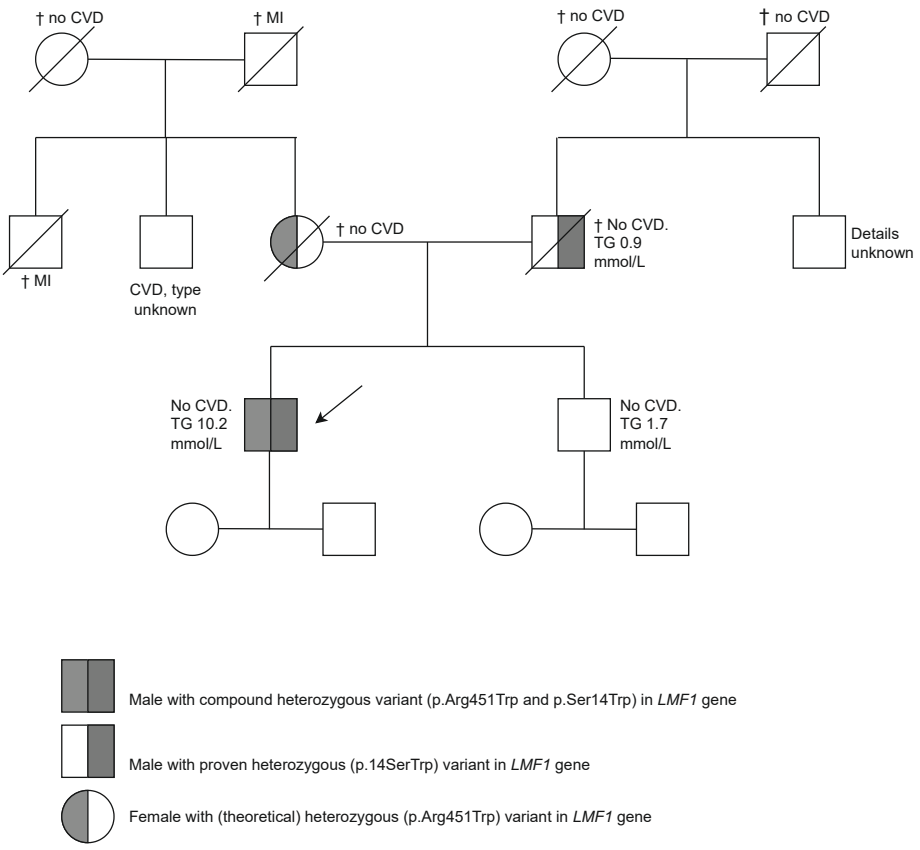

Figure 2. Pedigree of family of case 1

Square with arrow: index patient (proband). Squares or circles with diagonal line: deceased patient.

Case 3

With the interventions, his TGs stabilized between 5.0 and 6.0 mmol/L. Other lipids were remarkably low (TC 2.4 mmol/L, apoB 0.39 g/L, HDL-C <0.5 mmol/L, direct LDL-C 0.3 mmol/L). Other family members did not wish further analysis.

Discussion

Severe hypertriglyceridemia is often associated with impaired lipolysis, which is the process in which triglycerides are lipolyzed to free fatty acids and glycerol. The key protein responsible for intravascular lipolysis is LPL, with its lipolytic function being co-regulated by other proteins, such as apolipoprotein C2 (ApoC2), apolipoprotein A5

(apoA5), glycosylphosphatidylinositol-anchored HDL binding protein 1 (GPIHBP1) and lipase maturation factor 1 (LMF1). Variants in genes coding for these proteins influence plasma triglyceride concentration powerfully and critically when both alleles carry pathogenic variants.

The cause of chylomicronemia is in most cases polygenic, usually caused by clustering of common genetic variants, or heterozygosity for one of the genes involved in LPL mediated lipolysis, in combination with lifestyle factors.[6] Monogenic chylomicronemia is rare with an estimated prevalence 1-10 per 1 million persons in the general population.[2]

LPL is expressed and located in tissues that oxidize free fatty acids as energy source (heart and skeletal muscle) or store fatty acids (brown and white adipose tissue).[7,8] ApoC2, encoded by the *APOC2* gene, is present on TRLs and HDL and acts as an activator for LPL activity. Biallelic variants in *APOC2* cause a lipoprotein phenotype indistinguishable from homozygous LPL deficiency.[2] ApoA5, encoded by the *APOA5* gene, stabilizes the LPL enzyme complex and thereby promotes lipolysis.[9] *GPIHBP1,* codes for the endothelial protein GPIHBP1, which transports secreted LPL from the parenchymal cells to the endothelial cell surface, were lipolysis takes place.[2,10] Finally, the *LMF1* gene encodes for the LMF1 protein which assists maturation of LPL and hepatic lipase (HL). The LMF1 protein is a membrane-bound chaperone protein located in the endoplasmic reticulum and responsible for the post-translational maturation of nascent lipase polypeptides.[11] Proper lipase maturation involves the glycosylation, folding and assembly of these polypeptides and stabilization of the active dimeric lipases to fully active enzymes.[8,12] LMF1 deficiency is therefore associated with a lipase deficiency that affects both LPL and HL function.[12] Other proteins that are involved in TG metabolism are angiopoietin-like protein (ANGPTL) 3 and apolipoprotein C3 (apoC3). Both inhibit LPL activity and thereby lipolysis of TGs.[2]

Almost 95% of patients with monogenic chylomicronemia have pathogenic variants in the *LPL* gene, leading to partial or complete loss of LPL activity and a small minority have pathogenic variants in the other four genes.[2] Monogenic chylomicronemias are primarily associated with accumulation of TGs in large chylomicrons, as deficiency in LPL mediated lipolysis of TGs prevents conversion of chylomicrons to smaller lipoproteins. Severe chylomicronemia can be asymptomatic but manifestations may begin at an early age and include failure to thrive, eruptive xanthomas, lipemia retinalis, and gastrointestinal manifestations such as hepatosplenomegaly, and in particular acute pancreatitis, which can be life-threatening.[3,8,13] Hypertriglyceridemia-related pancreatitis is thought to be initiated by the release of free-fatty acids after partial lipolysis of

lipoproteins that prematurely activate trypsinogen, leading to auto-digestion of the pancreas.[2,14] Hypertriglyceridemia from a monogenic cause is usually not associated with CVD since chylomicrons contain little cholesterol and do not penetrate the arterial wall to cause atherosclerosis.[2,13] In contrast, if the same degree of hypertriglyceridemia would have been due to polygenic causes, smaller, cholesterol-richer, pro-atherogenic TRLs would be present because lipolysis is not completely disrupted.[13,15] Consequently, polygenic hypertriglyceridemia is associated with atherosclerosis and CVD,[2,13] in contrast to monogenic hypertriglyceridemia. The most effective and important therapy for severe hypertriglyceridemia is strict restriction of dietary fat intake, preferably with less than 10% of calories from fat. However, compliance with this type of diet is generally very difficult. Optimal management of lifestyle factors such as obesity and diabetes, and no use of alcohol or medication known to increase TG (such as estrogens, steroids or atypical antipsychotic drugs) is also essential.[2,3] In addition, statins, fibrates, and high dose of omega-3 fatty acids are often used in polygenic hypertriglyceridemia. However, these drugs are generally not effective enough to reduce TGs to safe levels in patients with monogenic chylomicronemia, because their effectiveness depends primarily on the presence of a lipolytic pathway. Statins do not add any value in the treatment of monogenic chylomicronemia, since they generally only lower LDL-C concentration. In addition, in specific subgroups the pancreatic lipase inhibitor orlistat, lomitapide or plasmapheresis could be an option.[16] New therapies targeting apoC3 and ANGPTL3 are being developed with the aim of specifically lowering TGs in patients without LPL activity.[2,3] Also, transfusion of human plasma can provide normal apoC2 to improve lipolysis of TRL to expedite control of hypertriglyceridemia.

As illustrated from the three cases and their families presented, there is heterogeneity in the clinical presentation of monogenic chylomicronemia. The patient in case 3, with a homozygous variant in *APOC2* had a severe clinical presentation with severe hypertriglyceridemia and life-threatening pancreatitis compared to the patient in case 1, who had a mild hypertriglyceridemia and compound heterozygous variants in *LMF1*. This is in line with other case reports about chylomicronemia in which patients with variants in the *APOC2* gene are generally younger at diagnosis, due to serious clinical manifestations such as failure to thrive or pancreatitis, than patients with pathogenic variants in their *LMF1* gene, who are generally diagnosed later in adulthood.[2] The dietary fat intake was not specifically evaluated in 3 cases but could have an influence on the risk of pancreatitis.

Another explanation for the difference in clinical presentation is the fact that the patient from case 3 was homozygous for the variant, the patients from case 2 were heterozygous for two different LPL related genes and the patient from case 1 was

a compound heterozygote. Homozygous patients usually have the most severe phenotype, while (compound) heterozygous variants usually lead to a milder phenotype, because in the latter some lipolysis is still possible.

Not all patients with monogenic chylomicronemia develop or present with pancreatitis. Case 3 (TG up to 66 mmol/L) and case 2 (TG up to 28 mmol/L) developed severe pancreatitis. The risk of pancreatitis increases when TG levels exceed 10 mmol/L and increases strongly when TG levels exceed 20 mmol/L,[17] which was the case in both patients.

Regarding CVD risk, none of the patients from the 3 family cases had CVD or other clinical signs of atherosclerosis. As mentioned previously, monogenic chylomicronemia is generally not associated with CVD, in contrast to polygenic chylomicronemia. Interestingly, CVD was present on the maternal side of the patient in case 1 with compound heterozygosity for the *LMF1* gene (*Figure 2*). This could be explained by a polygenic risk profile including the heterozygous p.Arg451Trp variant in the *LMF1* gene, in combination with lifestyle factors. The fact that patient 1 himself was free of atherosclerosis up to this point could be explained by the presence of another pathogenic variant in his *LMF1* gene leading to (almost) complete loss of LPL activity and therefore to larger lipoproteins that are generally less atherogenic. The extra pathogenic variant could therefore be protective of CVD, although exposing the patient to a high pancreatitis risk.

In summary, monogenic chylomicronemia is a group of rare genetic disorders associated with (severe) hypertriglyceridemia caused by variants in several genes associated with LPL metabolism. Clinical presentation and prognosis can vary widely among patients depending on the gene involved, the number of variants (i.e. homozygous, compound heterozygous, heterozygous), and the presence of other risk factors. This article highlights that in patients with hypertriglyceridemia, the absence of pancreatitis or the presence of mild hypertriglyceridemia does not exclude monogenic chylomicronemia. Owing to the high risk of pancreatitis, the good response to dietary fat restriction and the relative ineffectiveness of standard TG lowering medication in monogenic chylomicronemia, it is important to determine the etiology of hypertriglyceridemia. Genetic screening should be considered in patients with unexplained or severe hypertriglyceridemia, to determine appropriate treatment and follow-up.

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Chapter 2.2

Hyperalphalipoproteinemia in a patient with a heterozygous variant in the *LIPC* gene

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Manuscript draft

Abstract

Hyperalphalipoproteinemia is characterized by plasma high-density lipoprotein-cholesterol (HDL-C) levels above the age- and sex-specific 90th percentile or by cut-offs ranging from >1.9 to >2.6 mmol/L. HDL-C levels may be elevated due to secondary causes, polygenic susceptibility or pathogenic variants in individual genes associated with HDL metabolism. Genes involved in HDL metabolism are *CETP*, *SCARB1*, *APOC3*, *LIPG* and *LIPC*. Biallelic pathogenic variants in the *LIPC* gene, which encodes hepatic lipase (HL), are a very rare cause of hyperalphalipoproteinemia. HL plays a crucial role in the lipolysis of remnant lipoproteins and the remodeling of HDL and low-density lipoprotein (LDL). HL deficiency typically leads to accumulation of remnant lipoproteins and triglyceride-enriched HDL. The impact of heterozygous variants in the *LIPC* gene is largely unknown. This case report is of a female in her 5th decade with elevated HDL-C levels up to 3.5 mmol/L and with a new heterozygous variant in her *LIPC* gene. Standard homogenous assays to determine HDL-C might not be accurate in this situation with abnormal HDL.

Introduction

High-density lipoprotein (HDL) is the smallest and most dense class of circulating lipoproteins. HDL is a heterogeneous fraction: particles vary in size, density, composition and biological function.[1] Unlike all other lipoproteins, HDL particles do not have apolipoprotein B (apoB), but do have apolipoprotein A1 (apoA1) and/or apolipoprotein A2 (apoA2).[2] Hyperalphalipoproteinemia is characterized by elevated apoA1 and/or apoA2 concentrations. In clinical practice, hyperalphalipoproteinemia is usually caused by increased cholesterol levels in HDL (HDL-C), defined as above the age- and sex-specific 90th percentile or by cut-offs ranging from >1.9 to >2.6 mmol/L. Increased HDL-C levels are mostly caused by secondary factors, such as alcohol use, liver disease and certain medication, or by accumulation of common variants in genes, indicated by polygenic scores.[3-5] The minority of the cases is caused by monogenic variants in genes that influence critical proteins involved in reverse cholesterol transport including *CETP* (cholesterol ester transfer protein), *SCARB1* (scavenger receptor B1), *APOC3* (apolipoprotein C3), *LIPG* (lipase G or endothelial lipase) and *LIPC* (lipase C or hepatic lipase*)*. Hepatic lipase (HL) is a lipolytic enzyme, like lipoprotein lipase (LPL), that plays an essential role in the remodeling of triglyceride-rich lipoproteins (TRLs), HDL and LDL in the liver through hydrolysis of triglycerides (TG).[6,7] In HL deficiency, the limited lipolysis of remnant lipoproteins results in lipid and lipoprotein abnormalities similar to Familial Dysbetalipoproteinemia (FD) and causes TG-enriched HDL and LDL.[7,8] However, depending on the severity of the HL deficiency, the lipoprotein phenotype can be quite heterogeneous. The case presented here is that of a female in her 5th decade with extremely high HDL-C levels up to 3.5 mmol/L (99.5 percentile), with a very rare and not previously described heterozygous variant in her *LIPC* gene.

Case presentation

A woman in her 50s attended the outpatient clinic of the Vascular Department for analysis of increased plasma HDL-C values, initially of 3.5 mmol/L. She experienced recurrent transient ischemic attacks (TIAs) in her 40s. These TIAs were located in the vertebral arterial circulation and recurred despite the use of acetylsalicylic acid. Vascular imaging revealed no identifiable aneurysm or dissection of the carotid or vertebral arteries. There was also no evidence for cardiac arrhythmia. Her medical history revealed multinodular goiter without hyperthyroidism for which she received radioactive iodine twice and an adrenal adenoma, resulting in Cushing's syndrome for which she underwent adrenalectomy. Cardiovascular risk factors were not prominent: she quit smoking 16 years ago, had a normal weight (BMI 22 kg/m^2), normal blood

pressure and was normoglycemic and had an HbAc1 of 37 mmol/mol. Daily medication use was levothyroxine 25 microgram, atorvastatin 10 mg, omeprazole 10 mg, and clopidogrel 75 mg. Physical examination was normal except for a nodule in her thyroid gland. Her fasting lipid profile during her visit at the outpatient clinic was as follows (with reference values between brackets): total cholesterol (TC) 3.8 mmol/L (<6.5 mmol/L), TG 0.6 mmol/L (<2.0 mmol/L), LDL-C 1.3 mmol/L (<1.8 mmol/L), HDL-C 2.2 mmol/L (1.1–2.0 mmol/L) and ApoA1 1.83 g/L (1.0–2.0 g/L). Her father experienced a TIA at age 60 years and her mother was alive at age 94 years without cardiovascular disease (CVD) and with normal lipid levels (HDL-C: 1.3 mmol/L). Genetic testing was not performed. The index patients' half-brother and half-sister from maternal side experienced myocardial infarction at age 45 and 70 years, respectively (*Figure 1*). Their lipid profiles were not known. Excessive alcohol consumption was denied by the patient, who declared consuming nine alcoholic drinks per week. Her low TG levels supported this. The patient used atorvastatin and acetylsalicylic acid, both of which have been associated with a small increase in HDL-C levels, but she used no other drugs associated with raising HDL-C, such as estrogens or corticosteroids. A CT of her coronary and carotid arteries showed no plaque or calcification in her carotid arteries and the coronary calcification score (Agatston score) was 1, which is the 77th percentile for her age and sex. Next Generation Sequencing (NGS) was performed to identify a genetic cause for hyperalphalipoproteinemia. This revealed a heterozygous variant in the *LIPC* gene (c.1415A>T, p.Asp472Val; rs34596532 A>T). To further evaluate her lipoprotein species polyacrylamide gradient gel electrophoresis (PGGE) was performed. The gel of her apolipoprotein B (ApoB) containing lipoproteins showed polydisperse LDL (*Figure 2*). The gel of her HDL species showed that larger species of HDL (HDL2) predominated (*Figure 3*). Although the GGE confirmed increased staining of lipid in the size range compatible with (large) HDL, it could not specify whether this was cholesterol ester and/or TG because only neutral lipid was stained. Therefore, size exclusion chromatography was performed. This technique also separates lipoproteins by size, but provides suitable quantities for direct compositional analysis, and was compared to that of a control. The HDL contained TG, but, surprisingly, cholesterol was not detected (*Figure 4*). SDS-PAGE did not detect immunoglobulins, ruling out the presence of significant antibodies to HDL.

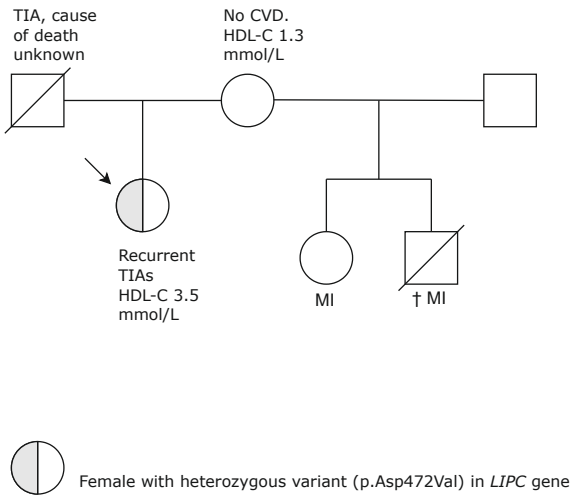

Figure 1. Pedigree of family

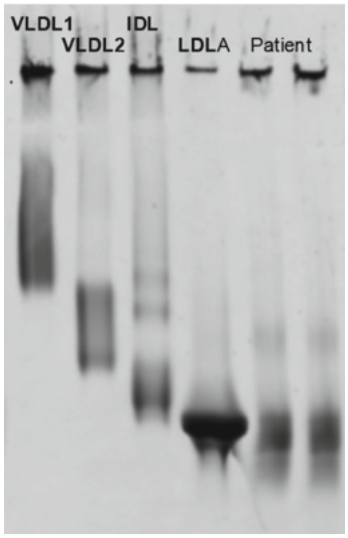

Figure 2. Gel electrophoresis of apolipoprotein B containing lipoproteins

Lanes with very-low-density lipoprotein 1 (VLDL1), very-low-density lipoprotein 2 (VLDL2), intermediate-density lipoprotein 1 (IDL), low-density lipoprotein (LDLA) markers (separated by ultracentrifugation, from controls) and duplicated patient sample. Patient; left lane: at 4 degree Celsius; patient's right lane: -80 degree Celsius.

There is a low concentration of small species of VLDL1. The prominent band in VLDL2 is probably an artefact from freezing. LDL is distributed over a wide size range (i.e. polydisperse) with the predominant species being small. There are also smaller species of LDL extending beyond the usual LDL size range. ApoB lipoproteins of the patient are predominantly in the small LDL range. Familial Dysbetalipoproteinemia phenotype is excluded.

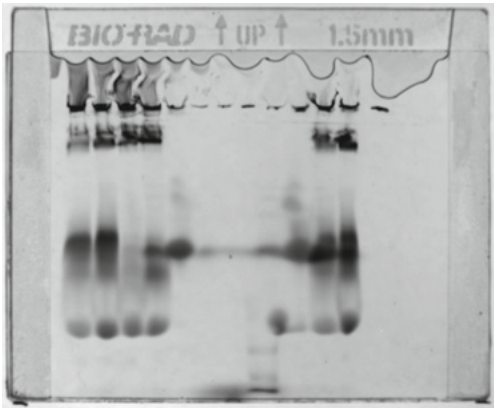

Figure 3. Gel electrophoresis of HDL species

Lane 1: Patient sample stored at 4 degree Celsius; Lane 2: Patient sample immediately after thawing from -80 degree Celsius; Lane 3: Low HDL-C control (male); Lane 4: Normal HDL-C control (female). The normal location of HDL3 and HDL2 subspecies can be seen in lane 4. Although there is no real difference between the two samples, the patient samples differ somewhat between the sample stored at 4 degree Celsius and -80 degree Celsius for a week after receipt. Both samples from the patient demonstrate predominantly (large) HDL2 with HDL3 as a minor species, but the intensity of staining differs.

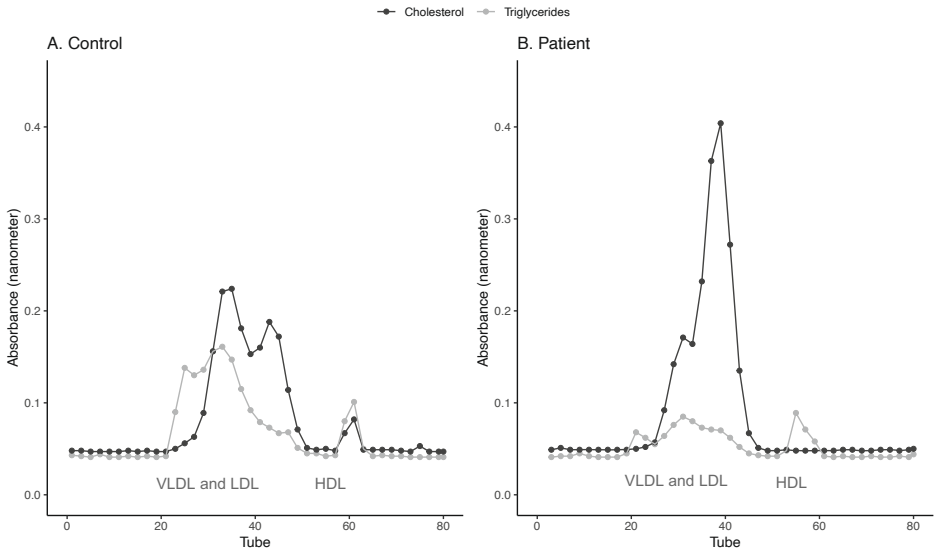

Figure 4. Size exclusion chromatography

VLDL and LDL zone from tube 0 to tube 50; HDL zone: tube 48 to 60.

A. Control sample: Predominance of large VLDL and significant cholesterol within the IDL-LDL zone. Both cholesterol and triglyceride are detected in HDL.

B. Patient sample: Patient sample shows much less triglyceride in VLDL range. Most cholesterol in the LDL range. No cholesterol is detected in HDL which does contain triglycerides.

Discussion

HDL particles consist of varying proportions of proteins, TG, phospholipids and cholesterol. HDL is classified as HDL3 (density 1.095-1.21g/mL) and HDL2 (density 1.063-1.095g/mL) by ultracentrifugation. HDL2 contains more cholesterol ester and TG while the smaller HDL3 is richer in protein. HDL2 usually contains apoA1 whereas HDL3 contains both apoA1 and apoA2.[2]

Hyperalphalipoproteinemia has several causes. The most common are alcohol abuse, certain medications, and chronic liver disease. About 10% of hyperalphalipoproteinemia cases are attributable to monogenic defects relating to reverse cholesterol transport, which is the most important physiological function of HDL. The best known gene associated with high HDL-C levels is *CETP,* which exchanges cholesteryl esters and TG between HDL particles and apoB containing lipoproteins.[9] CETP deficiency therefore leads to larger cholesterol ester-rich HDL particles (HDL2). Another important gene in HDL metabolism is the *SCARB1* gene, the hepatic clearance receptor for cholesterol from HDL species.[10] Pathogenic variants in the *SCARB1* gene lead to increased HDL-C levels through decreased hepatic HDL clearance.[11] ApoC3 is located on both TRLs and HDL. ApoC3 inhibits the biologic activity of lipoprotein lipase (LPL). Thus, in case of apoC3 deficiency, there is increased lipolysis of TRLs and increased HDL-C levels, leading to a favorable lipid profile.[12,13] Endothelial lipase (EL), encoded by *LIPG*, is a phospholipase that remodels HDL.[14] An EL deficiency leads to hyperalphalipoproteinemia, but the exact effect on HDL metabolism is not fully understood.[15] Another rare monogenic cause of hyperalphalipoproteinemia is biallelic pathogenic variants in the *LIPC* gene.[9] The *LIPC* gene is located on chromosome 15 and produces the mature 477-amino acid glycoprotein hepatic lipase (HL).[7] HL is a key enzyme for the hydrolysis of both TG and phospholipids in the liver and is therefore particularly important in the conversion of remnant lipoproteins to LDL and the conversion of large, TG rich HDL particles into intermediate-size HDL particles.[16] HL is synthesized and secreted from hepatocytes. It can bind extracellularly to heparan sulphate proteoglycans (HSPGs) in the space of Disse or circulate freely in the blood.[17]

HL deficiency is one of the rarest genetic dyslipidemias and has only been described in six families to date. HL deficiency is typically associated with a lipoprotein phenotype resembling FD (i.e. with accumulation of TRLs and presence of beta VLDL), in combination with TG-enrichment of HDL and LDL and increased HDL-C levels.[18] However, HL activity can vary greatly, from minimally reduced HL activity to complete HL deficiency when no protein is produced.[18] Consequently, this variation leads to a considerable heterogeneity in lipid and lipoprotein profiles ranging from a typical dysbetalipoproteinemia phenotype

and high levels of HDL, to no specific lipoprotein abnormalities. However, the most consistent finding in the few patients with pathogenic *LIPC* variants is TG enrichment of HDL and LDL and increased HDL2-cholesterol.[19] The degree of HL deficiency depends primarily on the location of the specific variant in the *LIPC* gene, the properties of the substituted amino acid(s) and a gene-dose effect. Homozygous or compound heterozygous subjects generally show more severe HL deficiencies. Heterozygous variants in the *LIPC* gene usually lead to partial HL deficiencies.[7,16,19]

Although we were unable to measure HL activity, the results suggest that the patients' HL is at least partially deficient for several reasons. First, she had large, TG-enriched HDL particles. A similar lipoprotein phenotype was documented in another patient who had partially deficient HL and a heterozygous variant in *LIPC*.[19] Second, the patient in the present case showed polydisperse LDL on the polyacrylamide gel, consistent with abnormal HL activity on lipoproteins. The results of the size exclusion chromatography suggest that the composition of HDL was abnormal with increased TG which is compatible with (partial) HL deficiency, but without a concomitant increase in cholesterol. This was in contrast with the regular lipid panel that showed elevated HDL-C levels in this patient. The direct (homogeneous) HDL-C measurement in routine clinical practice might therefore be erroneous in the case of a (partial) HL deficiency. It is possible that with partial HL deficiency the surface properties of HDL are sufficiently different to interfere with the homogenous assay. Although apoA1 concentrations were also relatively increased and compatible with elevated HDL-C concentrations, the rise in HDL-C was proportionally more, suggesting larger HDL particles as found in the GGE.

Previous studies showed conflicting results concerning the role of HL in the pathogenesis of atherosclerosis.[6,7,19-22] In general, it is thought that the atherogenicity of HL deficiency depends on the presence of circulating TRLs. In line with this, the index patients' TRLs were low and there was no evidence of atherosclerosis. However, we cannot rule out the possibility that dysfunctional HDL might have played a role in the development of her TIAs.

In conclusion, the patient in this case report with hyperalphalipoproteinemia was found to have a very rare heterozygous variant in her *LIPC* gene. From standard laboratory assays the patient appeared to have high HDL-C levels, but size exclusion chromatography showed that her HDL was rich in TG, compatible with (partial) HL deficiency, but no cholesterol. These findings suggest that the standard homogenous assays used in clinical practice to determine HDL-C, might not be accurate in some situations where HDL composition is affected by subtle changes in remodeling and lipolysis. Additional studies in patients with hyperalphalipoproteinemia, TG-enriched HDL and (heterozygous) variants in the *LIPC* gene are needed to confirm this observation.

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Chapter 3

The relation between VLDL-cholesterol and risk of cardiovascular events in patients with manifest cardiovascular disease

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Abstract

Introduction: Apolipoprotein B containing lipoproteins are atherogenic. There is evidence that with low plasma low-density lipoprotein cholesterol (LDL-C) levels residual vascular risk might be caused by triglyceride rich lipoproteins such as very-low-density lipoproteins (VLDL), chylomicrons and their remnants. We investigated the relationship between VLDL-cholesterol (VLDL-C) and recurrent major adverse cardiovascular events (MACE), major adverse limb events (MALE) and all-cause mortality in a cohort of patients with cardiovascular disease.

Methods: Prospective cohort study in 8057 patients with cardiovascular disease from the UCC-SMART study. The relation between calculated VLDL-C levels and the occurrence of MACE, MALE and all-cause mortality was analyzed with Cox regression models.

Results: Patients mean age was 60 ± 10 years, 74% were male, 4894 (61%) had coronary artery disease, 2445 (30%) stroke, 1425 (18%) peripheral arterial disease and 684 (8%) patients had an abdominal aorta aneurysm at baseline. A total of 1535 MACE, 571 MALE and 1792 deaths were observed during a median follow up of 8.2 years (interquartile range 4.5–12.2). VLDL-C was not associated with risk of MACE or all-cause mortality. In the highest quartile of VLDL-C the risk was higher for major adverse limb events (MALE) (HR 1.49; 95%CI 1.16–1.93) compared to the lowest quartile, after adjustment for confounders including LDL-C and lipid lowering medication.

Conclusion: In patients with clinically manifest cardiovascular disease plasma VLDL-C confers an increased risk for MALE, but not for MACE and all-cause mortality, independent of established risk factors including LDL-C and lipid-lowering medication.

Introduction

Apolipoprotein B (ApoB) containing lipoproteins are atherogenic and contribute to the development cardiovascular disease.[1-4] ApoB containing lipoproteins include chylomicrons, very-low-density lipoproteins (VLDL), their remnants, and low-density lipoproteins (LDL). Historically, the main focus has been on LDL-cholesterol (LDL-C) plasma levels in both risk prediction and as treatment target.[5] However, ApoB containing lipoproteins only consist of approximately 60% LDL-C. In recent years, non-high-density lipoprotein cholesterol (non-HDL-C) has been increasingly studied as risk predictor and as an alternative treatment target, especially in patients with (mild) hypertriglyceridemia.[5] Non-HDL-C reflects cholesterol in all ApoB containing lipoproteins and is calculated as total cholesterol (TC) minus HDL-C. Previous studies have shown that non-HDL-C is a better predictor of cardiovascular events than LDL-C and some guidelines therefore recommend using non-HDL-C in addition to LDL-C as treatment target.[6,7] In a fasting state, non-HDL-C levels contain LDL-C and VLDL-cholesterol (VLDL-C), including VLDL-remnants.

Remnants are the smaller residues of VLDL that remain after lipolysis of triglycerides (TG) as a result of lipoprotein lipase (LPL) activity. An easy approach to estimate VLDL-C levels is subtracting HDL-C and LDL-C from TC in a fasting state, since chylomicrons are only present in plasma postprandial. Together with chylomicrons and chylomicron-remnants, VLDL and VLDL-remnants are also often called triglyceride rich lipoproteins (TRLs). Of these, chylomicron- and VLDL-remnants are particularly atherogenic because of their reduced size and relatively high cholesterol content in addition to pro-inflammatory properties due to their triglyceride content.[8] These are small enough to enter the vascular wall where they get trapped in the intima, causing foam cell accumulation and low-grade inflammation, both contributing to the development of atherosclerosis.[4,9]

In patients with coronary artery disease (CAD) it is shown that TRLs are associated with cardiovascular disease.[3,10,11] A study in 10.001 patients with CAD receiving atorvastatin 10 mg showed that increased fasting calculated remnant cholesterol (VLDL-C) levels were associated with an increased risk of MACE for the highest VLDL-C quintile versus the lowest quintile.[3] Previous studies have shown that TRLs are associated with increased risk for cardiovascular events in the general population,[12-15] in patients with Familial Hypercholesterolemia (FH),[16] in patients with type 2 diabetes mellitus (T2DM) and in patients with chronic kidney disease (CKD).[17]

This raised the question whether VLDL-C is a risk factor for recurrent vascular disease and whether this effect is independent from LDL-C and lipid-lowering therapy in patients with clinical manifest vascular disease. Therefore, the aim of the present study is to establish the association between calculated VLDL-C and risk of major adverse cardiovascular events (MACE) major adverse limb events (MALE), the separate components of MACE (myocardial infarction (MI), stroke and cardiovascular mortality) and all-cause mortality in a cohort of patients with different clinical manifestations of arterial vascular disease.

Methods

Study design and patients

The Utrecht Cardiovascular Cohort - Secondary Manifestations of ARTerial disease (UCC-SMART) study is an ongoing, single-center, dynamic, prospective cohort of patients aged 18 to 80 referred to the University Medical Center Utrecht (UMC Utrecht) in the Netherlands, for management of cardiovascular risk factors or atherosclerotic cardiovascular disease. The study was approved by the Ethics Committee of the UMC Utrecht and all patients gave their written informed consent. The rationale and design has been published previously.[18]

For the present study, we used data of 8139 patients, enrolled in the UCC-SMART study between September 1996 and March 2017, with a history or recent diagnosis of clinically manifest arterial disease, including coronary artery disease (CAD), cerebrovascular disease (CeVD), peripheral artery disease (PAD) and/or aneurysm of the abdominal aorta (AAA). CAD was defined as either diagnosis of myocardial infarction (MI), angina pectoris or coronary stenosis in ≥1 major coronary artery, or self-reported history of MI, cardiac arrest or revascularization. CeVD was defined as either diagnosis of transient ischemic attack, ischemic stroke, amaurosis fugax or retinal infarction, or self-reported stroke or carotid artery operation in the past. PAD was defined as Fontaine stage of at least IIa (i.e. intermittent claudicatio and resting ankle-brachial index (ABI) <0.9 in at least one leg), or a self-reported history of amputation or vascular surgery of the lower extremities. AAA was defined as an aneurysm of the abdominal aorta (distal aortic diameter ≥3 cm) during screening or AAA surgery in the past. Patients could be classified in more than one vascular disease category at baseline. Patients with TG levels >9 mmol/L were excluded because in these patients LDL-C cannot reliably be estimated using the Friedewald formula (n=23).[19] In addition, known homozygotes of Apo ε2 genotype were excluded (n=59) since some of these patients might have Familial Dysbetalipoproteinemia (FD) and LDL-C cannot be accurately calculated in these patients.[20] In total, the cohort consisted of 8057 patients.

Screening at baseline

At baseline all patient characteristics were determined using a standardized screening protocol consisting of questionnaires, physical examination, laboratory testing, ankle-brachial index, and abdominal aortic and carotid ultrasound. TC, HDL-C and TG were measured using enzymatic colorimetric methods (AU5811 analysers, Beckman and Coulter). ApoB measurements were included from 2006 onwards and measured using a nephelometer. LDL-C was calculated using the Friedewald formula up to a plasma TG level of 9 mmol/L.[19,20] VLDL-C was calculated as fasting TC minus LDL-C minus HDL-C. T2DM was defined as a referral diagnosis of T2DM, self-reported use of glucose-lowering agents or insulin or fasting plasma glucose level ≥7.0 mmol/L at screening in combination with receiving glucose-lowering therapy within 1 year from screening. Medication use was self-reported. Lipid-lowering medication included use of statins, fibrates, bile acid sequestrants or nicotinic acid at baseline. Prescription of high intensity statins was defined as atorvastatin ≥40 mg or rosuvastatin ≥20 mg. Alcohol use was defined as self-reported current or recently stopped alcohol consumption and no alcohol use was defined as past or never alcohol consumption. Metabolic syndrome was defined according to the Adult Treatment Panel (ATP) III criteria[21] as having 3 or more of the following criteria: waist circumference (WC) >102 cm for males and >88 cm for females; TG ≥1.7 mmol/L; HDL-C <1.03 mmol/L for males and HDL-C <1.29 for females, systolic blood pressure (SBP) ≥130 mmHg or diastolic blood pressure (DBP) ≥85 mmHg; fasting plasma glucose ≥5.6 mmol/L.

Follow-up

The incidence of recurrent cardiovascular events was evaluated biannually in all patients with a questionnaire to obtain information about outpatient clinic visits and hospitalizations. Whenever a possible event was reported, all relevant data were collected. All events were evaluated by three independent physicians of the UCC-SMART Study Endpoint Committee. The primary outcome for this study was MACE, defined as non-fatal MI, non-fatal stroke and cardiovascular mortality. Secondary outcomes were MALE (major amputation or lower limb revascularization), the separate components of MACE: MI, stroke and cardiovascular mortality; and all-cause mortality. For detailed definitions of outcomes see *Supplementary Table 1*. Follow-up was defined as time between date of inclusion and the date of first cardiovascular event, death from any cause, lost to follow-up (n = 469), or end of follow-up in March 2017.

Data analyses

Patient characteristics are presented stratified in quartiles for VLDL-C. In the baseline table (*Table 1*) continuous variables are shown as mean with standard deviation (SD) or median with interquartile range (IQR) in case of a skewed distribution. Categorical variables are shown as number with percentage. Cox proportional hazard models were

used to calculate hazard ratios (HR) and corresponding 95% confidence intervals (95%CI) in quartiles with the lowest quartile serving as reference (*Table 2*) for the occurrence of vascular events. When a patient had multiple events, the first recorded event was used in the analyses. Patients were censored if they were lost to follow-up or if they died. Potential confounders were selected prior to the analyses based on causal diagrams. Two models were built, model I was adjusted for age and sex and model II was additionally adjusted for LDL-C, current smoking, waist circumference, creatinine level, systolic blood pressure, T2DM and use of lipid-lowering medication. Also, in exploratory analyses additional adjustments for HbA1c, HOMA-IR, HDL-C, hsCRP or alcohol use were performed. Linearity assumption was tested visually and statistically by adding continuous VLDL-C level as a restricted cubic spline function to the model (MACE p for linearity 0.92 and MALE p for linearity 0.22). The proportional hazard assumption, examined graphically by plotting scaled Schoenfeld residuals against time, was not violated. Formal testing of the PH assumption confirmed this with a *p*-value of 0.56. Cumulative incidence plots derived from a Kaplan-Meier curve were made for the incidence of MACE and MALE (*Figure 1*) and a histogram of the distribution of VLDL-C in the total population and in patients with and without metabolic syndrome was made (*Supplementary Figure 1*).

We tested for interaction of VLDL-C with LDL-C and use of lipid-lowering medication for MACE and MALE and stratified for LDL-C levels above and below 1.8 mmol/L according to secondary prevention guidelines[5] and use of lipid-lowering medication (*Supplementary Table 2*). In addition, we stratified for type of vascular disease (i.e. CAD, CeVD, PAD and AAA) at baseline (*Supplementary Table 3*). Single imputation was performed by bootstrapping and predictive mean matching, based on multiple regression to account for missing data. Missing values ranged from 0.21% for systolic blood pressure to 12.3% for waist circumference. For all analyses, a *p*-value of <0.05 was considered significant. R Studio, version 3.5.1, was used for the statistical analyses.

Results

Baseline characteristics

Baseline characteristics of the study population are presented according to quartiles of calculated VLDL-C as well as for the total study population in *Table 1*. In total, 74% of the cohort were males, and mean age was 60 (SD 10.3) years, 61% had a history of CAD, 30% of CeVD, 18% of PAD and 8% of AAA. Furthermore, 17% of the patients had T2DM and 52% metabolic syndrome. In higher quartiles of VLDL-C, the prevalence of the metabolic syndrome was higher i.e. 25% in the lowest quartile compared to 90% in

the highest quartile. Patients in the highest quartile had higher TC, LDL-C, non-HDL-C, TG, ApoB and hsCRP concentrations as compared to patients in the lowest VLDL-C quartile. The use of statins was 73% in the lowest quartile and 63% in the highest quartile. The distribution of VLDL-C in the total population and in patients with and without metabolic syndrome is shown in *Supplementary Figure 1*.

VLDL-C and risk of recurrent vascular events

A total of 1535 first MACE were observed, of which 559 were myocardial infarctions (MI), 431 strokes, 897 cardiovascular deaths. Furthermore, there were 571 MALE and all-cause mortality was 1792 during a total follow-up of 68.699 person-years with median follow-up of 8.2 (IQR 4.5–12.2) years.

Overall, in the highest VLDL-C quartile the HR was 1.49 (95%CI 1.16–1.93) for MALE and HR 1.64 (95%CI 1.26–2.14) for MI compared to the lowest VLDL-C quartile (*Table 2*). The risk for MACE, stroke, cardiovascular- and all-cause mortality was not significantly different in the highest compared to the lowest quartile. Exploratory analysis with additional adjustment for HbA1c, HOMA-IR, HDL-C, hsCRP or alcohol use did not change the results.

There was no effect modification by LDL-C or the use of lipid-lowering medication on the relationship between VLDL-C and vascular outcomes. The *p*-value for interaction of LDL-C was 0.50 for MACE and 0.09 for MALE (i.e. both no significant interaction) and for use of lipid-lowering medication the p's were 0.32 and 0.77 for MACE and MALE respectively.

Despite absence of effect modification by LDL-C levels and use of lipid-lowering medication, we evaluated the risk for recurrent events stratified for LDL-C treatment targets according to guidelines5 and use of lipid-lowering medication (*Supplementary Table 2*). Although not reaching statistical significance, the risk for MALE in the group with low LDL-C levels was similar to the group with high LDL-C levels (HR 1.33 95%CI 0.97–1.82 versus HR 1.26 95%CI 1.05–1.52).

Table 1. Baseline characteristics according to VLDL-C quartiles

	Quartile 1 n=2210	**Quartile 2 n=2085**	**Quartile 3 n=1882**	**Quartile 4 n=1880**	**Total n=8057**
VLDL-C (range, mmol/L)	0.09 - 0.45	0.46 - 0.63	0.64 - 0.90	0.91 - 4.01	0.09 - 4.01
VLDL-C (median, mmol/L)	0.36	0.54	0.76	1.21	0.63
Male	1592 (72%)	1512 (73%)	1394 (74%)	1450 (77%)	5948 (74%)
Age (years)	60 ± 11	61 ± 10	60 ± 10	57 ± 10	60 ± 10
BMI (kg/m^2)	25.6 ± 3.6	26.6 ± 4.0	27.5 ± 4.1	28.1 ± 4.1	26.9 ± 4.0
Waist circumference (cm)	92 ± 12	95 ± 12	98 ± 12	100 ± 12	96 ± 12
Systolic blood pressure (mmHg)	137 ± 20	139 ± 21	140 ± 21	142 ± 21	139 ± 21
Diastolic blood pressure (mmHg)	80 ± 11	81 ± 11	81 ± 12	82 ± 11	81 ± 11
Current smoker	555 (25%)	608 (29%)	589 (31%)	736 (39%)	2488 (31%)
Pack years[1]	11 (0 - 27)	14 (2 - 30)	16 (3 - 32)	23 (7 - 36)	14.6 (2.7 - 31.5)
Use of alcohol in the last year	1462 (66%)	1220 (59%)	944 (50%)	834 (45%)	4460 (56%)
> 20 units of alcohol every week	213 (10%)	184 (9%)	181 (10%)	253 (13%)	831 (10%)
Medical history					
• CAD	1287 (58%)	1277 (61%)	1174 (62%)	1156 (61%)	4894 (61%)
• CeVD	763 (35%)	606 (29%)	555 (29%)	521 (28%)	2445 (30%)
• PAD	291 (13%)	339 (16%)	348 (18%)	447 (24%)	1425 (18%)
• AAA	149 (7%)	185 (9%)	170 (9%)	180 (10%)	684 (8%)
• T2DM	247 (11%)	287 (14%)	348 (18%)	463 (25%)	1345 (17%)
• Metabolic syndrome	541 (25%)	767 (37%)	1218 (65%)	1679 (90%)	4205 (52%)
Medication					

Table 1. Continued

	Quartile 1 n=2210	Quartile 2 n=2085	Quartile 3 n=1882	Quartile 4 n=1880	Total n=8057
• Lipid-lowering medication	1625 (74%)	1430 (69%)	1251 (66%)	1192 (63%)	5498 (68%)
• Statins	1617 (73%)	1413 (68%)	1236 (66%)	1176 (63%)	5442 (68%)
• Fibrates	7 (0%)	11 (1%)	17 (1%)	26 (2%)	61 (1%)
• Bile acid sequestrant	1 (0%)	2 (0%)	3 (0%)	3 (0%)	9 (0%)
• Ezetemibe	77 (4%)	67 (3%)	56 (3%)	61 (4%)	261 (3%)
High intensity statins	187 (8%)	152 (7%)	137 (7%)	154 (8%)	630 (8%)
Blood pressure lowering medication	1559 (71%)	1558 (75%)	1436 (76%)	1456 (77%)	6009 (75%)
Platelet aggregation inhibitors	1791 (81%)	1618 (78%)	1408 (75%)	1364 (73%)	6181 (77%)
Laboratory measurements					
• Total cholesterol (mmol/L)	4.3 ± 1.0	4.7 ± 1.1	5.0 ± 1.1	5.4 ± 1.2	4.8 ± 1.2
• HDL-C (mmol/L)	1.4 ± 0.4	1.3 ± 0.3	1.2 ± 0.3	1.0 ± 0.3	1.2 ± 0.4
• LDL-C (mmol/L)*	2.5 ± 0.9	2.9 ± 1.0	3.0 ± 1.0	3.0 ± 1.1	2.8 ± 1.0
• Non-HDL-C (mmol/L)	2.9 ± 0.9	3.4 ± 1.0	3.8 ± 1.0	4.4 ± 1.2	3.6 ± 1.2
• Triglycerides (mmol/L)[1]	0.8 (0.7 - 0.9)	1.2 (1.1 - 1.3)	1.7 (1.5 - 1.8)	2.7 (2.3 - 3.3)	1.4 (1.0 - 2.0)
• ApoB (g/L)‡	0.7 ± 0.2	0.8 ± 0.2	0.9 ± 0.2	1.0 ± 0.3	0.8 ± 0.2
• HOMA-IR[1]	2.0 (1.3 - 2.9)	2.5 (1.7 - 3.8)	3.0 (2.0 - 4.6)	3.9 (2.5 - 6.3)	2.6 (1.7 - 4.2)
• HsCRP (mg/L)[1]	1.5 (0.7 - 3.3)	1.9 (0.9 - 4.2)	2.2 (1.1 - 4.8)	2.7 (1.4 - 5.5)	2.0 (1.0 - 4.4)
• eGFR (ml/min/1.73m^2)	79 ± 16	77 ± 17	76 ± 18	76 ± 20	77 ± 18

* Calculated with Friedewald formula up to triglycerides of 9 mmol/L

[1] Median with interquartile range

‡ Measured from January 2006

VLDL-C=very-low-density lipoprotein cholesterol; BMI=BodyMass Index; CAD=coronary artery disease; CeVD=cerebrovascular disease; PAD=peripheral artery disease; AAA=abdominal aortic aneurysm; T2DM=type 2 diabetesmellitus; HDL-C=high-density lipoprotein cholesterol; LDL-C=low-density lipoprotein cholesterol; non-HDL= non-high-density lipoprotein cholesterol; ApoB = Apolipoprotein B; HOMA-IR = Homeostasis Model Assessment – Insuline Resistance; HsCRP= high sensitivity C-reactive protein; eGFR = estimated glomerular filtration rate (calculated with Chronic Kidney Disease Epidemiology Collaboration [CKDEPI] formula).

Table 2. Risk of vascular events according to VLDL-C quartiles

			Quartile 1	**Quartile 2**	**Quartile 3**	**Quartile 4**
			n=2210	**n=2085**	**n=1882**	**n=1880**
		Model	HR (95% CI)	HR (95% CI)	HR (95% CI)	HR (95% CI)
Major adverse cardiovascular events (MACE)	# events		322	377	399	437
		I	Reference	1.12 (0.97 - 1.30)	1.27 (1.09 - 1.47)*	1.48 (1.27 - 1.70)
		II	Reference	1.00 (0.86 - 1.16)	1.04 (0.89 - 1.21)	1.10 (0.94 - 1.28)
Major adverse limb events (MALE)	# events		102	126	150	193
		I	Reference	1.23 (0.95 - 1.60)	1.58 (1.23 - 2.03)*	2.11 (1.66 - 2.69)*
		II	Reference	1.10 (0.85 - 1.44)	1.31 (1.01 - 1.70)*	1.49 (1.16 - 1.93)*
Myocardial infarction (MI)	# events		94	126	156	183
		I	Reference	1.29 (0.99 - 1.68)	1.68 (1.30 - 2.17)*	1.94 (1.51 - 2.50)*
		II	Reference	1.19 (0.91 - 1.56)	1.47 (1.12 - 1.92)*	1.64 (1.26 - 2.14)*
Stroke	# events		104	105	109	113
		I	Reference	0.98 (0.75 - 1.29)	1.09 (0.83 - 1.43)	1.20 (0.91 - 1.57)
		II	Reference	0.87 (0.66 - 1.15)	0.91 (0.69 - 1.21)	0.93 (0.69 - 1.23)
Cardiovascular mortality	# events		94	222	235	259
		I	Reference	1.16 (0.95 - 1.41)	1.31 (1.07 - 1.59)*	1.60 (1.32 - 1.94)*
		II	Reference	0.99 (0.81 - 1.21)	0.99 (0.81 - 1.21)	1.06 (0.87 - 1.30)
All-cause mortality	# events		396	437	472	487
		I	Reference	1.03 (0.89 - 1.17)	1.16 (1.02 - 1.33)*	1.30 (1.14 - 1.49)*
		II	Reference	0.90 (0.79 - 1.04)	0.94 (0.82 - 1.09)	0.95 (0.82 - 1.09)

Model I: age + sex

Model II: Model 1 + LDL-C + smoking + waist circumference + creatinine + systolic blood pressure + T2DM + use of lipid lowering medication

*$P<0.05$

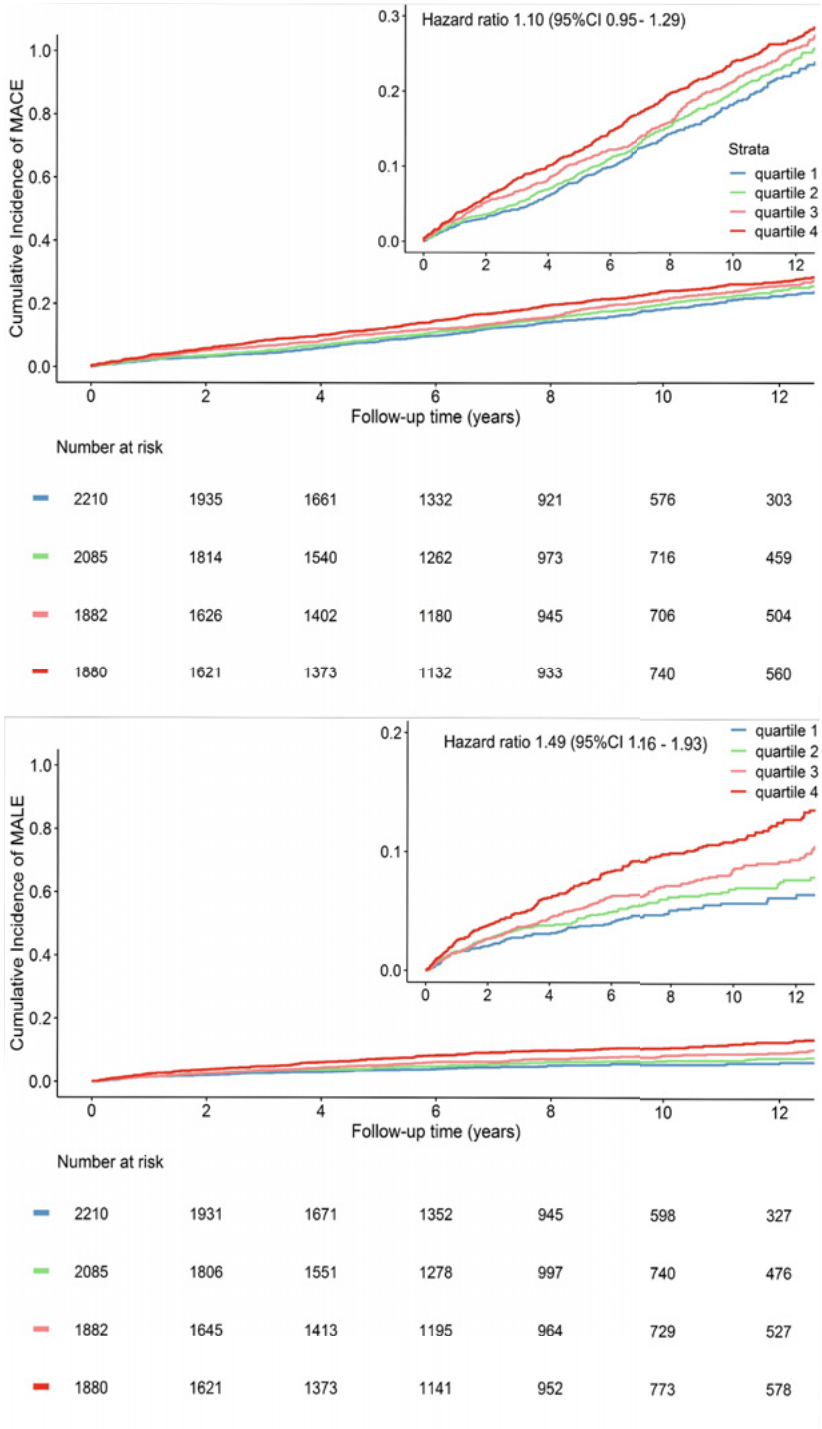

Figure 1. Cumulative incidence of MACE (A) and MALE (B) among quartiles of VLDL-C

Furthermore, we showed that even with use of lipid-lowering medication VLDL-C is a risk factor for MALE (HR 1.39 95%CI 1.13–1.72) and MI (HR 1.44 95%CI 1.20–1.73), but not for the other outcomes. In addition, we evaluated the risk of recurrent vascular events according to location of vascular disease at baseline (*Supplementary Table 3*). In patients with CAD a 1 mmol/L increase in VLDL-C was related to increased risk of MACE (HR 1.19, 95%CI 1.04–1.37), MALE (HR 1.30, 95%CI 1.03–1.65) and MI (HR 1.31, 95%CI 1.07–1.59). In patients with CeVD at baseline a 1 mmol/L higher VLDL-C was associated with an increased risk for MALE (HR 1.72, 95%CI 1.23–2.39) and MI (HR 1.68, 95%CI 1.20–2.35). Levels of VLDL-C in patients with PAD were not associated with other vascular outcomes including MALE. In a subgroup of 684 patients with AAA, VLDL-C was associated with incident MALE (HR 1.80, 95%CI 1.22–2.64) but not with other vascular outcomes.

Discussion

The present study shows that VLDL-C is associated with an increased risk of MALE, but not with MACE and all-cause mortality, independent of LDL-C and lipid-lowering medication in patients with cardiovascular disease.

A post hoc analysis of the TNT trial (in patients with CAD using atorvastatin 10 mg) showed that patients in the highest quintile of fasting calculated remnant cholesterol (VLDL-C) have a higher risk of MACE (composite of CHD death, nonfatal non–procedure-related myocardial infarction, resuscitated cardiac arrest, or fatal or nonfatal stroke) compared with patients in the lowest quintile (HR: 1.48 95%CI 1.15–1.92), independent of LDL-C levels.[3] We found no relation between high levels of VLDL-C and MACE, probably due to limited power since the continuous analyses showed a significant effect of VLDL-C on MACE (data not shown). A recent study showed that directly measured TRL-C is in particular associated with PAD in women from the general population.[15] A case control study in men with and without PAD also showed that remnant abnormalities play an important role in the development and severity of PAD[22] and another study showed that chylomicron- and VLDL-remnants are significantly increased in patients with intermittent limb claudication compared to controls.[23] The present study also showed a strong relationship between VLDL-C and the development of MALE. This relationship is most likely predominantly caused by VLDL-remnant cholesterol and the results indicate that remnant cholesterol might be a specific risk factor for the development of PAD. Furthermore, a study in patients with ischemic heart disease showed that patients in the highest tertile of calculated nonfasting remnant cholesterol (including chylomicrons and chylomicron

remnants) have an increased risk of all-cause mortality compared to the lowest tertile (HR 1.3, 95%CI 1.2–1.5).[24] These results are in contrast to the present study, however, these analyses were not adjusted for LDL-C levels and prescription of lipid-lowering medication.

Regarding the separate components of MACE, VLDL-C was only associated with an increased risk of MI, and no relation for stroke or cardiovascular mortality was observed. These results are in line with previous research which have shown that remnant cholesterol is a causal risk factor for CAD.[25,26] However, in contrast to the present study, research in population based cohorts also showed an increased risk for ischemic stroke[27] and all-cause mortality.[14] This difference could be due to differences in medication use or length of follow-up in the different study populations.

In line with previous research, this study showed that in patients with CAD, VLDL-C was related to a higher risk of recurrent cardiovascular events. This was not only due to the relatively large sample size of patients with CAD compared to other subgroups (and therefore reaching statistically significance more rapidly), the effect estimates of the hazard ratios are also higher in the CAD group compared to patients in other subgroups. This was also shown in a cohort of 560 patients with CAD and low LDL-C levels on lipid-lowering medication.[28] Hence, VLDL-C might attribute to the residual cardiovascular risk in patients with CAD. However, in the present study there was no association with MALE in patients with a history of PAD, which is possibly explained by index-event bias.

The formula to calculate VLDL-C (TC – HDL-C – LDL-C) is commonly used to give an estimation of cholesterol in VLDL in a fasting state. Several studies[14,24,27] use the formula to estimate VLDL-C in the non-fasting state where the calculated lipoprotein fraction also consists of chylomicrons and their remnants in addition to VLDL(remnants). The pro-atherogenic nature of the VLDL-C subfraction does not only depend on the cholesterol concentration but also on the size of particles, with smaller particles (VLDL-remnants) being more atherogenic than larger particles (VLDL). This means that atherogenicity of total VLDL-C may differ according to the proportion of VLDL-remnants. Similarly to LDL-C, cholesterol in remnant lipoproteins becomes trapped in the intima.[4] Unlike LDL-C, cholesterol in remnant lipoproteins does not require oxidation to be absorbed by macrophages.[29] Remnant lipoproteins are relatively cholesterol rich compared to larger TRLs due to lipolysis, and contain more cholesterol per particle compared to LDL particles.[2] Therefore, remnant lipoproteins can cause serious foam cell accumulation. On top of this, remnant lipoproteins are also associated with inflammation, where LDL-C is not.[9] A possible explanation for this is that hydrolysis of triglycerides in TRLs

will generate inflammation due to the release of free fatty acids that induce local endothelial inflammation.[30] In line with this, we found increasing levels of hsCRP across quartiles of remnant cholesterol (*Table 1*), indicating a higher level of inflammation with higher levels of VLDL-C.

In line with previous studies[3,11,28,31] this study showed that VLDL-C remains a risk factor for recurrent cardiovascular events, even when patients with vascular disease use lipid-lowering medication or achieve LDL-C treatment goals (*Supplementary Table 2*). This underlines the need for therapies specifically intervening with VLDL-C and TRL metabolism. Several new therapies are currently evaluated in clinical studies. Apolipoprotein C3 (ApoC3) is present on TRLs and promotes the assembly and secretion of TRLs[32] and inhibits LPL and hepatic lipase.[33] Loss-of-function ApoC3 mutations are associated with a reduced incidence of cardiovascular disease, an association mainly mediated by decreased remnant cholesterol levels.[34] In patients with Familial Chylomicronemia Syndrome it was shown that volanesorsen, an antisense oligonucleotide for ApoC3, lowered VLDL-C with 58%.[35] Angiopoietin-like protein 3 (ANGPTL3) reversibly inhibits LPL activity and is mainly active in the postprandial phase.[36] Loss-of-function mutations of ANGPTL3 are related to a decreased incidence of coronary artery disease and both antisense oligonucleotides for ANGPTL3 and a monoclonal antibody for ANGPTL3, evinacumab, have been shown to reduce TG by approximately 60%.[37]

Strengths of this study are the prospective study design, a large number of patients with different locations of vascular disease, and the long follow-up period and number of endpoints. Furthermore, calculated VLDL-C can easily be calculated from a conventional lipid panel and is therefore clinically available. Some study limitations should be considered. First, LDL-C levels were estimated with the Friedewald formula which uses a standard proportion of cholesterol versus triglycerides (5 triglycerides for 1 cholesterol molecule) to estimate LDL-C. Therefore VLDL-C is an approximation and not an absolute measurement. This could lead to a less precise estimation of VLDL-C. To address this we excluded all patients in which the Friedewald formula was not valid. Furthermore, VLDL-C consists of VLDL and VLDL-remnants and we were not able to evaluate the precise distribution of cholesterol in these lipoproteins. Second, plasma lipids were measured only once at baseline, so we could not account for natural variation or variation as a result of initiating lipid-lowering medication during the follow-up period. As the cohort started in 1996 only 68% of the patients in this cohort were prescribed statins at baseline, which could lead to an underestimation of the true risk for cardiovascular disease. Third, *APOE* genotyping was not available for the complete cohort (two third of the cohort was genotyped), possibly causing an incomplete exclusion of patients with an homozygous Apoε2 genotype.

In conclusion, in patients with clinically manifest cardiovascular disease plasma VLDL-C confers an increased risk for MALE, but not for MACE and all-cause mortality, independent of LDL-C and lipid-lowering medication. We therefore suggest to use also non-HDL-C in clinical practice and to pay attention to VLDL-C in patients who develop a vascular event despite low LDL-C levels or use of lipid-lowering medication.

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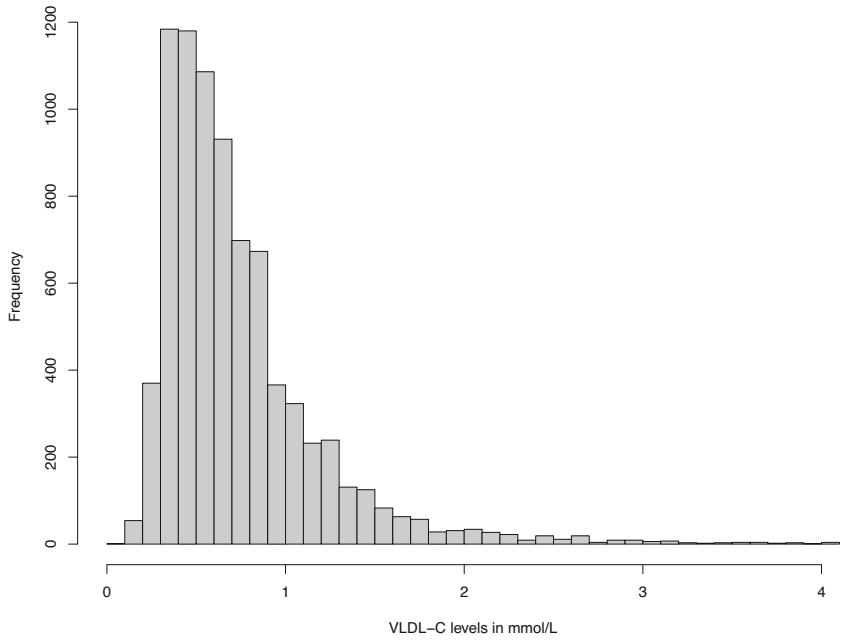
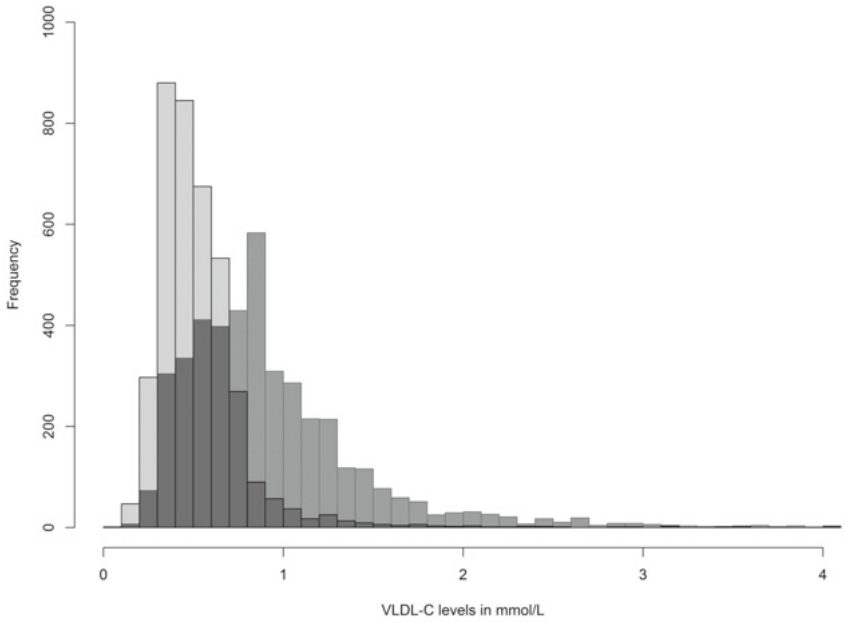
Supplementary Material

Supplementary Table 1. Definitions of vascular outcomes

Outcome	Defined as
Major Adverse Cardiovascular Events	• Nonfatal myocardial infarction • Nonfatal stroke (ischemic/hemorrhagic) • Vascular death (death from myocardial infarction, stroke, heart failure, or rupture of abdominal aortic aneurysm; vascular death from other causes; or sudden death (unexpected cardiac death occurring within 1 hour after onset of symptoms, or within 24 hours given convincing circumstantial evidence))
Major Adverse Limb Events	Major amputation (at level of the foot or more proximal) or lower limb revascularization (vascular intervention or thrombolysis)
Myocardial infarction	• (Non-)fatal myocardial infarction defined by ≥2 of the following: • Acute chest pain for at least 20 min • ST-elevation >1 mm in two adjacent leads or a left bundle branch block (LBBB) on ECG • Elevated troponin or elevated CK ≥2 times the normal value of CK and a MB-fraction >5% of the total CK; Or; • Coronary artery bypass graft (CABG) or percutaneous coronary intervention (PCI) • Sudden death (unexpected cardiac death occurring within 1 hour after onset of symptoms, or within 24 hours given convincing circumstantial evidence)
Stroke	(Non-) fatal ischemic or hemorrhagic stroke: Relevant clinical features for at least 24 hours causing an increase in impairment of at least one grade of the modified Rankin scale, with or without a new infarction or hemorrhage on CT or MRI
Cardiovascular mortality	Death from myocardial infarction, stroke, heart failure, or rupture of abdominal aortic aneurysm; vascular death from other causes; or sudden death (unexpected cardiac death occurring within 1 hour after onset of symptoms, or within 24 hours given convincing circumstantial evidence)
All-cause mortality	All deaths during follow-up, irrespective of the cause of death



Supplementary Figure 1A. Distribution of VLDL-C in the study population (n=8057)



Supplementary Figure 1B. Distribution of VLDL-C in patients with metabolic syndrome (MBS) (red, n=4223) and without MBS (n=3834, green)

Supplementary Table 2. Association between 1 mmol/L higher VLDL-C and risk of recurrent vascular events, stratified for LDL-C levels and use of lipid-lowering medication at baseline

			LDL-C < 1.8 mmol/l at baseline **n=1115**	**LDL-C ≥ 1.8 mmol/l at baseline** **n=6942**	**Prescription of lipid lowering medication at baseline** **n=5498**	**No prescription of lipid lowering medication at baseline** **n=2559**
		Model	HR (95% CI)	HR (95% CI)	HR (95% CI)	HR (95% CI)
Major adverse cardiovascular events (MACE)	# events		141	1394	880	655
		I	1.17 (0.92 - 1.48)	1.37 (1.22 - 1.54)*	1.33 (1.16 - 1.51)*	1.22 (1.05-1.43)
		II	1.06 (0.81 - 1.39)	1.14 (1.01 - 1.29)*	1.15 (0.99 - 1.33)	1.07 (0.90-1.27)
Major adverse limb events (MALE)	# events		59	512	318	253
		I	1.75 (1.34 - 2.27)*	1.60 (1.35 - 1.89)*	1.66 (1.37 - 2.01)*	1.50 (1.21 - 1.86)*
		II	1.33 (0.97 - 1.82)	1.26 (1.05 - 1.52)*	1.39 (1.13 - 1.72)*	1.35 (1.08 - 1.68)*
Myocardial infarction (MI)	# events		47	512	512	198
		I	1.05 (0.67 - 1.63)	1.59 (1.34 - 1.88)*	1.59 (1.34 - 1.88)*	1.32 (1.02 - 1.72)*
		II	1.16 (0.70 - 1.92)	1.44 (1.20 - 1.73)*	1.44 (1.20 - 1.73)*	1.16 (0.87 - 1.55)
Stroke	# events		43	388	244	187
		I	1.08 (0.71 - 1.67)	1.17 (0.93 - 1.47)	1.04 (0.78 - 1.39)	1.24 (0.93 - 1.64)
		II	0.97 (0.59 - 1.61)	0.98 (0.76 - 1.26)	0.89 (0.64 - 1.23)	1.16 (0.86 - 1.57)
Cardiovascular mortality	# events		78	819	445	452
		I	1.35 (1.01 - 1.81)*	1.43 (1.23 - 1.66)*	1.39 (1.15 - 1.68)*	1.29 (1.07 - 1.56)*
		II	1.17 (0.82 - 1.65)	1.10 (0.93 - 1.30)	1.09 (0.87 - 1.35)	1.12 (0.91 - 1.37)
All-cause mortality	# events		181	1611	907	885
		I	1.11 (0.89 - 1.39)	1.30 (1.17 - 1.46)*	1.32 (1.15 - 1.51)	1.11 (0.96 - 1.28)
		II	0.94 (0.72 - 1.22)	1.05 (0.92 - 1.18)	1.07 (0.92 - 1.25)	0.99 (0.84 - 1.14)

Model I: age + sex

Model II: Model 1 + LDL-C + smoking + waist circumference + creatinine + systolic blood pressure + T2DM + use of lipid lowering medication.

*P<0.05

Supplementary Table 3. Relation between 1 mmol/L higher VLDL-C and risk of recurrent vascular events, total and stratified for location of vascular disease at baseline

			Total	**Patients with coronary artery disease**	**Patients with cerebrovascular disease**	**Patients with peripheral artery disease**	**Patients with AAA**
			n=8057	**n=4894**	**n=2445**	**n=1425**	**n=684**
		Model	HR (95% CI)	HR (95% CI)	HR (95% CI)	HR (95% CI)	HR (95% CI)
Major adverse cardiovascular events (MACE)	# events		1535	927	449	381	226
		I	1.31 (1.19 - 1.45)*	1.42 (1.25 - 1.60)*	1.26 (1.05 - 1.52)*	1.13 (0.94 - 1.36)	1.12 (0.85 - 1.47)
		II	1.12 (1.01 - 1.26)*	1.19 (1.04 - 1.37)*	1.05 (0.85 - 1.30)	1.05 (0.86 - 1.27)	1.03 (0.74 - 1.42)
Major adverse limb events (MALE)	# events		571	251	113	373	69
		I	1.62 (1.41 - 1.87)*	1.69 (1.37 - 2.08)*	2.05 (1.54 - 2.71)*	1.00 (0.82- 1.22)	1.93 (1.36 - 2.73)*
		II	1.32 (1.13 - 1.55)*	1.30 (1.03 - 1.65)*	1.72 (1.23 - 2.39)*	0.92 (0.75 - 1.14)	1.80 (1.22 - 2.64)*
Myocardial infarction (MI)	# events		559	402	118	113	59
		I	1.44 (1.24 - 1.67)*	1.40 (1.17 - 1.67)*	1.79 (1.33 - 2.42)*	1.29 (0.95 - 1.75)	0.83 (0.45 - 1.53)
		II	1.34 (1.13 - 1.58)*	1.31 (1.07 - 1.59)*	1.68 (1.20 - 2.35)*	1.27 (0.91 - 1.79)	0.72 (0.36 - 1.44)
Stroke	# events		431	211	200	93	45
		I	1.17 (0.96 - 1.43)	1.35 (1.03 - 1.76)*	1.06 (0.77 - 1.45)	1.03 (0.69 - 1.52)	1.18 (0.66 - 2.09)
		II	1.02 (0.81 - 1.27)	1.12 (0.82 - 1.52)	0.89 (0.62 - 1.28)	0.92 (0.60 - 1.40)	1.20 (0.61 - 2.38)
Cardiovascular mortality	# events		897	488	315	258	183
		I	1.40 (1.23 - 1.59)*	1.58 (1.33 - 1.87)*	1.26 (0.99 - 1.61)	1.14 (0.91 - 1.41)	1.26 (0.93 - 1.71)
		II	1.11 (0.96 - 1.29)	1.19 (0.97 - 1.45)	0.97 (0.73 - 1.28)	1.00 (0.79- 1.25)	1.13 (0.79 - 1.63)
All-cause mortality	# events		1792	945	597	536	305
		I	1.25 (1.13 - 1.38)*	1.38 (1.21 - 1.57)*	1.10 (0.91 - 1.32)	1.08 (0.92 - 1.27)	1.15 (0.90 - 1.47)
		II	1.04 (0.93 - 1.16)	1.08 (0.93 - 1.25)	0.87 (0.71 - 1.07)	0.98 (0.83 - 1.17)	0.98 (0.74 - 1.32)

Model I: age + sex

Model II: Model 1 + LDL-C + smoking + waist circumference + creatinine + systolic blood pressure + T2DM + use of lipid lowering medication.

*P<0.05

Chapter 4

Genetic variants associated with low-density lipoprotein cholesterol and systolic blood pressure and the risk of recurrent cardiovascular disease in patients with established vascular disease

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Abstract

Background and aims: Polygenic risk scores (PRSs) can be used to quantify the effect of genetic contribution to LDL-cholesterol (LDL-C) and systolic blood pressure (SBP). Several PRSs for LDL-C and SBP have been shown to be associated with cardiovascular disease (CVD) in the general population. This study aimed to evaluate the effect of an LDL-C PRS and an SBP PRS on the risk of recurrent CVD in patients with CVD.

Methods: Genotyping was performed in 4,416 patients included in the UCC-SMART study. Weighted LDL-C PRS (279 LDL-C-related SNPs) and SBP PRS (425 SBP-related SNPs) were calculated. Linear regression models were used to evaluate the relation between both PRSs and LDL-C and SBP. The effects of the LDL-C PRS and SBP PRS, and its combination on the risk of recurrent CVD (stroke, myocardial infarction, and vascular death) were analyzed with Cox proportional-hazard models.

Results: Per SD increase in LDL-C PRS, LDL-C increased by 0.18 mmol/L (95%CI 0.15–0.21). Per SD increase in SBP PRS, SBP increased by 3.19 mmHg (95%CI 2.60–3.78). During a follow-up of 11.7 years (IQR 9.2–15.0) 1,198 recurrent events occurred. Neither the LDL-C nor the SBP PRS were associated with recurrent CVD (HR 1.05 per SD increase in LDL-C PRS (95%CI 0.99–1.11) and HR 1.04 per SD increase in SBP PRS (95%CI 0.98–1.10)). The combination of both scores was neither associated with recurrent CVD (HR 1.09; 95%CI 0.93–1.28).

Conclusions: In patients with vascular disease, LDL-C PRS and SBP PRS, both separately and in combination, were not significantly associated with recurrent CVD.

Introduction

Increased low-density lipoprotein cholesterol (LDL-C) and systolic blood pressure (SBP) are among the most important risk factors for the development and progression of cardiovascular disease.[1] SBP and LDL-C are highly heritable traits, involving a large set of genes contributing to disease.[2] Hundreds of single nucleotide polymorphisms (SNPs) associated with plasma LDL-C and SBP have been identified through genome-wide association studies (GWAS) and this is still increasing.[3-5] These genetic variants represent lifelong exposure to LDL-C or SBP in which the small individual effects of each SNP are assumed to be cumulative. Polygenic risk scores (PRSs) aggregate the modest effects of multiple SNPs into a single score as a proxy for lifelong exposure to a given trait.[6] As demonstrated earlier, including genetic information in risk models could potentially contribute to the improvement of personalized cardiovascular risk prediction or to the identification of high-risk patients who might benefit from stricter treatment goals through treatments.[7-9] Previous studies in the general population showed that a PRS for LDL-C and SBP is associated with an increased risk of incident cardiovascular events.[8,10-12] However, very few studies have reported on the association between such PRSs and recurrent cardiovascular events. So far, only one study evaluated the effect of an LDL-C PRS in a selected study population that underwent carotid endarterectomy.[13] This study found no significant association between the LDL-C PRS and the occurrence of cardiovascular events including cardiovascular death, non-fatal stroke, non-fatal myocardial infarction, or vascular interventions. Treatment with lipid-lowering- and antihypertensive medications could modulate the effects of genetic variants on LDL-C and SBP in patients with stable vascular disease. In addition, the effects of these genetic variants on recurrent vascular events may be different compared to first events, because patients with few risk alleles may have other risk factors that caused the first event that also increase the risk of recurrent vascular events.[14] The aim of the present study is therefore twofold. First, to replicate the effect of PRSs for known genetic variants associated with LDL-C or SBP on these risk factors within a cohort of patients with established vascular disease. Second, to evaluate the effect of these PRSs for LDL-C and SBP on the risk of recurrent cardiovascular events in this high-risk patient population.

Methods

Study population

Data from patients enrolled in the Utrecht Cardiovascular Cohort – Second Manifestations of Arterial Disease (UCC-SMART) study were used. The UCC-SMART study is an ongoing, single-center, prospective cohort at the tertiary referral center University Medical Center Utrecht (UMCU) in the Netherlands. Patients aged 18-80 years, referred to the UMCU with established cardiovascular disease (coronary artery disease (CAD), cerebrovascular disease (CeVD), peripheral arterial disease (PAD) or abdominal arterial aneurysm (AAA)), underwent vascular screening. A description of the study rationale has been published previously.[15] The UCC-SMART study was approved by the Medical Ethics Committee of the UMCU, and all patients provided written informed consent prior to inclusion. For the current study, data of patients that were included between September 1996 and August 2010 were used, as these patients were genotyped (n=6,971).

Baseline measurements

At baseline, all patients underwent a standardized vascular screening protocol including a health questionnaire, physical examination, laboratory testing, ankle-branchial index, and an abdominal, aortic and carotid ultrasound. Office blood pressure measurements were performed with automated blood pressure monitors (Iso-Stabil 5; Speidel & Keller, Jungingen, Germany) on the arm with the highest blood pressure. The mean of 3 measurements on that arm was recorded. Smoking, alcohol use, and medication use were self-reported. Lipid-lowering medication included use of statins, fibrates, bile acid sequestrants or nicotinic acid. Prescription of high intensity statins was defined as atorvastatin ≥40 mg or rosuvastatin ≥20 mg. Antihypertensive medications were grouped based on drug class (angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, beta-blockers, alpha-blockers, calcium antagonists, diuretics, aldosterone antagonists, central acting antihypertensives, direct vasodilators). Type 2 diabetes mellitus (T2DM) was defined as either a referral or self-reported diagnosis of T2DM, or a fasting plasma glucose ≥7 mmol/L at study inclusion with initiation of glucose-lowering treatment within 1 year, or baseline use of hypoglycemic agents or insulin.

Laboratory measurements

Laboratory blood testing was performed in the fasting state. Total cholesterol (TC) and triglycerides (TG) were measured with a commercial enzymatic dry chemistry kit (Johnson & Johnson, New Brunswick, USA). High-density lipoprotein cholesterol (HDL-C) was measured with a commercial enzymatic kit (Boehringer, Mannheim, Germany) and

LDL-C was calculated using the Friedewald formula up to triglyceride levels of 9 mmol/L to reduce missing values in this analysis.[16,17] The estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula.[18]

Genotyping and quality control

Genotyping of the cohort was performed using the Illumina GSA array. All SNPs went through a thorough quality control (QC) check using PLINK v. 1.9.[19] Genotype imputation has been performed using IMPUTE2 v2.3.0. After imputation 91.3 million SNPs were available. SNPs with an imputation quality (R2) <0.3 (n=36.8 million), a minor allele frequency below 0.1% (n=71.2 million) and SNPs with a Hardy-Weinberg equilibrium *p*-value <1 × 10−6 (n=90) were also excluded, resulting in 19.9 million imputed SNPs available. Patients of non-European ancestry (n=543), with low quality genotyping (n=212) or those who were related to each other (n=203) were excluded. In case of the latter, the patient with the most recent date of inclusion was excluded. Other reasons for exclusion during quality control were samples with likely sample contamination based on high degree of relatedness with other samples (n=37), or when samples were >5 standard deviations from median for inbreeding coefficient (n=32), with a sex mismatch between genotype and phenotype (n=18), and samples without phenotype data available (n=43). In total, 1,088 patients were excluded after quality control, resulting in 5,883 patients. Lastly, patients without established cardiovascular disease were excluded (n=1,467) resulting in 4,416 patients with vascular disease eligible for the analyses.

SNP selection and calculation of the polygenic risk scores

To identify SNPs for both PRSs, we first retrieved the most recent (at the time of conducting the analysis) meta-analyses of GWAS describing genetic variants associated with either LDL-C[5] or SBP[3,4,20] at genome-wide level of significance (*p* <5×10−8). From these meta-analyses, a total of 444 SNPs and 616 SNPs were identified as potentially relevant for the construction of each PRS. To remove highly correlated variants, we performed LD pruning on the summary data of these SNPs extracted from the Pan-ancestry genetic analysis of the UK biobank[21] using PLINK v.1.9.[22] To this end, we used the '--indep-pairwise 1,000 10 0.2' flag in PLINK, which means that we used a window of 1,000 SNPs, calculated LD between each pair of SNPs in the window, removed one of a SNP pair if LD was greater than r2=0.2, shifted the window 10 SNPs forward and then repeated the procedure. This resulted in a final selection of 279 and 425 SNPs associated with LDL-C and SBP, respectively.

For each patient, two weighted PRSs were calculated by summing the dosages of effect alleles (labeled as the alternate alleles; ranging from 0 to 2) of an individual patient at each SNP multiplied by the β-coefficient of the respective alternate allele. Because the UCC-SMART study population is from European descent, we used the β-coefficients from European ancestry sub-analysis of the Pan-UKB. These β-coefficients were adjusted for use of medication (row 4,491 for LDL-C and row 4,519 for SBP).[23] A list of genetic variants and their β-coefficients used to derive both PRSs is provided in *Supplementary Tables 1A and B*.

Follow-up

Follow-up duration was defined as time from inclusion in the cohort until development of first cardiovascular event, death, loss to follow-up or the preselected date of July 1, 2019. From 1996 till July 1, 2019, 360 patients were lost to follow-up (8%). During follow-up, patients received questionnaires on hospital admissions and outpatient clinic visits twice a year. If an event was reported, all relevant hospital documents, and laboratory and radiologic findings were collected. All events were audited independently by three physicians of the UCC-SMART endpoint committee. The primary outcome for this study was the combination of non-fatal and fatal vascular events, consisting of non-fatal myocardial infarction (MI), non-fatal stroke and vascular death. Secondary outcomes were the separate components of the composite outcome (non-fatal MI, non-fatal stroke and vascular death). For detailed description of the outcomes see *Supplementary Table 2*.

Data analyses

Baseline characteristics are presented in four groups, according to the median of both polygenic risk scores (the distributions of both PRSs are displayed in *Supplementary Figure 1*); one reference group with genetically lower LDL-C and SBP (LDL-C PRS ≤ median and SBP PRS ≤ median), one group with genetically higher SBP (LDL-C PRS ≤ median, SBP PRS >median), one group with genetically higher LDL-C (LDL-C PRS >median, SBP PRS ≤ median), and one group with both genetically higher SBP and LDL-C (LDL-C PRS >median, SBP PRS >median). The organization of patients according to both PRSs is provided in *Supplementary Figure 2*.

Baseline data are presented as number and percentage for categorical variables, mean ± standard deviation (SD) for normally distributed variables or median with interquartile range (IQR) in case of a skewed distribution. For the association between the LDL-C PRS and LDL-C and the SBP PRS and SBP values, respectively, linear regression models were fitted. Three models were built. The first model was adjusted for age, sex, and the first five principal components. The second model was additionally adjusted for BMI,

T2DM, smoking, alcohol use, eGFR, and triglycerides. The third model was additionally adjusted for use of lipid-lowering- or antihypertensive medication. For these analyses the LDL-C - and SBP PRS were standardized. Hence, the beta coefficient corresponds to the change per SD increase in the PRS. In addition, the beta-coefficients derived from the linear regression models were plotted according to quartiles of the LDL-C and SBP PRS.

Cox proportional hazard models were used to determine the relationship between the (standardized) LDL-C PRS and SBP PRS and recurrent events. Linearity of the relationships between LDL-C PRS and SBP PRS with recurrent vascular events was assessed with restricted cubic splines. The Cox proportional hazard assumption was visually checked and confirmed by plotting Schoenfeld residuals against time. Two models were built. The first model was adjusted for age, sex, and the first five principal components. The second model was additionally adjusted for body mass index (BMI), T2DM, smoking, alcohol use, eGFR, triglycerides, and systolic blood pressure and lipid-lowering medication (in model for LDL PRS), or LDL-C and antihypertensive medication (in model for SBP PRS). Additionally, to evaluate potential effect modification between the LDL-C and SBP PRS Cox models were fitted between the combined LDL-C and SBP PRS groups and recurrent cardiovascular events. To evaluate whether several key characteristics (T2DM, sex, age, type of vascular disease at baseline, and use of lipid-lowering- and antihypertensive medication) might modify the association between both PRSs and recurrent vascular events, we included interaction terms into the models.

Several sensitivity analyses were performed. To assess whether a different distribution of patient groups would influence the results, we classified patients according to the highest quintile and decile of both PRSs and compared the hazard of recurrent MACE in those with genetically higher LDL-C and SBP (top quintiles and top deciles of both PRSs) versus all others. Also, to evaluate whether the results were influenced by pleiotropy, we performed a sensitivity analysis by excluding SNPs that were significantly associated with either SBP or LDL-C PRS (*p*-value adjusted for multiple testing=0.018 for LDL-C and *p*-value adjusted for multiple testing=0.012 for SBP, *Supplementary Tables 7 and 8*).

To improve statistical accuracy, missing values of variables of interest [BMI (n=9; 0.2%), smoking status (n=17, 0.4%), eGFR (n=19, 0.4%), triglycerides (n=28, 0.6%), systolic blood pressure (n=9, 0.2%), LDL-C (n=38, 0.9%)] were completed by single regression imputation using predictive mean matching.[24] There were no missing values for age, sex, T2DM, lipid-lowering- and antihypertensive medication. All analyses were performed with R statistical software (Version 3.5.1; R foundation for Statistical Computing, Vienna, Austria).

Results

Baseline characteristics

Baseline characteristics of the patients stratified according to the medians of both PRSs are shown in *Table 1*. The mean age was 61 ± 10 years and 75% of the patients were male, 61% had a history of CAD, 27% of CeVD, 21% of PAD, and 9% of AAA. Compared to the reference group (genetically lower LDL-C and SBP), the group with genetically higher LDL-C and SBP had a higher mean SBP (143 ± 21 mmHg versus 139 ± 20 mmHg) and a higher mean LDL-C (3.02 ± 1.07 mmol/L versus 2.87 ± 1.04 mmol/L). This group also had a higher prescription rate for lipid-lowering- (68% versus 59%) and antihypertensive medications (75% versus 70%) compared to the reference group. There were no clinically relevant differences with respect to the other variables at baseline among the four groups.

Relation between polygenic risk scores and traits

LDL-C polygenic risk score and LDL-C

Supplementary Table 3 shows that the LDL-C PRS was significantly associated with LDL-C (per SD increase in PRS, LDL-C increased by 0.11 mmol/L; 95%CI 0.08–0.14). Additional adjustment for the use of lipid-lowering medication further strengthened this relation (β-coefficient per SD 0.18 mmol/L; 95%CI 0.15–0.21). To evaluate whether the effect of PRS was different in patients with or without lipid-lowering, we added use of lipid-lowering medication as an interaction term in the model (p=0.08). *Figure 1* shows mean LDL-C levels according to LDL-C PRS quartiles stratified for use of lipid-lowering medication after adjustment for age, sex, BMI, SBP, smoking, alcohol use, T2DM, eGFR, triglycerides, and the first 5 principal components. Mean LDL-C levels were higher in patients without lipid-lowering medication in all quartiles.

SBP polygenic risk score and SBP

The SBP PRS was significantly associated with SBP, as shown in *Supplementary Table 4*. One SD increase in the SBP PRS corresponded to an increment of 3.15 mmHg (95%CI 2.56–3.74) in SBP. Additional adjustment for use of antihypertensive medication did not change the results meaningfully (β 3.19; 95% CI 2.60–3.78). *Figure 2* shows mean SBP according to SBP PRS quartiles, stratified for the use of antihypertensive medication after adjustment for age, sex, BMI, LDL-C, smoking, alcohol use, T2DM, eGFR, triglycerides, and the first 5 principal components. SBP levels were similar in patients with and without antihypertensive medication indicating that the effect of the SBP does not depend on the use of antihypertensive drugs, which was confirmed by the non-significant interaction between SBP PRS and use of antihypertensive drugs (p=0.17).

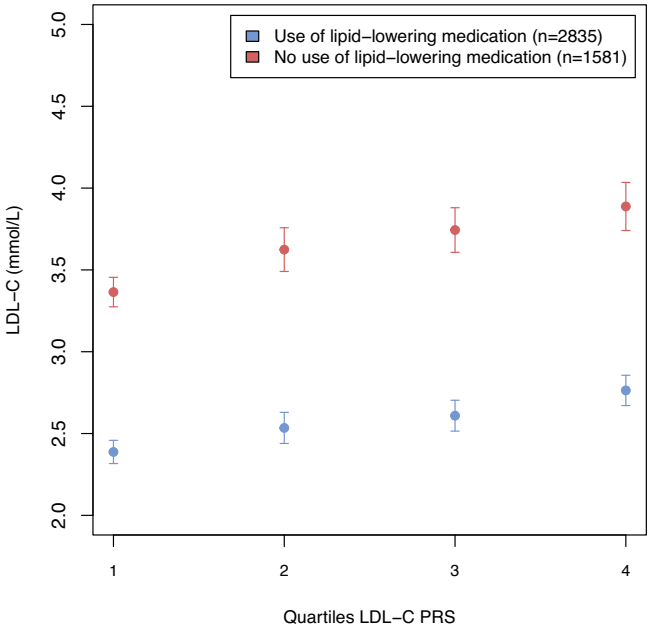

Figure 1. Relation LDL-C polygenic risk score and LDL-C values in quartiles in patients with and without use of lipid-lowering medication

Linear regression analyses describing the association between mean LDL-C level and use of lipid-lowering-specific quartile of LDL-C PRS. Models were adjusted for age, sex, BMI, SBP, smoking, alcohol use, T2DM, eGFR, triglycerides, and the first 5 principal components.

Relation between polygenic risk scores and recurrent cardiovascular events

During a median follow-up of 11.7 years (IQR: 9.2–15.0 years; 51,991 person-years), the composite outcome (consisting of non-fatal myocardial infarction, non-fatal stroke, and vascular death) occurred in 1,198 patients.

LDL-C polygenic risk score and recurrent cardiovascular events

After adjustment for traditional cardiovascular risk factors including age, sex, BMI, T2DM, smoking, alcohol use, eGFR, triglycerides, SBP, and lipid-lowering medication, LDL-C PRS was not associated with the risk of recurrent cardiovascular events (hazard ratio (HR) per one SD increase in PRS; 1.05; 95%CI 0.99–1.11) (*Table 2*). There was no interaction with use of lipid-lowering medication (*p* for interaction=0.39). Also, there was no effect modification by age, sex, T2DM and type of vascular disease at baseline in the relation between LDL-C PRS and recurrent cardiovascular events (*p* for all interactions>0.05). Exploratory analyses examining secondary outcomes showed similar results (non-fatal MI (HR 1.05; 95%CI 0.96–1.16), non-fatal stroke (HR 1.00; 95%CI 0.90–1.12), and vascular death (HR 1.05; 95%CI 0.98–1.13) (*Supplementary Table 5*).

Table 1. Baseline characteristics according to combined LDL-C and SBP polygenic risk score

	Reference group	**LDL-C PRS ≤ median,SBP PRS > median**	**LDL-C PRS > median, SBP PRS ≤ median**	**LDL-C PRS and SBP PRS > median**	**Total**
	n=1123	**n=1085**	**n=1085**	**n=1123**	**n=4416**
Male sex	840 (75%)	808 (74%)	815 (75%)	831 (74%)	3294 (75%)
Age (years)	61 ± 10	61 ± 10	60 ± 10	60 ± 10	61 ± 10
Current smoker	402 (36%)	348 (32%)	372 (34%)	354 (32%)	1476 (33%)
Current alcohol use	550 (49%)	536 (49%)	548 (51%)	577 (51%)	2211 (50%)
Body mass index (kg/m^2)	26.8 ± 3.8	26.9 ± 3.9	26.7 ± 4.0	26.7 ± 3.9	26.7 ± 3.9
Systolic blood pressure (mmHg)	139 ± 20	144 ± 22	138 ± 21	143 ± 21	141 ± 21
Diastolic blood pressure (mmHg)	81± 11	82 ± 11	80 ± 12	83 ± 11	81 ± 11
History of vascular disease					
Diabetes mellitus type 2	173 (15%)	199 (18%)	156 (14%)	177 (16%)	705 (16%)
Coronary artery disease	651 (58%)	632 (58%)	702 (65%)	720 (64%)	2705 (61%)
Peripheral artery disease	231 (21%)	251 (23%)	217 (20%)	237 (21%)	936 (21%)
Cerebrovascular disease	338 (30%)	305 (28%)	260 (24%)	300 (27%)	1203 (27%)
Abdominal aortic aneurysm	107 (10%)	90 (8%)	95 (9%)	101 (9%)	393 (9%)
Laboratory values					
Total cholesterol (mmol/L)	4.82 ± 1.19	4.84 ± 1.21	5.03 ± 1.23	5.04 ± 1.31	4.93 ± 1.24
HDL-cholesterol (mmol/L)	1.23 ± 0.36	1.21 ± 0.38	1.20 ± 0.35	1.21 ± 0.37	1.21 ± 0.36
LDL-cholesterol (mmol/L)	2.87 ± 1.04	2.89 ± 1.08	3.08 ± 1.08	3.02 ± 1.07	2.97 ± 1.07
Triglycerides (mmol/L)	1.3 (0.9 - 1.9)	1.4 (1.0 - 2.0)	1.4 (1.0 - 2.0)	1.5 (1.1 - 2.2)	1.4 (1.0 - 2.0)
Estimated GFR (ml/min/1.73m^2)	75 ± 17	74 ± 18	76 ± 17	76 ± 18	75 ± 18
hsCRP (mg/L)	2.2 (1.0 - 4.6)	2.3 (1.0 - 4.9)	1.9 (0.9 - 4.3)	2.0 (1.0 - 4.4)	2.1 (1.0 - 4.5)

Table 1. Continued

	Reference group	LDL-C PRS ≤ median,SBP PRS > median	LDL-C PRS > median, SBP PRS ≤ median	LDL-C PRS and SBP PRS > median	Total
	n=1123	n=1085	n=1085	n=1123	n=4416
Medication use					
Lipid lowering medication	660 (59%)	641 (59%)	770 (71%)	764 (68%)	2835 (64%)
High intensity statins	54 (5%)	61 (6%)	85 (8%)	79 (7%)	279 (6%)
Antihypertensive medication	789 (70%)	819 (75%)	783 (72%)	845 (75%)	3236 (73%)
Number of antihypertensive drugs	1.2 [0 - 5]	1.4 [0 - 7]	1.3 [0 - 5]	1.4 [0 - 6]	1.3 [0 - 7]
Platelet inhibitors	819 (73%)	796 (73%)	813 (75%)	864 (77%)	3292 (75%)

Data are presented as number (%), mean ± standard deviation, median (interquartile range) or mean [range].

Abbreviations: HDL; high-density lipoprotein, LDL; low-density lipoprotein, SBP; systolic blood pressure, GFR; glomerular filtration rate, hsCRP; high sensitivity C-reactive protein

Table 2. LDL-C and SBP polygenic risk score and recurrent cardiovascular events (non-fatal MI, non-fatal stroke and vascular death)

			LDL-C PRS n=4416	**SBP PRS n=4416**
		Model	HR per SD increase in PRS (95% CI)	HR per SD increase in PRS (95% CI)
Recurrent cardiovascular events	#events		1198	1198
		I	1.02 (0.96 - 1.08)	1.04 (0.99 - 1.10)
		II	1.05 (0.99 - 1.11)	1.04 (0.98 - 1.10)

Model I: adjusted for age and sex, and the first five principal components.
Model II:
LDL-C PRS:
Model I + additional adjustment for BMI, type 2 diabetes mellitus, smoking, alcohol use, eGFR, triglycerides, first 5 principal components, SBP, and lipid-lowering medication
SBP PRS:
Model I + additional adjustment for BMI, type 2 diabetes mellitus, smoking, alcohol use, eGFR, triglycerides, first 5 principal components, LDL-C, and antihypertensive medication

Table 3. Combined LDL-C and SBP polygenic risk score and recurrent cardiovascular events (non-fatal MI, non-fatal stroke and vascular death)

			LDL-C PRS ≤ median, SBP PRS < median (Reference group) n=1123	**LDL-C PRS ≤ median, SBP PRS > median n=1085**	**LDL-C PRS > median, SBP PRS ≤ median n=1085**	**LDL-C PRS > median, SBP PRS > median n=1123**
		Model	HR (95% CI)	HR (95% CI)	HR (95% CI)	HR (95% CI)
Recurrent cardiovascular events	# events		295	320	280	303
		I	Reference	1.08 (0.92 - 1.26)	0.98 (0.83 - 1.15)	1.06 (0.91 - 1.25)
		II	Reference	1.06 (0.90 - 1.24)	1.03 (0.87 - 1.22)	1.09 (0.93 - 1.28)

Model I: adjusted for age, sex, and the first 5 principal components
Model II: Model I + additionally adjusted for BMI, type 2 diabetes mellitus, smoking, alcohol use, eGFR, triglycerides, lipid-lowering medication, antihypertensive medication

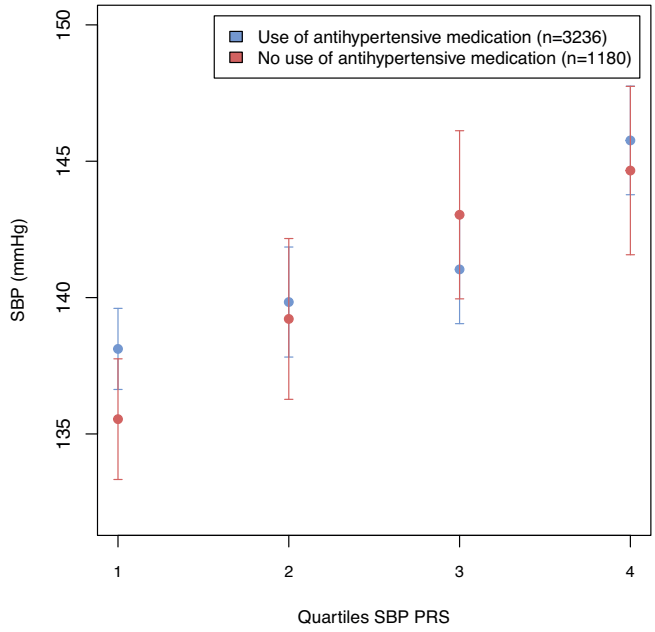

Figure 2. Relation SBP polygenic risk score and SBP values in quartiles in patients with and without use of antihypertensive medication

Linear regression analyses describing the association between mean SBP and use of antihypertensives-specific quartile of SBP PRS. Models were adjusted for age, sex, BMI, LDL-C, smoking, alcohol use, T2DM, eGFR, triglycerides, and the first 5 principal components

SBP polygenic risk score and recurrent cardiovascular events

The SBP PRS was not associated with recurrent cardiovascular events (HR 1.04 per one SD increase in PRS; 95%CI 0.98-1.10) (*Table 2*). The effects were similar in patients with or without antihypertensive mediation (*p* for interaction=0.79). No interaction was observed with age, sex, T2DM and type of vascular disease at baseline (*p* for all interactions >0.05). Analyses examining secondary outcomes also found no statistically significant association between SBP PRS and non-fatal MI (HR 1.03; 95%CI 0.94-1.13) and non-fatal stroke (HR 0.99; 95%CI 0.89-1.10), but did find a significant association with vascular death (HR 1.11; 95%CI 1.03-1.19) (*Supplementary Table 5*).

Combined polygenic risk scores and recurrent cardiovascular events

Patients with a genetically higher LDL-C and SBP experienced 303 recurrent cardiovascular events during follow-up (incidence rate 25.2 per 1,000 person-years). Patients with a genetically lower LDL-C and SBP experienced 295 recurrent cardiovascular events (incidence rate 24.8 per 1,000 person-years). Compared

to patients with a genetically lower LDL-C and SBP, there was no statistically significant difference in the risk of recurrent cardiovascular events in patients with a genetically higher LDL-C and SBP (HR 1.09; 95%CI 0.93–1.28) (*Table 3*). Also, there was no significant difference in the risk of the separate cardiovascular outcomes (non-fatal MI (HR 1.10; 95%CI 0.84–1.44), non-fatal stroke (HR 1.02; 95%CI 0.75–1.39) and vascular death (HR 1.14; 95%CI 0.93–1.40)) when comparing both groups (*Supplementary Table 6*).

Sensitivity analyses

Repeating the analyses after classification of patients according to the highest quintile and decile of both PRSs showed comparable results (*Supplementary Tables 9–10*). Furthermore, to determine whether the results were influenced by pleiotropy, we performed a sensitivity analysis in which we excluded SNPs that were significantly associated with both LDL-C and SBP. For the LDL-C PRS, a total of 81 SNPs were excluded, and for the SBP PRS, a total of 77 SNPs. Exclusion of these SNPs from both PRSs did not change the estimates meaningfully (*Supplementary Tables 11–14*).

Discussion

In this prospective cohort study of patients with vascular disease, we replicated the association of a PRS for LDL-C and a PRS for SBP with these risk factors, constructed by SNPs identified through the latest large-scale genome-wide association studies. However, no statistically significant association was observed between these PRSs and recurrent cardiovascular events.

Results of the current study are in line with the results from a study that investigated an LDL-C PRS in patients undergoing carotid endarterectomy. This study also found no association between an LDL-C PRS and recurrent cardiovascular events within a follow-up of three years (HR per one SD increase 1.03 (95%CI 0.92–1.15)).[13]

To our knowledge, the combined effect of a PRS for LDL-C and a PRS for SBP on cardiovascular events only has been evaluated in apparently healthy individuals enrolled in the UK biobank.[10] In contrast to our study, this study found that relatively small absolute differences in combined exposure to genetically lower LDL-C and SBP translated into a large difference in the risk for major coronary events (odds ratio (OR) 0.61 (95%CI 0.59–0.64)).[10] Although a direct comparison of PRS effect sizes may be challenging due to use of varying (number of) SNPs and outcomes, it remains

somewhat notable that the present study found no effect of either PRSs on the risk of recurrent cardiovascular events, also given the abundant evidence on LDL-C and SBP as causal contributors to cardiovascular risk. Several mechanisms may explain why no association was observed in this study.

First, the present study was conducted in a relatively small cohort compared to previous studies evaluating a PRS.[10,11] This may have resulted in limited power to demonstrate a genuine lack of associations, especially when the magnitude of the effect is small. This is supported by the ambivalent results we obtained: both PRSs were not associated with the primary outcome, but we did observe a nominally significant association between the PRS for SBP and the secondary outcome vascular death. Hence, before drawing any definitive conclusions, replication in larger cohorts of patients with vascular disease is needed. Second, index-event bias has been proposed as an explanation for differences in associations of PRS in patients with cardiovascular events compared to patients without prior cardiovascular disease.[25] This can be understood by considering the onset of vascular events as the sum of the effect of multiple causal factors. If one important causal risk factor (such as a high genetically determined LDL-C or SBP (reflected in a high LDL-C or SBP PRS)) is already present, less effect of other factors is required for disease onset. Subsequently, comparing patients with a genetically unfavorable LDL-C and/or SBP profile to patients with a genetically favorable LDL-C and/or SBP profile who already have developed vascular disease, leads to a relatively healthy risk profile in the former compared to the latter and hence a bias of the results towards null. This type of bias is recently investigated in a study using data from the UK biobank.[26] The authors demonstrated that associations of a CAD PRS with incident cardiovascular outcomes were greatly attenuated among those with established CAD compared to those without CAD. Nonetheless, the estimates did not change after adjustment for most known risk factors for vascular disease, making index event bias a less likely explanation.

Finally, use of lipid-lowering- or antihypertensive medication and healthy lifestyle may have contributed to the lack of an association between both PRSs and recurrent vascular events. As demonstrated in the baseline table, patients with both the LDL-C PRS and SBP PRS above the median had a higher prescription rate for lipid-lowering- and antihypertensive medication compared to patients with both PRS below median. Moreover, patients with a genetically higher LDL-C and SBP may be more likely to be treated more intensively with these type of medications and potentially adopt a more healthy lifestyle during follow-up, which eventually compensates for the higher genetically determined LDL-C and SBP levels. Moreover, these types of medication and the change to a healthy lifestyle may be more effective in patients with genetically higher LDL-C and SBP. This concept is supported by previous studies showing that both

statins, Proprotein Convertase Subtilisin-Kexin type 9 (PCSK9) monoclonal antibodies, and also a healthy lifestyle are able to modify the risk of (recurrent) cardiovascular events associated with a high PRS.[27-30]

This study shows that genetically determined LDL-C and SBP do not explain differences in residual cardiovascular risk in patients with established vascular disease. Although this is an etiologic study, these results support the recommendations in international guidelines not to routinely collect genetic information for CVD risk stratification. In general, the position of genetic risk scores in clinical practice is under debate. Currently, PRSs are considered of limited use for the prediction of CVD events.[31] Moreover, in the scenario that PRSs will play an important role in clinical practice in the future, it is likely that its greatest value lies in the first decades of life, prior to clinical events and even prior to definable plaque burden by imaging.

Strengths of the present study include the prospective cohort study design reflecting clinical practice of patients with vascular disease being treated according to national guidelines, the substantial follow-up duration and the large number of validated clinically relevant outcomes. Also, genotyping and quality control were performed according to a highly standardized protocol by experts in the field. Lastly, elaborate sensitivity analyses were performed to further investigate the main findings of this study.

Some limitations need to be considered. In the present study two PRSs were used based on 704 different SNPs related to either LDL-C or SBP identified through GWAS in the general population. Some have argued that such PRSs are of limited value in populations with established vascular disease and advocate the design and use of dedicated GWAS of disease progression.[26,32,33] However, this study demonstrated a robust effect of the selected SNPs on plasma LDL-C and SBP levels in patients with vascular disease, independent of the use of lipid-lowering- or antihypertensive medication. Moreover, differences in LDL-C and SBP levels when stratified for LDL-C or SBP PRS, were comparable with the differences observed in the general population.[7,8] In addition, the allele frequencies of the selected SNPs in the current study population were comparable to the allele frequencies found in the general European population (*Supplementary Table 1*). Another important limitation is that use of medication such as lipid-lowering- and antihypertensive medication was only recorded at baseline. Although the use of these types of medication probably increased during follow-up, since treatment advice was part of the screening for this study, we were not able to account for these changes in the analyses. Lastly, the PRSs used in this study are only applicable to populations of European descent, which may limit the generalizability of the results and poses an ethical dilemma.[34,35]

In conclusion, in patients with established cardiovascular disease, we replicated the known association of PRSs for LDL-C and SBP with these risk factors. We found no statistically significant association between an LDL-C PRS and an SBP PRS, nor in combination, and recurrent cardiovascular events. These results suggests that genetically determined LDL-C and SBP do not explain the differences in residual cardiovascular risk in patients with established vascular disease.

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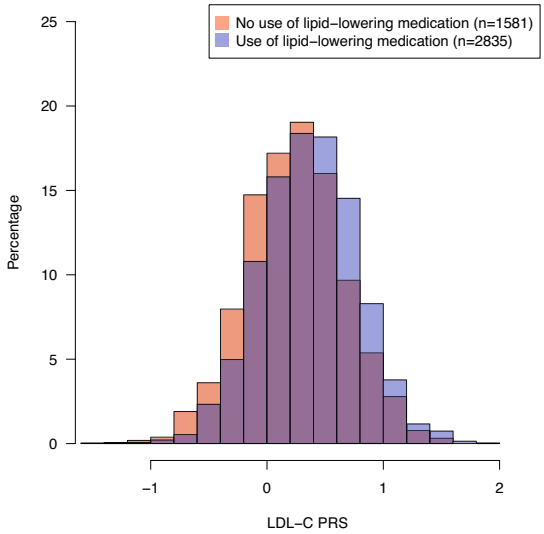
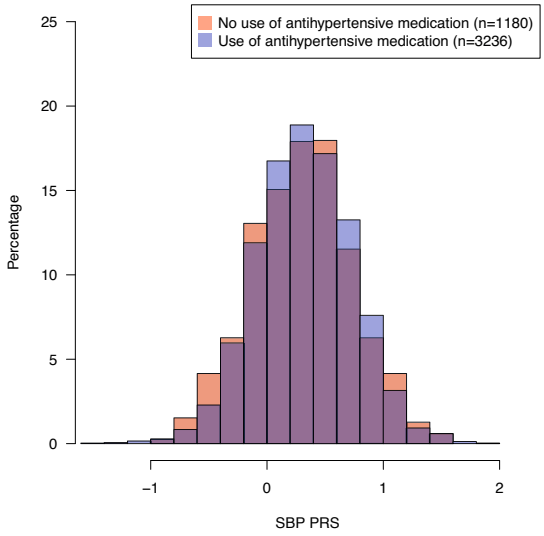
Supplementary Material

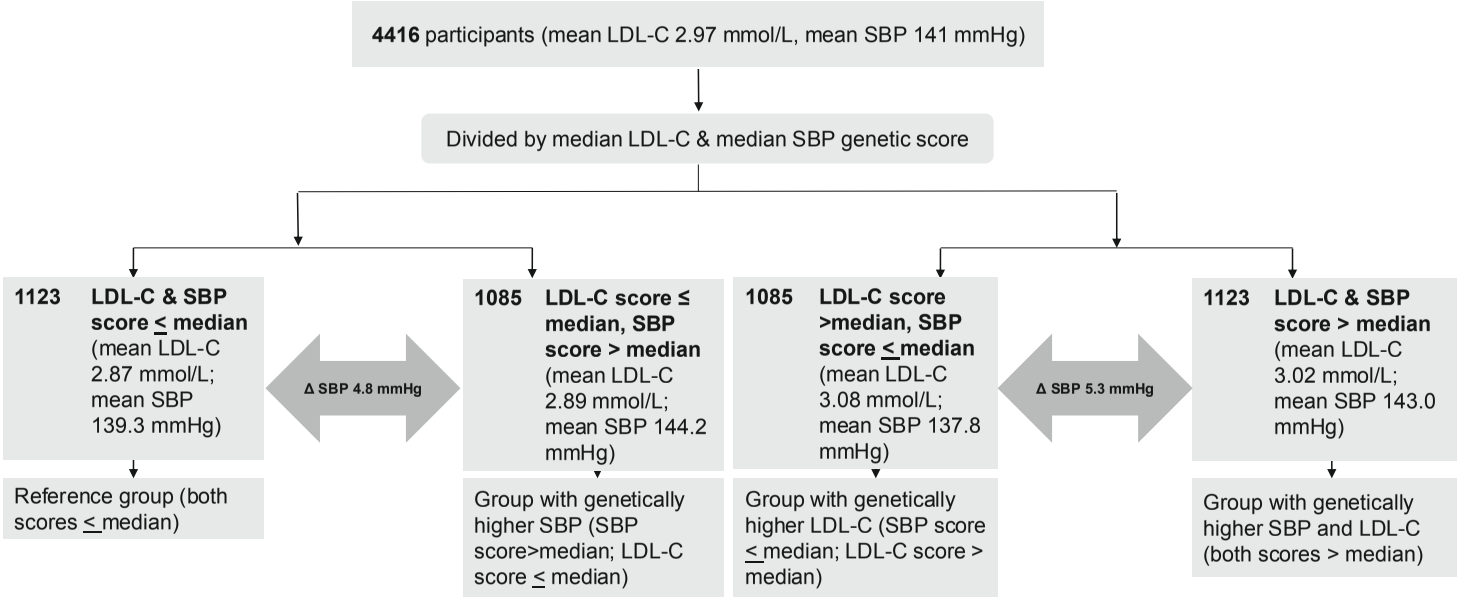
Supplementary Tables 1A and 1B:
Please find these Supplementary Tables online with the following internet link:
https://www.sciencedirect.com/science/article/pii/S0021915022001265?via%3Dihub#appsec1



Supplementary Figure 1A. Distribution of LDL-C PRS



Supplementary Figure 1B. Distribution of SBP PRS



Supplementary Figure 2. Organization of patients by both PRSs

Supplementary Table 2. Definitions of vascular outcomes

Outcome	Defined as
Myocardial infarction	• (Non-)fatal myocardial infarction defined by ≥2 of the following: • Acute chest pain for at least 20 min • ST-elevation >1 mm in two adjacent leads or a left bundle branch block (LBBB) on ECG • Elevated troponin or elevated CK ≥2 times the normal value of CK and a MB-fraction >5% of the total CK; Or; • Coronary artery bypass graft (CABG) or percutaneous coronary intervention (PCI) • Sudden death (unexpected cardiac death occurring within 1 hour after onset of symptoms, or within 24 hours given convincing circumstantial evidence)
Stroke	(Non-) fatal ischemic or hemorrhagic stroke: Relevant clinical features for at least 24 hours causing an increase in impairment of at least one grade of the modified Rankin scale, with or without a new infarction or hemorrhage on CT or MRI
Vascular mortality	Death from myocardial infarction, stroke, heart failure, or rupture of abdominal aortic aneurysm; vascular death from other causes; or sudden death (unexpected cardiac death occurring within 1 hour after onset of symptoms, or within 24 hours given convincing circumstantial evidence))
Major Adverse Cardiovascular Events (MACE)	Composite of the above mentioned outcomes
All-cause mortality	All deaths during follow-up, irrespective of the cause of death

Supplementary Table 3. Relation LDL-C polygenic risk score and LDL-C values per SD in patients with and without use of lipid-lowering medication

	All patients	**No lipid-lowering medication**	**Any lipid-lowering medication**	
	n=4416	**n=1581**	**n=2835**	
	Beta (mmol/L) per SD increase in PRS (95% CI)	Beta (mmol/L) per SD increase in PRS (95% CI)	Beta (mmol/L) per SD increase in PRS (95% CI)	*p*-interaction
Model I	0.11 (0.08 - 0.15)	0.22 (0.17 - 0.27)	0.16 (0.13 - 0.20)	
Model II	0.11 (0.08 - 0.14)	0.22 (0.17 - 0.27)	0.15 (0.12 - 0.19)	0.08
Model III	0.18 (0.15 - 0.21)	-	-	

Model 1: adjusted for age, sex, and the first 5 principal components
Model II: model I + additional adjustment for BMI, T2DM, smoking, alcohol use, systolic blood pressure, eGFR, triglycerides
Model III: model II + additional adjustment for lipid-lowering medication

Supplementary Table 4. Relation SBP polygenic risk score and SBP values per SD in patients with and without use of antihypertensive medication

	All patients	**No antihypertensive medication**	**Any antihypertensive medication**	
	n=4416	**n=1180**	**n=3236**	
	Beta (mmHg) per SD increase in PRS (95% CI)	Beta (mmHg) per SD increase in PRS (95% CI)	Beta (mmHg) per SD increase in PRS (95% CI)	*p*-interaction
Model I	3.18 (2.58 - 3.77)	3.85 (2.75 - 4.95)	3.01 (2.30 - 3.71)	
Model II	3.15 (2.56 - 3.74)	3.94 (2.85 - 5.03)	2.95 (2.24 - 3.65)	0.17
Model III	3.19 (2.60 - 3.78)	-	-	

Model 1: adjusted for age, sex, first 5 principal components
Model II: model I + additional adjustment for BMI, T2DM, smoking, alcohol use, LDL-cholesterol, eGFR, triglycerides
Model III: model II + additional adjustment for antihypertensive medication

Supplementary Table 5. LDL-C and SBP polygenic risk score and separate components of MACE

			LDL-C PRS	**SBP PRS**
			n=4416	**n=4416**
		Model	HR per SD (95% CI)	HR per SD (95% CI)
Myocardial infarction	# events		427	427
		I	1.04 (0.94 - 1.14)	1.04 (0.94 - 1.14)
		II	1.05 (0.96 - 1.16)	1.03 (0.94 - 1.13)
Stroke	# events		339	339
		I	0.98 (0.88 - 1.09)	0.99 (0.89 - 1.10)
		II	1.00 (0.90 - 1.12)	0.99 (0.89 - 1.10)
Vascular death	# events		714	714
		I	1.00 (0.93 - 1.08)	1.12 (1.04 - 1.20)
		II	1.05 (0.98 - 1.13)	1.11 (1.03 - 1.19)

Model I: adjusted for age, sex, and the first 5 principal components

Model II:

LDL-C PRS:

Model I + additional adjustment for BMI, T2DM, smoking, alcohol, eGFR, triglycerides, SBP and lipid-lowering medication

SBP PRS:

Model I + additional adjustment for BMI, T2DM, smoking, alcohol, eGFR, triglycerides, LDL-C, antihypertensive medication

Supplementary Table 6. Combined effect of LDL-C and SBP polygenic risk score and separate components of MACE

			LDL-C PRS < median, SBP PRS < median (Reference group)	**LDL-C PRS ≤ median, SBP PRS > median**	**LDL-C PRS > median, SBP PRS ≤ median**	**LDL-C PRS > median, SBP PRS > median**
			n=1123	**n=1085**	**n=1085**	**n=1123**
		Model	HR (95% CI)	HR (95% CI)	HR (95% CI)	HR (95% CI)
Myocardial infarction	# events		105	111	96	115
		I	Reference	1.08 (0.83 - 1.41)	0.94 (0.71 - 1.24)	1.09 (0.83 - 1.42)
		II	Reference	1.06 (0.81 - 1.39)	0.96 (0.73 - 1.27)	1.10 (0.84 - 1.44)
Stroke	# events		84	87	86	82
		I	Reference	1.05 (0.78 - 1.42)	1.11 (0.82 - 1.50)	1.00 (0.74 - 1.36)
		II	Reference	1.05 (0.77 - 1.42)	1.14 (0.84 - 1.54)	1.02 (0.75 - 1.39)
Vascular death	# events		184	200	144	186
		I	Reference	1.13 (0.93 - 1.38)	0.90 (0.72 - 1.12)	1.12 (0.91 - 1.37)
		II	Reference	1.11 (0.91 - 1.36)	0.97 (0.78 – 1.21)	1.14 (0.93 - 1.40)

Model I: adjusted for age, sex, and the first 5 principal components

Model II: Model I + additionally adjusted for BMI, T2DM, smoking, alcohol use, eGFR, triglycerides, lipid-lowering medication, antihypertensive medication

Supplementary Tables 7 and 8:
Please find these Supplementary Tables online with the following internet link:
https://www.sciencedirect.com/science/article/pii/S0021915022001265?via%3Dihub#appsec1

Supplementary Table 9. Combined LDL-C and SBP polygenic risk score (based on top quintile of both PRS) and recurrent cardiovascular events (non-fatal MI, non-fatal stroke and vascular death)

		Genetically lower LDL-C and SBP (Reference group)	**Genetically higher LDL-C and SBP (both scores in top quintile)**
		n=4244	**n=172**
		HR (95% CI)	HR (95% CI)
Recurrent cardiovascular events	# events	1156	42
		Reference	1.01 (0.74 - 1.37)

Model adjusted for age, sex, BMI, T2DM, smoking, eGFR, triglycerides, alcohol use, lipid-lowering medication, antihypertensive medication, and the first 5 principal components

Supplementary Table 10. Combined LDL-C and SBP polygenic risk score (based on top decile of both PRS) and recurrent cardiovascular events (non-fatal MI, non-fatal stroke and vascular death)

		Genetically lower LDL-C and SBP (Reference group)	**Genetically higher LDL-C and SBP (both scores in top decile)**
		n=4375	**n=41**
		HR (95% CI)	HR (95% CI)
Recurrent cardiovascular events	# events	1188	10
		Reference	0.94 (0.51 - 1.76)

Model adjusted for age, sex, BMI, T2DM, smoking, eGFR, triglycerides, alcohol use, lipid-lowering medication, antihypertensive medication, and the first 5 principal components

Supplementary Table 11. Sensitivity analyses with an LDL-C PRS excluding SNPs that were significantly associated with SBP (after correction for multiple testing)

	All patients	**No lipid-lowering medication**	**Any lipid-lowering medication**	
	n=4416	**n=1581**	**n=2835**	
	Beta (mmol/L) per SD increase in PRS (95% CI)	Beta (mmol/L) per SD increase in PRS (95% CI)	Beta (mmol/L) per SD increase in PRS (95% CI)	*p*-interaction
Model I	0.09 (0.06 – 0.12)	0.20 (0.15 - 0.25)	0.14 (0.11 – 0.17)	
Model II	0.10 (0.07 – 0.13)	0.21 (0.16 - 0.25)	0.13 (0.1 – 0.16)	0.05
Model III	0.16 (0.13 – 0.19)	-	-	

Model 1: adjusted for age, sex, and the first 5 principal components
Model II: model I + additional adjustment for BMI, T2DM, smoking, alcohol use, systolic blood pressure, eGFR, triglycerides.
Model III: model II + additional adjustment for lipid-lowering medication

Supplementary Table 12. Sensitivity analyses with an SBP PRS excluding SNPs that were significantly associated with LDL-C (after correction for multiple testing)

	All patients	**No antihypertensive medication**	**Any antihypertensive medication**	
	n=4416	**n=1180**	**n=3236**	
	Beta (mmHg) per SD increase in PRS (95% CI)	Beta (mmHg) per SD increase in PRS (95% CI)	Beta (mmHg) per SD increase in PRS (95% CI)	*p*-interaction
Model I	2.64 (2.05 – 3.24)	3.17 (2.07 – 4.26)	2.5 (1.79 – 3.21)	
Model II	2.59 (2.00 – 3.19)	3.12 (2.03 – 4.21)	2.47 (1.76 – 3.17)	0.36
Model III	2.63 (2.04 – 3.22)	-	-	

Model 1: adjusted for age, sex, first 5 principal components
Model II: model I + additional adjustment for BMI, type 2 diabetes mellitus, smoking, alcohol use, LDL-cholesterol, eGFR, triglycerides
Model III: model II + additional adjustment for antihypertensive medication

Supplementary Table 13. Sensitivity analyses evaluating the relation between an LDL-C and SBP PRS (excluding SNPs that were significantly associated with SBP or LDL-C after correction for multiple testing) and recurrent cardiovascular events

			LDL-C PRS	**SBP PRS**
			n=4416	**n=4416**
		Model	HR per SD increase in PRS (95% CI)	HR per SD increase in PRS (95% CI)
Recurrent cardiovascular events	#events		1198	1198
		I	1.01 (0.95 - 1.07)	1.04 (0.99 - 1.11)
		II	1.04 (0.98 - 1.10)	1.04 (0.98 - 1.10)

Model I: adjusted for age and sex, and the first five principal components

Model II:

LDL-C PRS:

Model I + additional adjustment for BMI, T2DM, smoking, alcohol use, eGFR, triglycerides, first 5 principal components, SBP, and lipid-lowering medication

SBP PRS:

Model I + additional adjustment for BMI, T2DM, smoking, alcohol use, eGFR, triglycerides, first 5 principal components, LDL-C, and antihypertensive medication

Supplementary Table 14. Sensitivity analyses evaluating the relation between the combined LDL-C and SBP polygenic risk score (excluding SNPs that were significantly associated with both SBP and LDL-C) and recurrent cardiovascular events

			LDL-C PRS ≤ median, SBP PRS < median (Reference group)	**LDL-C PRS ≤ median, SBP PRS > median**	**LDL-C PRS > median, SBP PRS ≤ median**	**LDL-C PRS > median, SBP PRS > median**
			n=1123	**n=1085**	**n=1085**	**n=1123**
		Model	HR (95% CI)	HR (95% CI)	HR (95% CI)	HR (95% CI)
Recurrent cardiovascular events	# events		295	320	280	303
		I	Reference	1.07 (0.91 - 1.26)	1.08 (0.92 - 1.27)	1.07 (0.91 - 1.26)
		II	Reference	1.06 (0.90 - 1.24)	1.14 (0.97 - 1.35)	1.10 (0.93 - 1.29)

Model I: adjusted for age, sex, and the first 5 principal components

Model II: Model I + additionally adjusted for BMI, T2DM, smoking, alcohol use, eGFR, triglycerides, lipid-lowering medication, antihypertensive medication

Chapter 5

The association between a genetic variant in the *SULF2* gene, metabolic parameters and vascular disease in patients at high cardiovascular risk

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In revision

Abstract

Background: Clearance of triglyceride rich lipoproteins (TRLs) is mediated by several hepatic receptors, including the low-density lipoprotein-receptor (LDL-R) and heparan sulfate proteoglycans (HSPGs). *SULF2* is an important gene in the regulation of HSPG. A specific single nucleotide polymorphism (SNP) in the *SULF2* gene, rs2281279, is associated with (postprandial) triglyceride (TG) levels and insulin resistance. Carrying at least one minor G allele in this SNP is associated with a favorable metabolic profile.

Aim: To determine the relationship between rs2281279, metabolic parameters and vascular events and type 2 diabetes mellitus (T2DM) in patients at high cardiovascular risk.

Methods: Patients (n=4386) at high cardiovascular risk from the UCC-SMART study were included. Patients were stratified according their rs2281279 genotype in three groups: 2438 patients had an AA genotype, 1642 patients had an AG genotype and 306 patients had a GG genotype. Effects of rs2281279 genotype on metabolic parameters and vascular events and T2DM were analyzed with linear regression and Cox proportional hazard models using an additive model.

Results: In 4386 patients there was no relation between rs2281279 genotype and triglycerides, non-HDL-cholesterol, insulin and quantitative insulin sensitivity check index. During a median follow-up of 11.8 years (IQR 9.3–15.5), 1026 non-fatal and fatal cardiovascular events and 320 limb events occurred. Presence of the G allele in rs2281279 did not affect the risk of vascular events (HR 1.03; 95%CI 0.94–1.14) or limb events (HR 0.92; 95%CI 0.77–1.10). In patients without diabetes at baseline (n=3289) 395 newly T2DM cases were diagnosed. Presence of the G allele in rs2281279 did not affect the risk of T2DM (HR 1.09; 95%CI 0.94–1.27).

Conclusions: Rs2281279 genotype is not associated with metabolic parameters, including TRL metabolism and does not increase the risk of vascular events or T2DM in patients at high risk for cardiovascular disease.

Introduction

Postprandial hypertriglyceridemia is a hallmark of metabolic syndrome and type 2 diabetes mellitus (T2DM) and an independent risk factor for cardiovascular disease (CVD).[1,2] In the postprandial phase, triglycerides are mainly present in chylomicrons, very-low-density lipoproteins (VLDL) and their highly atherogenic remnant lipoproteins (collectively called triglyceride rich lipoproteins (TRLs)). Important determinants of TRL metabolism are lipoprotein lipase (LPL) mediated lipolysis and clearance by several hepatic receptors. LPL is a key enzyme for lipolysis of triglycerides (TGs) to nonesterified fatty acids.[3] Clearance of TRLs by the liver is achieved by receptors located in the space of Disse, comprising the low-density lipoprotein receptor (LDL-R), LDLR-related protein (LRP) and heparan sulfate proteoglycans (HSPGs).[4] HSPGs are a diverse group of proteoglycans and are involved in several physiological processes throughout the body.[5] Syndecan-1 is the primary HSPG that mediates clearance of TRLs in the liver.[6,7] It contains a single transmembrane protein to which sugar polymers are attached, heparan sulfates, that bind TRLs and subsequently clear them from the circulation.[6]

A study in mice identified that dysregulation of the heparan sulfate glucosamine-6-O-endosulfatase-2 (SULF2)-gene disrupts HSPG structure.[8] This gene encodes the sulf2 enzyme that decreases the sulfation grade of the heparan sulfate chains by removing essential 6-O sulfate residues, thereby preventing binding and clearance of TRLs from the circulation by HSPGs. In obese and diabetic mice, sulf2 overexpression results in TRL accumulation in plasma.[8,9] Furthermore, liver biopsies from obese subjects showed a significant positive association between sulf2 mRNA expression and fasting plasma TG levels.[10] The rs2281279 single nucleotide polymorphism (SNP) in the *SULF2* gene (with an estimated allele frequency of 28% Europeans[11]) was evaluated in different studies, showing conflicting results with regard to metabolic parameters and CVD.[9,10,12-14]

Proper HSPG function is particularly relevant in subjects homozygous for the ε2 allele in the *APOE* gene, approximately 1% of the general population.[15] Compared to other *APOE* genotypes, the ε2ε2 genotype results in a decreased affinity of Apolipoprotein E2 (<2%) to the LDL-R, thereby greatly impairing TRL clearance through this receptor.[16] Binding of ApoE2 protein to HSPG is also decreased but the remaining binding capacity is considered sufficient for adequate TRL uptake.[17] Subjects with the ε2ε2 genotype are at risk to develop Familial Dysbetalipoproteinemia (FD), a highly atherogenic disorder characterized by (postprandial) TRL accumulation and premature cardiovascular disease.[18] For the development of FD a second metabolic

hit – notably adiposity or insulin resistance – is required. It is hypothesized that this is caused by overexpression of sulf2, causing degradation of HSPG, which is the critical remnant clearance receptor in subjects with an ε2ε2 genotype.[19]

In the present study in patients at high cardiovascular risk, we aim to evaluate whether presence of the minor G allele of rs2281279, is associated with favorable metabolic parameters and a decrease in the risk of vascular events and T2DM. Furthermore we aim to evaluate whether *APOE* genotype modifies the relation between the presence of the minor allele G in rs2281279 and metabolic parameters.

Methods

Study population

Patients originated from the Utrecht Cardiovascular Cohort – Second Manifestations of Arterial Disease (UCC-SMART) study. The UCC-SMART study is an ongoing, single-center, prospective cohort study including newly referred patients to the University Medical Center Utrecht from 18 years of age with established cardiovascular disease or cardiovascular risk factors. A description of the study protocol has been published elsewhere.[20] The UCC-SMART study was approved by the local Medical Ethics Committee and written informed consent was obtained from all patients. To date, in total 13.667 patients are included in the UCC-SMART study. For the present study, data were used of all patients (patients with either established cardiovascular disease or patients with hypertension, T2DM or dyslipidemia) enrolled in the UCC-SMART study between September 1996 and August 2010, because patients included in this period were genotyped (n=6970).

Vascular screening at baseline

After inclusion, all patients underwent a vascular screening protocol, including health questionnaires, physical examination, laboratory testing, ankle-branchial index, and abdominal, aortic and carotid ultrasound. Vascular disease at baseline was defined as presence of coronary artery disease (CAD), cerebrovascular disease (CeVD), peripheral arterial disease (PAD) or abdominal arterial aneurysm (AAA). For definitions of these types of CVD at baseline see *Supplementary Table 1*. T2DM was defined as either a referral or self-reported diagnosis of T2DM, or a fasting plasma glucose ≥7 mmol/L at study inclusion with initiation of glucose-lowering treatment within 1 year, or baseline use of hypoglycemic agents or insulin. Medication use, smoking and alcohol use were self-reported. Lipid-lowering medication included use of statins, fibrates, bile acid sequestrants or nicotinic acid at baseline. Prescription of high intensity statins was

defined as atorvastatin ≥40 mg or rosuvastatin ≥20 mg. Patients with hypertension were defined as those who were prescribed antihypertensive medication and/or had an office systolic BP of ≥140 or diastolic BP of ≥90 mmHg. Obesity was defined as an BMI ≥30 kg/m^2. Metabolic syndrome (MetS) was defined according to the National Cholesterol Education Program Adult Treatment Panel III definition.[21]

Imputation of rs2281279

A subset of the total UCC-SMART cohort was included in this study (n=5959). Samples were genotyped using the Illumina Infinium Global Screening Array and extensive sample and SNP quality control was conducted according to community standards to remove low quality samples and SNPs. In addition, only samples from European descent were kept, and related samples were excluded by keeping only a single sample from each pair. Since the SNP of interest (rs2281279) was not genotyped in this cohort, imputation of this SNP was performed using the 1000 Genomes Project (1KG) phase 3 and Genome of the Netherlands Consortium (GoNL) v5 as reference panels (PMID: 24974849, 20981092). SHAPEIT2 was used for phasing and IMPUTE2 for subsequent imputation. Imputation quality (info score, r^2) of this SNP was 0.836. Genotype probabilities were transformed to best-guess genotypes using a cut-off of 0.1 (from 0, 1 and 2). For this cut-off 10% uncertainty was considered acceptable. This cut-off is also used in standard GWAS software (PLINK and OCTOOL/SNPTEST). Patients with a high probability of having an AA, AG or GG genotype for rs2281279 (imputation value ≤0.10 (AA), value between >0.90 and ≤1.10 (AG) and value >1.90 (GG)) were included. Patients with a low probability (genotype probability between >0.10 and ≤0.90 and between >1.10 and ≤1.90 were excluded (n=1573), resulting in 4386 patients eligible for the analyses.

Laboratory measurements

Baseline lipid levels were obtained in a fasting state. Total cholesterol (TC), triglycerides (TG) and glucose were measured with a commercial enzymatic dry chemistry kit (Johnson & Johnson, New Brunswick, USA). HDL-cholesterol (HDL-C) was measured with a commercial enzymatic kit (Boehringer, Mannheim, Germany) and LDL-cholesterol (LDL-C) was calculated using the Friedewald formula up to triglyceride levels of 9 mmol/L.[22,23] Non-HDL-cholesterol (Non-HDL-C) was calculated as fasting TC minus HDL-C. From 2003 onwards, insulin was measured with an immunometric technique on an IMMULITE 1000 Analyzer (Diagnostic Products Corporation, LA, USA). Prior to 2003, insulin was not measured. The quantitative insulin sensitivity check index (QUICKI) was used to express insulin sensitivity. It was calculated using the formula: 1/(log(insulin in mU/L) minus log(glucose in mg/dL)).[24] In patients without T2DM, the Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) was calculated as follows: Glucose(in mmol/L) * insulin (in mU/L) / 22.5.[25]

Follow-up

During follow-up patients received questionnaires to evaluate possible cardiovascular events twice a year. Outcomes of interest for this study was the combination of non-fatal and fatal vascular events, limb events and T2DM. T2DM was not routinely assessed prior to July 2006. All patients without diabetes mellitus at baseline that were included before July 2006 received a questionnaire in 2006, to assess if they had been diagnosed with T2DM since inclusion. If an event was reported, hospital discharge letters, relevant laboratory results and radiologic examinations were collected and the event was classified independently by three physicians of the UCC-SMART endpoint committee. For detailed definitions of outcomes see *Supplementary Table 2*. Follow-up was defined as time from inclusion until development of first event, death, loss to follow-up or the preselected date of 1 July 2019.

Data analyses

To prevent loss of statistical power and potential bias,[26] missing data were imputed by single regression imputation. Missing values were <1.0%, except for use of high intensity statins (10%) and waist- and hip circumference (16%). Insulin was not imputed because this was not measured before 2003, therefore 1919 (44%) patients had missing insulin, and consequently also QUICKI, values. Linear regression models were fitted to determine the cross-sectional association between rs2281279 genotype and triglycerides, non-HDL-C, insulin and QUICKI, with adjustment for age and sex. For triglycerides and insulin linearity was obtained after log transformation. Effect modification by vascular disease was tested and rejected (p=0.69) by adding an interaction term to the models.

Cox proportional hazard models were fitted to determine the effect of rs2281279 genotype on cardiovascular events, limb events and T2DM. Restricted cubic splines showed a linear relationship between rs2281279 genotype and outcomes. The Cox proportional hazard assumption was visually checked by plotting Schoenfeld residuals against time. Cox models were adjusted for age and sex. Potential effect modification by presence of vascular disease at baseline was tested (p=0.48). For vascular events data from 4386 patients were used. For the T2DM event data from 3289 patients without DM at baseline for whom follow-up data on T2DM were available were used. For all regression analyses an additive model with rs2281279 genotype as continuous determinant was used an wild type (AA) was used as the reference category. For all analyses, a *p*-value of <0.05 was considered significant. Analyses were performed using RStudio (version 3.5.1, R Foundation for Statistical Computing, Vienna, Austria).

Sensitivity analyses

To evaluate whether the association between the rs2281279 genotype, metabolic parameters and outcomes was influenced by presence of T1DM, hypertriglyceridemia (TG >9 mmol/L) or vascular disease at baseline the analyses were repeated excluding these specific subgroups. In order to increase certainty with regard to the rs2281279 genotype a stricter cut-off (0.05 instead of 0.10) to transform genotype probabilities to best-guess genotypes was used (*Supplementary Table 4* and *5*). The effect of rs2281279 was also evaluated in patients stratified for T2DM status at baseline (*Supplementary Table 6* and *7*). Furthermore, the effect of potential effect modifiers (BMI, age, sex, hypertension, metabolic syndrome) in the relation between rs2281279 genotype and (log)triglycerides and cardiovascular events was examined.

***SULF2* genotype and *APOE* genotype**

Descriptive analyses are provided for patients with either an *APOE* ε2ε2 or ε3ε3 genotype in combination with an AA or GG genotype for rs2281279. We hypothesize that an ε2ε2 genotype in combination with an AA genotype in rs2281279 might mimic an FD model, because in that case both TRL clearing pathways are not properly functioning. Patients with an ε2ε2 genotype in combination with a GG genotype in rs2281279 G might in that case mimic 'healthy' ε2ε2 subjects, ε3ε3-subjects in combination with an AA genotype of rs2281279 might mimic decreased HSPG function only, and finally, an ε3ε3 genotype and an GG genotype in rs2281279 might mimic a healthy model with two functioning TRL clearance pathways.

Results

Baseline characteristics

In total 4386 patients at high risk for cardiovascular disease were included. Baseline characteristics stratified for rs2281279 genotype are shown in *Table 1*. Mean age was 57.9 ± 12.2 years and 69% were male, 45% had a history of CAD, 16% of CeVD, 20% of PAD and 7% of AAA. Furthermore, 16% of the patients had T2DM and 50% fulfilled the criteria for metabolic syndrome. The AA genotype was present in 2438 (55%) patients, and 1642 patients (37%) had an AG genotype and 306 patients (7%) had a GG genotype. The minor allele frequency was 26% and comparable with the frequency earlier reported in the European population. There were no relevant differences at baseline across rs2281279 genotypes, including age and sex. There were also no differences in use of lipid lowering medication, including use of high intensity statins, across the three groups.

Table 1. Baseline characteristics according to rs2281279 genotype

	AA **n=2438**	**AG** **n=1642**	**GG** **n=306**	**All patients** **n=4386**
Male sex (n, %)	1687 (69%)	1103 (67%)	218 (72%)	3008 (69%)
Age	58.0 ± 12.1	57.5 ± 12.4	58.8 ± 11.5	57.9 ± 12.2
Type of vascular disease				
• Coronary heart disease (n, %)	1106 (45%)	733 (45%)	147 (48%)	1986 (45%)
• Peripheral vascular disease (n, %)	399 (16%)	245 (15%)	56 (18%)	700 (16%)
• Cerebrovascular disease (n, %)	481 (20%)	323 (20%)	65 (21%)	869 (20%)
• Abdominal aortic aneurysm (n, %)	166 (7%)	117 (7%)	15 (5%)	298 (7%)
Diabetes mellitus type 2 (n, %)	409 (17%)	261 (16%)	48 (16%)	718 (16%)
Medication use				
• Lipid lowering treatment	1368 (56%)	891 (54%)	180 (59%)	2439 (56%)
• High intensity statins	120 (5%)	91 (6%)	17 (6%)	228 (5%)
• Antihypertensive treatment	1631 (67%)	1087 (66%)	206 (67%)	2924 (67%)
• Platelet inhibitors	1418 (58%)	957 (58%)	191 (62%)	2566 (59%)
Current smoking	776 (32%)	534 (33%)	101 (33%)	1411 (32%)
Current alcohol consumption	1215 (50%)	790 (48%)	157 (51%)	2162 (49%)
Body Mass Index (kg/m^2)	26.8 ± 4.3	26.5 ± 4.2	26.9 ± 4.5	26.7 ± 4.3
Waist circumference (cm)	95 ± 13	94 ± 12	95 ± 13	94 ± 12
Waist-to-hip ratio	0.91 ± 0.08	0.90 ± 0.09	0.91 ± 0.09	0.91 ± 0.08
Metabolic syndrome	1226 (50%)	817 (50%)	168 (55%)	2211 (50%)
Systolic blood pressure (mmHg)	142 ± 21	141 ± 22	141 ± 22	141 ± 22
Diastolic blood pressure (mmHg)	83 ± 12	83 ± 12	83 ± 12	83 ± 12
Laboratory values				
• Total cholesterol (mmol/L)	5.17 ± 1.45	5.17 ± 1.36	5.19 ± 1.39	5.17 ± 1.41
• HDL-cholesterol (mmol/L)	1.23 ± 0.39	1.24 ± 0.39	1.21 ± 0.33	1.23 ± 0.38
• Non-HDL-cholesterol (mmol/L)	3.93 ± 1.46	3.94 ± 1.37	3.98 ± 1.38	3.94 ± 1.42
• LDL-cholesterol (mmol/L)	3.14 ± 1.23	3.15 ± 1.20	3.19 ± 1.26	3.15 ± 1.22
• Triglycerides (mmol/L)	1.40 (1.00 - 2.10)	1.44 (1.00 - 2.10)	1.41 (1.00 - 2.30)	1.40 (1.00 - 2.10)
• Insulin (mU/L)	9 (6 - 14)	9 (6 - 15)	9 (6 - 15)	9 (6 - 14)
• Glucose (mmol/L)	6.3 ± 2.1	6.3 ± 2.0	6.4 ± 1.8	6.3 ± 2.0

HDL; high-density lipoprotein. LDL; low-density lipoprotein. All data in n (%), mean with standard deviation or median (interquartile range).

Relation between rs2281279 and metabolic parameters

Fasting triglyceride levels were not different across genotypes (AA 1.40 (1.00–2.10) mmol/L; AG 1.44 (1.00–2.10) mmol/L; GG 1.41 (1.00–2.30), p=0.52). There was no effect of rs2281279 genotype on (log) triglyceride levels (β 0.004; 95%CI -0.008–0.016) or non-HDL-C (β 0.013 95%CI; -0.054–0.080). There was also no effect on (log) insulin levels (β -0.008; 95%CI; -0.026–0.010) and QUICKI (β 0.001; 95%CI -0.001–0.003) (*Table 2*). Estimates did not change in an unadjusted model or in a model additionally adjusted for BMI, use of lipid lowering medication, SBP, smoking and T2DM.

Table 2. Additive effect of presence of rs2281279 (G allele) on metabolic parameters (n=4386)

	Beta (95%CI)
(log)Triglycerides	0.004 (-0.008 - 0.016)
Non-HDL-cholesterol	0.013 (-0.054 - 0.080)
(log)Insulin[a]	-0.008 (-0.026 - 0.010)
QUICKI[a]	0.001 (-0.001 - 0.003)

HDL; high-density lipoprotein, QUICKI; quantitative insulin sensitivity check index, CI; confidence interval.

[a] n=2467

Model adjusted for age + sex.

Relation between rs2281279 and risk of vascular events and T2DM

In patients at high cardiovascular risk (n=4386), median follow-up was 11.8 (IQR 9.3–15.5) years in which 1026 cardiovascular events and 320 limb events occurred. Rs2281279 genotype did not affect the risk of (recurrent) cardiovascular events (HR 1.03; 95%CI 0.94–1.14) or (recurrent) limb events (HR 0.92 (0.77–1.10) compared to patients with an AA genotype. In patients without diabetes at baseline for whom follow-up data were available (n=3289), 395 newly T2DM cases were diagnosed during a median follow-up of 12.3 (IQR 10.0–16.0) years. Rs2281279 genotype did not change the risk (HR 1.09; 95%CI 0.94–1.27) for T2DM during follow-up (*Table 3*). Results were similar in an unadjusted model and in a model additionally adjusted for BMI, use of lipid lowering medication, SBP, smoking and T2DM.

Table 3. Additive effect of presence of rs2281279 (G allele) on vascular events and T2DM

	Number of patients	**Number of events (%)**	**HR (95%CI)**
Cardiovascular events	4386	1026 (23%)	1.03 (0.94 – 1.14)
Limb events	4386	320 (7%)	0.92 (0.77 – 1.10)
T2DM	3289	395 (12%)	1.09 (0.94 – 1.27)

T2DM; type 2 diabetes mellitus HR; hazard ratio, CI; confidence interval.

Model adjusted for age + sex.

Sensitivity analyses

Analyses when excluding patients with T1DM (n=96), patients with triglycerides >9 mmol/L (n=36) or patients without vascular disease at baseline (n=1144) did not change the results. Furthermore, when using a stricter cut-off of 0.05 to transform genotype probabilities to best-guess genotypes, 3930 patients (AA; n=2225, AG; n=1445, GG; n=260) were included. Using this cut-off did not change the results (*Supplementary Table 3* and *4*). T2DM was an effect modifier in the relation between rs2281279 genotype and (log)triglycerides (p for interaction 0.04). The effect of rs2281279 genotype on (log)triglycerides in patients without T2DM was β 0.010 (95%CI; -0.002–0.023) and in patients with T2DM β -0.020 (95%CI; -0.051–0.011). Median TG levels in patients without T2DM in combination with an GG genotype (n=258) were 1.40 (IQR 1.00–2.01) and TG levels in patients with T2DM and an GG genotype (n=48) were 1.74 (IQR 1.28–2.33). There was no interaction of T2DM status in the relation between rs2281279 genotype and non-HDL-C, (log)insulin or QUICKI. In addition, there was no interaction with T2DM in the relation between rs2281279 and vascular events (*Supplementary Table 5* and *6*). Furthermore, there was no interaction with BMI, age, sex, hypertension and MetS in the relationship between rs2281279 genotype and (log)TG and rs2281279 genotype and cardiovascular events, respectively (p for all interactions >0.05).

Association between *SULF2* genotype and *APOE* genotype

The SULF AA + ε2ε2 group included 29 patients and the SULF GG + ε2ε2 group consisted of 4 patients. Comparing these ε2ε2 subgroups, there was a remarkable difference in systolic blood pressure (149 ± 22 mmHg in SULF AA *versus* 135 ± 6 mmHg in SULF GG), total cholesterol (in SULF AA 6.20 ± 3.52 mmol/L *versus* 4.78 ± 1.58 mmol/L in SULF GG), triglycerides (2.48 (IQR 1.75–4.11) mmol/L in SULF AA *versus* 2.31 (IQR 1.85–2.68) mmol/L in SULF GG), apoB levels (0.52 ± 0.16 g/L in SULF AA *versus* 0.67 ± 0.11 g/L in SULF GG) and use of lipid lowering therapy (69% in SULF AA *versus* 25% in SULF GG) (*Supplementary Table 7*). The SULF2 AA + ε3ε3 group included 1318 patients and the SULF GG + ε3ε3 included 166 patients. There were no important differences between the ε3ε3 subgroups. The most important differences when comparing the two ε2ε2 subgroups with the two ε3ε3 subgroups were age, BMI and plasma apoB and TG levels.

Discussion

The present study, in 4386 patients at high cardiovascular risk, demonstrates that rs2281279 genotype is not associated with metabolic parameters, including TRL metabolism and insulin resistance, and does not increase the risk for vascular events or T2DM. These results were similar in patients with or without T2DM and there was no effect of age, sex, BMI, hypertension or MetS in these relationships

Rs2281279 is a SNP in the *SULF2* gene that codes for the sulf2 enzyme, which causes degradation of HSPGs in the liver and increased TRL in the plasma. The effect of the sulf2 enzyme was first investigated in mice. This study showed that livers from T2DM mice compared to livers from control mice expressed an eleven-fold increase in sulf2 mRNA levels, and that sulf2 overexpression results in TRL accumulation in the circulation.[8,9] In addition, it was demonstrated that use of antisense oligonucleotides, selectively inhibiting hepatic sulf2 mRNA expression completely abolished postprandial hypertriglyceridemia in these diabetic mice.[9] Also in obese subjects, it was shown in liver biopsies that sulf2 expression was significantly associated with increased fasting plasma TG levels and an increased HOMA-IR,[10] making sulf2 an attractive target for intervention.

The association of rs2281279 with fasting TG levels was first identified in a cohort comprising 210 patients with T2DM and subsequently replicated in an independent cohort consisting of 1308 patients with T2DM.[10] These associations, however, were unadjusted, and in both cohorts only borderline significant. Subsequent studies that investigated the relation between this SNP and metabolic outcomes in several study populations showed varying and conflicting results. In 29 obese patients with T2DM, as well as in 68 normolipidemic healthy subjects it was found that carrying at least one minor G allele was associated with a significantly lower postprandial TG response.[10,12] In addition, both studies found significant differences in fasting glucose and TG levels according to rs2281279 status. However, the analyses in obese and T2DM patients were unadjusted, had a small sample size and the study in healthy subjects combined the hetero- and homozygotes for the minor G allele. In contrast, another study in 165 non-diabetic subjects from a population based cohort found no differences in both fasting TG levels and postprandial TG response according to rs2281279 genotype.[14] In addition, a Finnish prospective cohort consisting of 339 subjects with hypertension and 441 controls found no association between rs2281279 genotype and any of the variables (including lipids and hypertension).[13] Analyses in patients with obesity or hypertension did not change the results in this study. The results of the present study are in line with the two largest studies evaluating the metabolic effects of this specific SNP in the *SULF2* gene demonstrating that there was no effect of rs2281279 on metabolic parameters. Although not significant, there is a negative trend for the additive effect of presence of the G allele for metabolic parameters in patients with T2DM.

There are two potential explanations for these findings. First, it could be hypothesized that rs2281279 has limited effect on HSPG related metabolic changes, this may be the case if the HSPG functions as a low affinity but high capacity mechanism of the clearance of remnant lipoproteins. In addition, given the frequency of the minor allele G

in rs2281279 of 28% in Europeans[11] (meaning that 72% carry at least one A allele), that this A allele alone is not (very) pathogenic because it is very common in the general population.[27] In general, when one SNP has limited effects on metabolic parameters and outcomes, genetic risk scores (combine the modest effects of multiple SNP) provide more information and are increasingly used.[28] Rs2281279 however is the only studied SNP in *SULF2* in association with TRL metabolism.

Third, it could be hypothesized that HSPG itself has limited effect in TRL metabolism. One small study in humans evaluated the potential additive effect of HSPG on LDL-R in postprandial TRL clearance. The postprandial response in 11 healthy controls *versus* 22 patients with heterozygous loss of function variants in the *LDLR* gene (patients with Familial Hypercholesterolemia) was compared.[29] Patients with FH were stratified according to a HSPG genetic risk score, consisting of variants in genes affecting HSPG synthesis, but the *SULF2* gene was not included. The authors found no difference between the 2 FH groups in the postprandial TG response (incremental area under the curve (iAUC)), but did find significantly increased postprandial retinyl ester response (iAUC) in FH subjects with a high HSPG genetic score, suggesting a delay in TRL clearance in patients with many unfavorable variants in HSPG genes.[29] Based on these results the authors concluded that there is a minor, yet additive role of HSPG on the LDL-R in postprandial TG clearance in humans. This hypothesis is not supported by other studies that clearly demonstrate that HSPGs play a substantial role in the hepatic clearance of TRLs in human. This becomes particularly apparent in the evaluation of pathogenic variants in the *APOE* gene. Pathogenic variants located in the HSPG binding domain of the *APOE* gene lead to impaired binding of the ApoE protein to HSPG at hepatic cell surface. It has been shown that the impaired HSPG binding is associated with the severity of remnant accumulation in FD.[17,30] Moreover, pathogenic variants in the HSPG binding domain in the *APOE* gene are generally associated with a dominant pattern of inheritance and higher penetrance of FD compared to the homozygous ε2 variant in the *APOE* gene, characterized by decreased LDL-R binding only, which is recessively inherited.[19]

A second aim of this study was to evaluate whether *APOE* genotype modifies the relation between rs2281279 genotype and metabolic parameters. Therefore patients with either an SULF AA or GG and ε2ε2 and ε3ε3 genotype were stratified. Due to the rarity of the ε2ε2 genotype, it was only possible to provide descriptive statistics. In addition, these analyses were under the assumption that genotype serves as a proxy for (dys)function of the HSPG receptor, which is not proven. Furthermore, subjects were all included in the UCC-SMART cohort, and therefore do not reflect healthy subjects from the general population. Although speculative, involving only a small subgroup of

ε2ε2 genotypes, it seems that the SULF AA + ε2ε2 group compared to the SULF GG + ε2ε2 group had a less favorable profile, reminiscent of an FD metabolic profile, possibly due to an interaction of the *SULF2* genotype with the ε2 allele.

A previous study in Turkish people evaluated several SNPs in the *GLCE* (glucuronic acid epimerase) gene, another HSPG biosynthesis enzyme, and its interaction with the ε2 allele in the *APOE* gene. In this study the authors used the ε2 allele as a proxy for decreased HSPG function. The authors found that the association between these SNPs in *GLCE* and TG and HDL-C in subjects with an ε2ε3 genotype was stronger compared to subjects with an ε3ε3 or ε3ε4 genotype.[31] The ε2 allele indeed has a reduced binding affinity of 40% for the HSPG compared to ε3, but this is considered sufficient for adequate clearance of TRLs.[17]

Previous studies showed that TRL accumulation has a strong relationship with CVD in general and peripheral artery disease in particular.[32,33] However, in this study, rs2281279 was not associated with vascular events, including limb events, or T2DM. A Finnish cohort consisting of healthy controls and subjects with hypertension, also found no association for rs2281279 with CVD outcomes (cerebrovascular events and ischemic heart disease) during follow-up of approximately 10 years.[13] This could be due to the fact that the effect of rs2281279 on TRL is too small to render clinically relevant effects on CVD, or because, like in our study, there is no effect of the rs2281279 on TRL in first place.

The strengths of this prospective cohort study include the large number of patients at high CVD risk and the long follow-up. This is the largest cohort evaluating the metabolic effects of rs2281279 as well as its effects on vascular events.

Several limitations of this study need to be considered. First, for the assessment of the genotype of rs2281279 imputation was used. However, this is a generally acceptable method in genetics[34] and the imputation quality of this variant was high (0.836). In addition, sensitivity analyses considering a more strict cut-off to assign best-guess genotypes did not change the results. Second, in this cohort there is no information on postprandial responses. Nonetheless, fasting TG and presence of T2DM are predictors of postprandial hypertriglyceridemia and can therefore be used as a proxy for postprandial response.[35] Third, in this study it was assumed that genotype of rs2281279 was as a proxy for (dys)function of HSPG, but this is not substantiated by functional tests. Therefore levels of the sulf2 enzyme or tests into HSPG function itself would provide more insight in the effect of this SNP in the *SULF2* gene on metabolic parameters. Fourth, it cannot be ruled out that the sample size used in this study is still too small to detect an effect of this specific SNP.

In conclusion, in patients with a high cardiovascular risk, rs2281279 genotype was not associated with metabolic parameters, including TRL metabolism. In addition, rs2281279 was not related with an increased risk of vascular events or development of T2DM.

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Supplementary Material

Supplementary Table 1. Definitions of CVD at baseline

CVD at baseline	Definition
Coronary artery Disease (CAD)	• Myocardial infarction • Angina pectoris • Coronary stenosis in ≥1 major coronary artery • Self-reported history of MI, cardiac arrest or revascularization
Peripheral artery Disease (PAD)	• Fontaine stage of at least IIa (i.e. intermittent claudication and resting ankle-brachial index (ABI) <0.9 in at least one leg) • Self-reported history of amputation or vascular surgery of the lower extremities
Cerebrovascular Disease (CeVD)	• Transient ischemic attack • Ischemic stroke • Amaurosis fugax • Retinal infarction • Self-reported stroke or carotid artery operation in the past
Abdominal aortic aneurysm (AAA)	• Aneurysm of the abdominal aorta (distal aortic diameter ≥3 cm) during screening • AAA surgery in the past

Supplementary Table 2. Definitions of outcomes

Outcome	Definition
Cardiovascular events	• Nonfatal myocardial infarction • Nonfatal stroke (ischemic/hemorrhagic) • Retinal infarction/hemorrhage • Vascular death (death from myocardial infarction, stroke, heart failure, or rupture of abdominal aortic aneurysm; vascular death from other causes; or sudden death (unexpected cardiac death occurring within 1 hour after onset of symptoms, or within 24 hours given convincing circumstantial evidence))
Limb events	• Major amputation (at level of the foot or more proximal) • Lower limb revascularization (vascular intervention or thrombolysis)
Type 2 diabetes mellitus (T2DM)	• A self-reported diagnosis and/or the use of glucose-lowering agents. Patients who reported new-onset T2DM were sent an additional questionnaire for confirmation and detailed information of the diagnosis, including the date of diagnosis, initial and current treatment, and family history of diabetes

Supplementary Table 3. Additive effect of presence of rs2281279 (G allele) on metabolic parameters with cut-off 0.05 (n=3930)

	Beta (95%CI)
(log)Triglycerides	0.002 (-0.010 - 0.015)
Non-HDL-cholesterol	0.012 (-0.060 - 0.083)
(log)Insulin[a]	-0.011 (-0.031 - 0.008)
QUICKI[a]	0.002 (-0.001 - 0.004)

HDL; high-density lipoprotein, QUICKI; quantitative insulin sensitivity check index, CI; confidence interval.
[a] n=2202.
Model adjusted for age + sex.

Supplementary Table 4. Additive effect of presence of rs2281279 (G allele) on vascular events and T2DM with cut-off 0.05

	Number of patients	**Number of events (%)**	**HR (95%CI)**
Cardiovascular events	3930	933 (24%)	1.07 (0.97 – 1.19)
Limb events	3930	281 (7%)	0.96 (0.79 – 1.16)
T2DM	2945	357 (12%)	1.08 (0.92 – 1.28)

T2DM; type 2 diabetes mellitus HR; hazard ratio, CI; confidence interval.
Model adjusted for age + sex.

Supplementary Table 5. Additive effect of rs2281279 (G allele) on metabolic parameters, stratified for T2DM status at baseline (n=4386)

	In patients without T2DM n=3668 Beta (95%CI)	**In patients with T2DM n=718 Beta (95%CI)**	***P*-value for interaction**
(log)Triglycerides	0.010 (-0.002 – 0.023)	-0.020 (-0.051 – 0.011)	0.04
Non-HDL-cholesterol	0.022 (-0.049 – 0.094)	-0.019 (-0.198 – 0.159)	0.52
(log)Insulin[a]	-0.007 (-0.027 – 0.013)	-0.011 (-0.056 – 0.033)	0.88
QUICKI[a]	0.001 (-0.002 – 0.003)	0.002 (-0.003 – 0.008)	0.74
(log)HOMA-IR[a]	0.009 (-0.032 – 0.014)	NA	NA

T2DM; type 2 diabetes mellitus, HDL; high-density lipoprotein, QUICKI; quantitative insulin sensitivity check index, HOMA-IR; Homeostatic Model Assessment for Insulin Resistance, CI; confidence interval.
[a] Patients without T2DM (n=2063) and patients with T2DM (n=404)
Model adjusted for age + sex

Supplementary Table 6. Additive effect of presence of rs2281279 (G allele) on vascular events, stratified for T2DM status at baseline (n=4386)

	In patients without T2DM		**In patients with T2DM**		***P*-value for interaction**
	n=3668		**n=718**		
	Number of events (%)	HR (95%CI)	Number of events (%)	HR (95%CI)	
Cardiovascular events	802 (22%)	1.00 (0.90 – 1.12)	224 (31%)	1.16 (0.94 – 1.42)	0.25
Limb events	237 (6%)	0.93 (0.76 – 1.15)	83 (12%)	0.89 (0.62 – 1.27)	0.85

HR; hazard ratio; CI; confidence interval. Model adjusted for age + sex.

Supplementary Table 7. Baseline characteristics, stratified for *SULF2* and *APOE* genotype

	SULF2 AA + ε2ε2 n=29 HSPG – / LDLR – FD model	**SULF2 GG + ε2ε2 n=4 HSPG + / LDLR – 'Healthy' ε2ε2 model**	**SULF2 AA + ε3ε3 n=1318 HSPG – / LDLR + Decreased HSPG**	**SULF2 GG + ε3ε3 n=166 HSPG + / LDLR + Healthy model**
Male sex (n, %)	18 (62%)	3 (75%)	907 (69%)	116 (70%)
Age	56.8 ± 14.5	52.7 ± 20.7	58.1 ± 12.3	58.5 ± 11.6
Vascular disease (n,%)	20 (69%)	2 (50%)	989 (75%)	128 (77%)
Diabetes mellitus type 2 (n, %)	4 (14%)	0 (0%)	221 (17%)	34 (20%)
Medication use				
• Lipid lowering treatment	20 (69%)	1 (25%)	744 (56%)	95 (57%)
• High intensity statins	1 (3%)	0 (0%)	69 (5%)	10 (6%)
Current smoking	11 (38%)	1 (25%)	403 (31%)	51 (31%)
Current alcohol consumption	9 (31%)	1 (25%)	646 (49%)	77 (46%)
Body Mass Index (kg/m^2)	28.3 ± 5.5	32.0 ± 12.9	26.9 ± 4.23	26.9 ± 4.3
Waist circumference (cm)	98 ± 14	95 ± 22	95 ± 12	95 ± 12
Waist-to-hip ratio	0.93 ± 0.11	0.85 ± 0.07	0.91 ± 0.08	0.92 ± 0.09
Metabolic syndrome	17 (59%)	2 (50%)	684 (52%)	93 (56%)
Systolic blood pressure (mmHg)	149 ± 22	135 ± 6	141 ± 21	141 ± 21
Diastolic blood pressure (mmHg)	86 ± 12	76 ± 12	82 ± 12	83 ± 11
Laboratory values				
• Total cholesterol (mmol/L)	6.20 ± 3.52	4.78 ± 1.58	5.15 ± 1.42	5.26 ± 1.37
• HDL-cholesterol (mmol/L)	1.39 ± 0.53	1.16 ± 0.12	1.24 ± 0.39	1.21 ± 0.35
• Non-HDL-cholesterol (mmol/L)	4.81 ± 3.62	3.62 ± 1.69	3.92 ± 1.42	4.05 ± 1.35
• Apolipoprotein B (g/L)	0.52 ± 0.16	0.67 ± 0.11	0.90 ± 0.28	0.94 ± 0.28
• LDL-cholesterol (mmol/L)	3.01 ± 2.00	2.62 ± 1.59	3.15 ± 1.20	3.24 ± 1.23
• Triglycerides (mmol/L)	2.48 (1.75 - 4.11)	2.31 (1.85 - 2.68)	1.35 (1.00 - 2.00)	1.49 (1.04 - 2.31)
• Glucose (mmol/L)	6.8 ± 3.4	6.0 ± 0.57	6.3 ± 2.1	6.6 ± 2.1

HDL; high-density lipoprotein, LDL; low-density lipoprotein.

Part II

The *APOE* gene and Familial Dysbetalipoproteinemia

Chapter 6

Adiposity and the development of dyslipidemia in *APOE* ε2 homozygous subjects: A longitudinal analysis in two population-based cohorts

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Abstract

Background and aims: Familial Dysbetalipoproteinemia (FD), characterized by remnant lipoprotein accumulation and premature cardiovascular disease, occurs in homozygous carriers of the *APOE* ε2 allele, but genetic predisposition alone does not suffice for the clinical phenotype. Cross-sectional studies suggest that a second metabolic hit – notably adiposity or insulin resistance – is required, but the association between these risk factors and development of FD has not been studied prospectively.

Methods: For this study, we evaluated 18,987 subjects from two large prospective Dutch population-based cohorts (PREVEND and Rotterdam Study) of whom 118 were homozygous *APOE* ε2 carriers. Of these, 69 subjects were available for prospective analyses. Dyslipidemia – likely to be FD – was defined as fasting triglyceride (TG) levels >3 mmol/L in untreated subjects or use of lipid lowering medication. The effect of weight, body mass index (BMI), waist circumference, type 2 diabetes mellitus and non-TG metabolic syndrome on development of dyslipidemia was investigated.

Results: Eleven of the 69 ε2ε2 subjects (16%) developed dyslipidemia – likely FD – during follow-up. Age-, sex- and cohort-adjusted risk factors for the development of FD were BMI (OR 1.19; 95%CI 1.04–1.39), waist circumference (OR 1.26 95%CI 1.01–1.61) and presence of non-TG metabolic syndrome (OR 4.39; 95%CI 1.04–18.4) at baseline. Change in adiposity during follow-up was not associated with development of dyslipidemia.

Conclusions: Adiposity increases the risk of developing an FD-like lipid phenotype in homozygous *APOE* ε2 subjects. These results stress the importance of healthy body weight in subjects at risk of developing FD.

Introduction

The apolipoprotein E gene (*APOE*) codes for the apoE protein, which plays a crucial role in lipoprotein metabolism by effecting hepatic clearance of triglyceride rich lipoproteins (TRLs) comprising chylomicrons, very-low-density lipoprotein (VLDL) and their remnants.[1,2] There are three *APOE* variants designated *APOE*-ε3, -ε4, and -ε2, with corresponding allele frequencies of approximately 78%, 14% and 8%, respectively.[3] Subjects with an *APOE* ε2ε2 genotype generally have lower plasma total cholesterol, lower low-density lipoprotein cholesterol (LDL-C) and lower apolipoprotein B (apoB) plasma levels[3,4] and are therefore, on average, at lower risk of cardiovascular disease (CVD) compared to subjects with other *APOE* genotypes.[3,5,6] However, approximately 15% of ε2 homozygotes develop Familial Dysbetalipoproteinemia (FD), which is characterized by increased remnant lipoprotein plasma concentrations.[7] These cholesterol-enriched remnant lipoproteins cause foam cell accumulation and low-grade inflammation in the vascular wall of arteries, contributing to the process of atherosclerosis. Hence, in FD, the protective ε2 lipid profile transforms to a highly atherogenic lipoprotein phenotype. This 'switch' from the favorable hypolipidemic to dysbetalipoproteinemic state is most likely caused by secondary metabolic abnormalities, in addition to the genetic predisposition. Several additional risk factors, including adiposity and insulin resistance, have been postulated to be associated with FD lipid phenotype in ε2ε2 subjects. However, the direction of this association between adiposity and insulin resistance and the development of FD is unclear, considering the majority of the studies were limited to a cross-sectional study design.[8-13] The exact underlying pathophysiological mechanism is uncertain, but might relate to hepatic overproduction of VLDL particles and impaired triglyceride (TG) lipolysis due to insulin resistance.[14] In ε2 homozygotes, the altered conformation of the apoE2 protein decreases the affinity for the low-density lipoprotein receptor (LDL-R) compared to apoE3 by >98%.[14] In ε2 homozygotes, remnant lipoproteins cannot be cleared efficiently from the circulation by the LDL-R, but in most subjects this is of little consequence because the second remnant clearing receptor, the heparan sulphate proteoglycan receptor (HSPG-R), functions normally. However, studies in mice have shown that, in an insulin resistant state, the HSPG-R is degraded by upregulation of sulfatase 2 (Sulf2).[15] This mechanism could be causally implicated in the extensive remnant accumulation seen in FD.[16-19] Furthermore, it has been shown that ε2 heterozygotes could also develop a typical FD lipoprotein phenotype, demonstrated with ultracentrifugation.[20] The aim of this study was to prospectively evaluate the association between adiposity, type 2 diabetes mellitus (T2DM), non-TG metabolic syndrome (MetS) and the development of dyslipidemia – likely FD – in ε2ε2 subjects from the general population.

Methods

Study population

Subjects from two large Dutch population-based, prospective cohorts were included: the Prevention of Renal and Vascular End-Stage Disease cohort and the Rotterdam Study. Details of the study design and recruitment have been described in previous reports.[21-23] In brief, the PREVEND cohort investigates renal and vascular damage in the general population. In 1997–1998, all inhabitants of the city of Groningen, aged 28–75 years (n=85,421), were asked to complete a short questionnaire for collection of demographics and cardiovascular morbidity and to provide a sample of early morning urine. Of the responders, all subjects with a urinary albumin concentration ≥10 mg/L were invited for a baseline visit and 6000 were enrolled. Additionally, a randomly selected group with a urinary albumin concentration of <10 mg/L was invited for a baseline visit and 2592 were enrolled. In total, 8592 subjects completed the baseline visit.

The Rotterdam Study aims to unravel the etiology and natural history of chronic diseases in mid-life and late-life, including cardiovascular, endocrine, hepatic and neurological diseases, among inhabitants of the Ommoord district in the city of Rotterdam. This ongoing prospective cohort started in 1990, and initially all inhabitants above 55 years were invited for participation. The cohort was subsequently expanded in 2000 and again in 2005, with inclusion of subjects above 45 years. Subjects are invited for an interview and an extensive set of examinations every 3–4 years. From the Rotterdam Study, we included all subjects who attended the research center between 1997 and 2006 for the third examination cycle of the first cohort, and the baseline examination of both expansion cohorts (n=10.395).

For the present study, we combined both studies resulting in 18,987 subjects. Thereafter, we excluded subjects without an *APOE* ε2 genotype (n=17.924) or subjects without *APOE* genotype measurement (n=945), resulting in 118 homozygous subjects (0.6%) with the *APOE* ε2 genotype. There were no important differences in participant (age, sex, body mass index (BMI), waist and blood pressure) and clinical (CVD, T2DM, total cholesterol (TC) and TG) characteristics between subjects with and without *APOE* genotyping. The median time interval between baseline and follow-up in the PREVEND cohort was 4.2 (IQR 4.0–4.3) years and in the Rotterdam Study 10.4 (IQR 5.6–10.7) years. For the prospective analyses in this study, ε2ε2 subjects with FD-like lipid phenotype at baseline (n=23) were excluded. Of the remaining 95 subjects, 69 were re-examined during follow-up. See *Supplementary Figure 1* for a flowchart of subjects in- or excluded in this study. All subjects gave written informed consent and the Ethics Committee of the institutions approved the studies.

Baseline and follow-up measurements in PREVEND and Rotterdam Study

In both cohorts, examinations were performed as part of a standardized screening protocol as previously described.[24,25] BMI was calculated as weight in kilograms (kg) divided by height in meters (m) squared. Alcohol consumption in PREVEND was defined as self-reported current alcohol consumption (≥10 gram every month) and no alcohol use was defined as rare (<10 gram/every month) or no alcohol consumption. In the Rotterdam Study, alcohol consumption was defined as minimum alcohol intake of 1 gram/day and no alcohol use was defined as <1 gram/day. Smoking was defined as current smoking. In PREVEND, information on medication use was based on questionnaires and combined with information from a pharmacy-dispensing registry, which has complete information on drug usage for >95% of subjects. In the Rotterdam Study, medication use was assessed by interview at every visit. T2DM was defined as a fasting blood glucose concentration ≥7.0 mmol/L, a non-fasting blood glucose concentration ≥11.1 mmol/L (when fasting samples were unavailable), or the use of blood glucose-lowering drugs. MetS was defined according to the National Cholesterol Education Program Adult Treatment Panel III criteria.[26] For non-TG metabolic syndrome (non-TG MetS), the criterion for MetS was used by replacing the criterion of elevated TG with elevated high sensitivity C-reactive protein (hsCRP) (≥2 mg/L), because TG was used to define dyslipidemia – likely FD. This was based on previous works in which waist circumference was replaced by hsCRP in the definition of MetS.[27,28] This implies that subjects must fulfill ≥3 individual criteria of non-TG MetS, which is not necessarily the hsCRP criterion, just like in the original MetS criterion. In PREVEND, previous coronary artery disease (CAD) and stroke were based on interview at baseline. CAD was defined as myocardial infarction or coronary revascularization and stroke was defined as previous ischemic or hemorrhagic stroke. In the Rotterdam Study, history of myocardial infarction and stroke was assessed by interview and confirmed by medical records (from general practitioner and/or hospital). CAD was defined as previous myocardial infarction and stroke was defined as previous ischemic or hemorrhagic stroke. Both studies instructed subjects to have their blood samples taken in a fasting state and lipids were determined by standard analytical methods.[24,25]

Outcome

In this study, dyslipidemia – likely FD – or FD-like lipid phenotype was defined as fasting plasma TG levels >3 mmoL/L or use of lipid lowering medication. This definition was used as the reference standard for the diagnosis of FD (ultracentrifugation)[29] is not part of standard laboratory analyses. To overcome this, measurement of apolipoprotein B (apoB) can distinguish between other causes of mixed hyperlipidemia or hypertriglyceridemia and FD.[30] However, currently there are no prospectively validated

algorithms to screen for FD. In addition, previously developed apoB algorithms were all validated in cohorts with dyslipidemic patients, while the current study consists of subjects from the general population. Furthermore, apoB levels were only measured in half of the study population.

Analyses

Baseline characteristics are presented for the total study population. Baseline data are presented as number and percentage for categorical variables, mean and standard deviation (SD) for normally distributed variables or median with interquartile range (IQR) in case of unevenly distributed variables. For the cross-sectional analyses, we included 118 subjects with a homozygous *APOE* ε2 genotype and evaluated the association with risk factors and the presence of FD-like lipid phenotype at baseline. For the prospective analyses, 69 ε2ε2 subjects without FD-like lipid phenotype at baseline and with a follow-up visit were included. Lipid measures were only evaluated during the first and last follow-up visit of the Rotterdam Study. Baseline characteristics and difference in change of these characteristics in subjects who did and did not develop dyslipidemia – likely FD – during follow-up were evaluated. Thereafter, the effect of baseline characteristics and change in clinical characteristics between baseline and follow-up was assessed with logistic regression models adjusted for age, sex and cohort. The models assessing change between baseline and follow-up were additionally adjusted for baseline values. HsCRP at the follow-up measurement, and therefore change in non-TG MetS status, was not available in half of the cohort (Rotterdam Study). Missing data (with a maximum of 18% for use of lipid-lowering- and antihypertensive mediation in PREVEND and with a maximum of 17% for alcohol use in the Rotterdam Study) were imputed by single imputation using predictive mean matching. All analyses were conducted in R statistical software, version 3.5.1. For all analyses, a *p*-value <0.05 was considered statistically significant.

Results

Study population

Baseline characteristics of 118 subjects with an *APOE* ε2 genotype are presented in *Table 1*. In total, 46% were male, age 58 ± 14 years. Their mean BMI was 26.7 ± 4.7 kg/m^2 and waist circumference was 92 ± 14 cm. CAD was present in 5% of the subjects and 3% had a previous stroke. Furthermore, 10% had T2DM and 37% non-TG MetS at baseline. To compare the clinical variables of these ε2 homozygotes with the general population (including carriers of an ε3 and ε4 allele), an overview of both cohorts is given in *Supplementary Table 1*. This table shows that clinical variables at baseline of ε2 homozygotes are very similar compared to subjects with other *APOE* genotypes.

Table 1. Baseline characteristics of 118 subjects with an *APOE* ε2ε2 genotype

	n=118
Male sex (n)	54 (46%)
Age (years)	58 ± 14
Weight (kg)	78 ± 16
Body mass index (kg/m2)	26.7 ± 4.7
Systolic blood pressure (mmHg)	136 ± 21
Diastolic blood pressure (mmHg)	77 ± 11
Waist circumference (cm)	92 ± 14
Current smoking (n)	34 (29%)
Alcohol consumption (n)	80 (68%)
Coronary heart disease (n)	6 (5%)
Stroke (n)	3 (3%)
Diabetes mellitus type 2 (n)	12 (10%)
Metabolic syndrome (n)	42 (36%)
Non-TG metabolic syndrome (n)[a]	44 (37%)
Lipid lowering medication (n)	10 (8%)
Antihypertensives (n)	26 (22%)
Total cholesterol (mmol/L)	5.23 ± 1.69
HDL-cholesterol (mmol/L)	1.39 ± 0.39
Non-HDL-cholesterol (mmol/L)	3.84 ± 1.78
Triglycerides (mmol/L)[b]	1.57 (1.07 - 2.29)
hsCRP (mg/L)[b]	1.5 (0.7 - 2.7)
Creatinine (umol/L)[b]	79 (70 - 89)

[a] Adaptation of original criterion for MetS by replacing the criterion of elevated TG for elevated high sensitivity C-reactive protein (hsCRP) (≥2 mg/L).

[b] Median with interquartile range.

Abbreviations: APOE = Apolipoprotein E TG = triglycerides; HDL = high-density lipoprotein; non-HDL = non-high-density lipoprotein cholesterol; HsCRP = high sensitivity C-reactive protein.

Association between baseline characteristics and presence of FD-like lipid phenotype

At baseline, 19% (n=23) of the subjects had dyslipidemia – likely FD – and 81% (n=95) did not (*Supplementary Table 2*; cross-sectional analyses are presented in the Supplementary Materials because the focus of this study are the prospective analyses). In general, subjects with dyslipidemia at baseline were more often male and had an older age. Subjects with dyslipidemia at baseline had higher body weight (OR 1.24 95%CI 1.05–1.47), BMI (OR 1.14 95% CI 1.03–1.28), waist circumference (OR 1.35 95%CI

1.11–1.69) and more often non-TG MetS (OR 14.90 (95% CI 4.64–57.5) (*Supplementary Figure 2*). The latter association with non-TG MetS was driven by glucose (≥5.6 mmol/L), systolic blood pressure (≥130 mmHg), waist circumference (>102 cm for men and >88 cm for women) and HDL-C (≤1.01 for men and ≤1.10 for women) components from the non-TG MetS definition (*Supplementary Table 3A*).

Association between baseline characteristics and development of FD-like lipid phenotype

Of the 95 homozygous *APOE* ε2 subjects without dyslipidemia – likely FD – at baseline, 69 (73%) were re-examined during follow-up. Eleven of the 69 ε2ε2 subjects (16%) developed dyslipidemia between baseline and follow-up while 58 (84%) subjects did not (*Table 2*). Homozygous *APOE* ε2 subjects who developed dyslipidemia between baseline and follow-up had a higher weight, BMI and waist circumference at baseline compared to subjects without development of dyslipidemia. Subjects that developed dyslipidemia between baseline and follow-up had 15% more T2DM, and 29% more non-TG MetS at baseline, compared to subjects who did not develop dyslipidemia. In subjects who developed dyslipidemia, lipids at baseline, including total cholesterol, non-HDL-C and TGs, were higher compared to subjects without dyslipidemia during follow-up. *Figure 1* shows the association between baseline characteristics and development of dyslipidemia – likely FD – between baseline and follow-up in ε2ε2 carriers adjusted for age, sex and cohort. BMI (OR 1.19, 95%CI 1.04–1.39), waist circumference (OR 1.26 95% CI 1.01–1.61) and non-TG MetS (OR 4.39 95%CI 1.04–18.4) at baseline were associated with the development of dyslipidemia during follow-up. Non-TG MetS was mainly driven by glucose and HDL-C components from the non-TG MetS definition (*Supplementary Table 3B*). Weight (OR 1.17 95%CI 0.97–1.43) and presence of T2DM at baseline (OR 7.95 95%CI 0.76–89.5) did not show statistically significant associations with development of dyslipidemia – likely FD – between baseline and follow-up.

Association between change in baseline characteristics during follow-up and development of FD-like lipid phenotype

During follow-up, subjects gained 1.7 kg in weight on average. Weight gain was less pronounced in subjects who developed dyslipidemia – likely FD – than in those who did not (1.1 kg versus 1.8 kg *Table 3*). In subjects who developed dyslipidemia between baseline and follow-up, total cholesterol and non-HDL-C levels decreased during this time interval, and the use of lipid lowering medication increased by 73%. Lipid levels in subjects without development of dyslipidemia did not change substantially. Furthermore, 2 subjects developed T2DM during follow-up but did not switch to an FD-like lipid phenotype, while development of an FD-like lipid phenotype was not accompanied by the development of T2DM. *Figure 2* shows the odds ratios for the association

between change in baseline characteristics and development of dyslipidemia – likely FD – between baseline and follow-up. No statistically significant or clinically relevant associations were seen. Furthermore, additional analyses to evaluate the development of dyslipidemia – likely FD – in ε2ε3 subjects were performed, of the 1329 subjects with an ε2ε3 genotype in this cohort, 146 (11%) developed dyslipidemia. These analyses show that differences in baseline characteristics in ε2ε3 subjects with and without development of dyslipidemia are less prominent compared to subjects with an ε2ε2 genotype (*Supplementary Tables 5–7* and *Supplementary Figures 3* and *4*).

Table 2. Baseline characteristics in ε2ε2 subjects who did and did not develop FD-like lipid phenotype between baseline and follow-up

	No FD-like lipid phenotype during follow-up **n=58**	**FD-like lipid phenotype during follow-up** **n=11**
Male sex (n)	23 (40%)	4 (36%)
Age (years)	54 ± 13	56 ± 14
Weight (kg)	74 ± 15	83 ± 21
BMI (kg/m^2)	25.2 ± 3.7	29.5 ± 6.7
Waist circumference (cm)	87 ± 14	96 ± 17
Diabetes mellitus type 2 (n)	2 (3%)	2 (18%)
Metabolic syndrome (n)	6 (10%)	8 (72%)
Non-TG metabolic syndrome (n)[a]	9 (16%)	5 (45%)
Use of lipid lowering medication (n)	0 (0%)	0 (0%)
Total cholesterol (mmol/L)	4.63 ± 1.11	6.65 ± 1.94
Non-HDL-cholesterol (mmol/L)	3.06 ± 1.13	5.48 ± 1.85
Triglycerides (mmol/L)[b]	1.20 (0.87 - 1.59)	2.48 (1.98 - 2.65)

[a.] Adaptation of original criterion for MetS by replacing the criterion of elevated TG for elevated high sensitivity C-reactive protein (hsCRP) (≥2 mg/L).

[b.] Median with interquartile range.

Abbreviations: BMI = Body Mass Index; TG = triglycerides; non-HDL = non-high-density lipoprotein.

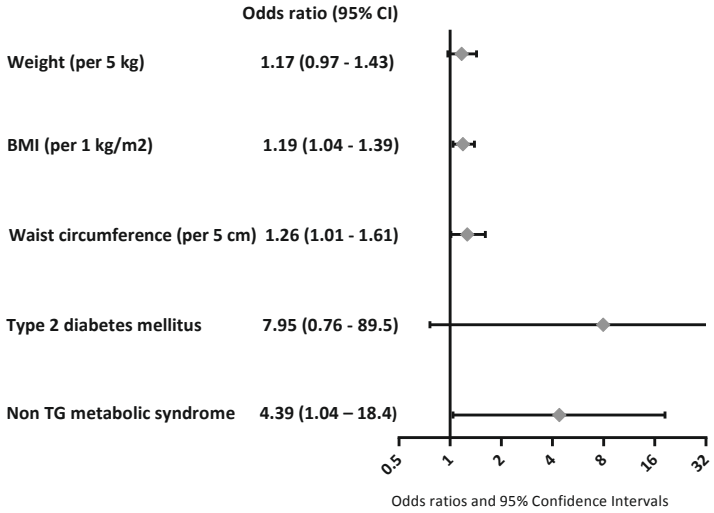

Figure 1. Logistic regression analyses showing association between baseline characteristics and development of FD-like lipid phenotype between baseline and follow-up in ε2ε2 subjects

Models adjusted for age + sex + cohort

Table 3. Change in baseline characteristics in ε2ε2 subjects and development of FD-like lipid phenotype between baseline and follow-up

	No FD-like lipid phenotype during follow-up **n=58**	**FD-like lipid phenotype during follow-up** **n=11**
Change in weight (kg)	1.8 ± 3.8	1.1 ± 3.3
Change in BMI (kg/m^2)	0.6 ± 1.3	0.8 ± 2.4
Change in waist circumference (cm)	2.6 ± 4.9	2.6 ± 4.8
Change in diabetes mellitus type 2 status (n)	2 (3%)	0 (0%)
Change in metabolic syndrome status (n)	2 (3%)	-2 (-18%)
Change in use of lipid lowering medication (n)	0 (0%)	8 (73%)
Change in total cholesterol (mmol/L)	0.09 ± 0.69	-1.43 ± 2.55
Change in non-HDL-cholesterol (mmol/L)	0.14 ± 0.74	-1.48 ± 2.58
Change in triglycerides (mmol/L)[a]	0.09 (-0.25 – 0.36)	0.20 (-0.54 – 0.84)

[a] Median with interquartile range.

Abbreviations: BMI = Body Mass Index; TG = triglycerides; non-HDL = non-high-density lipoprotein

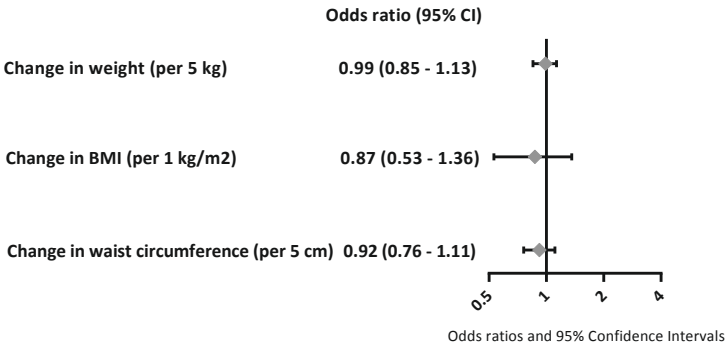

Figure 2. Logistic regression analyses showing association between change in baseline characteristics and development of FD-like lipid phenotype between baseline and follow-up in ε2ε2 subjects

Models adjusted for age + sex + cohort + baseline value

Discussion

In this prospective study, baseline adiposity increased the risk of developing dyslipidemia – likely FD – in ε2ε2 subjects from the general population. BMI, waist circumference and non-TG MetS at baseline were associated with development of dyslipidemia during follow-up, but *change* in these clinical variables did not significantly influence the risk for development of dyslipidemia – likely FD – in ε2ε2 subjects.

Previously, several cross-sectional studies were performed that evaluated the association between adiposity and presence of FD. In line with our finding that adiposity increases the risk of presence of dyslipidemia – likely FD – in ε2ε2 subjects, it was previously observed that high BMI and hyperinsulinemia were more prevalent in hyperlipidemic ε2ε2 subjects compared to normolipidemic ε2ε2 subjects form the general population.[10,31] Furthermore, recent Bayesian network analysis confirmed that insulin resistance (indirectly) increases the prevalence of FD in ε2ε2 subjects from the general population.[11] Another study in patients with an ε2ε2 genotype and vascular disease showed that adiposity measures and MetS were associated with the presence of FD.[9] In the present study, presence of T2DM appears to be associated with development of dyslipidemia – likely FD, with an OR of 7.95 (95%CI 0.76–89.5), but its wide confidence interval resulted in non-significant associations, probably due to insufficient power.

The only other prospective study was performed in 1999 in 10 men with an ε2ε2 genotype evaluated total cholesterol, TG and BMI values at baseline and after 10 years and found no significant changes.[31] However, this study did not report the presence or development of FD lipid phenotype.

The potential mechanism behind the relation between adiposity and development of FD may be degradation of the HSPG-R, an important hepatic remnant clearance receptor, because the affinity of the apoE2 ligand is very low for the other remnant-clearing receptor (LDL-R) in ε2ε2 subjects, thereby severely limiting remnant lipoprotein clearance.[15] In obese and diabetic mice, it was shown that lower HSPG-R status in an insulin resistant state is caused by Sulf2, an extracellular sulphatase and heparin sulphate remodeling enzyme that disrupts the structure of HSPG-R by removing 6-O sulphate groups.[16,18]

In the present study, it was observed that obesity at baseline was associated with development of dyslipidemia – likely FD – between baseline and follow-up, but *change* in obesity during follow-up was not. This suggests that the 'switch' to an FD-like lipid phenotype is preceded by a slow and gradual process of increasing adiposity, insulin resistance and remnant accumulation, which probably takes longer than the time between baseline and follow-up in this study (median follow-up 4.2 (IQR 4.0–4.3) years in PREVEND and 10.4 (IQR 5.6–10.7) years in Rotterdam Study). Mean age of ε2ε2 subjects at baseline in the present study was 59 years, and the metabolic changes that lead to the development of dyslipidemia – likely FD – probably start already at younger age. In line with this, it could be hypothesized that the HSPG-R remnant clearance system functions normally for a long time, even when part of the HSPG-receptors are damaged by Sulf2 upregulation due to adiposity or insulin resistance. In that case, the 'switch' to FD will only take place when a certain threshold of damage to the number of HSPG-R occurs (in combination with a certain threshold of remnant accumulation by VLDL overproduction).

This increase in remnant accumulation due to insulin resistance may also be relevant for patients that have a high cardiovascular risk despite low levels of LDL-C, as remnant cholesterol is an important CVD risk factor.[32] In patients without ε2ε2 genotype, obesity may lead to insulin resistance and remnant lipoprotein accumulation by similar mechanisms as in ε2ε2 and FD patients, although the remnant accumulation will be less severe because the LDL-R clearing system functions normally in non-ε2ε2 subjects. Previously, it was shown that in healthy individuals, and patients with obesity and T2DM, genetic variants in HSPG and Sulf2 influenced postprandial remnant clearance.[17,33] Therefore, the Sulf2 and HSPG pathway may be an attractive target for future pharmacological interventions.

The findings in the present study emphasize the importance of a healthy lifestyle in ε2ε2 subjects. This has clinical implications for healthy people with an *APOE* ε2ε2 genotype, in particular relatives of FD patients identified with cascade screening. For these subjects, maintaining a healthy weight may contribute to the prevention of FD.

Strengths of the study are the combination of two large well-defined population-based cohorts from different areas in the Netherlands and the prospective cohort design with a long follow-up period, although a longer follow-up period would be ideal but such studies are not yet available.

Some limitations should also be considered. First, due to the lack of apoB measurement in the total study population, the definition of FD-like lipid phenotype in this study could only be based on fasting TG >3.0 mmoL/L or use of lipid lowering medication. The cut-off of triglycerides >3.0 mmoL/L is assumed to be acceptable as TG levels >3.0 mmoL/L are high enough not be a random finding and low enough to diagnose potential primary disorders in triglyceride metabolism. However, this could have resulted in misclassification of the diagnosis of FD-like lipid phenotype, especially in subjects with TG levels around 3.0 mmoL/L due to natural variations of TG levels, which is partly based on dietary influences. Also, subjects with an ordinary hypertriglyceridemia and subjects with the presence of a cholesterol-enriched triglyceride rich lipoprotein fraction, characteristic of FD, could not be distinguished. Although the use of lipid-lowering medication in ε2ε2 subjects is very likely to be influenced by FD, as ε2ε2 genotype is usually associated with hypocholesterolemia. Furthermore, more detailed information about (changes in) alcohol consumption or diet was not available to evaluate more precisely the influence of diet and alcohol on the development of dyslipidemia during follow-up. It is uncertain whether individual dietary patterns remain stable over prolonged periods. Furthermore, there was no information about the type of lipid lowering medication use, however, a considerable part of the population that is defined as having dyslipidemia – likely FD – was not aware of the diagnosis, as *APOE* genotype was performed in a research setting years after inclusion of the subjects. Therefore, treatment decisions for these patients in clinical practice were not based or influenced by *APOE* genotype. It is also important to emphasize that lipid levels in subjects allocated as having dyslipidemia – likely FD – are on average reduced due to the use of lipid-lowering medication. Second, aggregating cohorts with over 18,000 subjects still yielded no more than 118 ε2ε2 subjects, emphasizing the challenge to obtain sufficient statistical power to investigate the preclinical disease course of FD in the population. This also leads to small numbers of subjects with, for example, T2DM, which is also indicated by the large confidence intervals of the odds ratios, leading to less precision of the estimate, making firm conclusions based on these numbers difficult.

Third, by design it was unknown if the change in risk factors occurred before or after the onset of FD-like lipid phenotype, which might have resulted in an underestimation of the true effect of the change in risk factors over time. Fourth, 32 of the 118 subjects did not have a follow-up visit, however, as shown in *Supplementary Table 4* there were, except for age, no important differences in baseline characteristics of subjects with or without follow-up, thereby confirming a limited effect of potential selection bias. Fifth, in this study dominant variants in the *APOE* gene causing 10% of the FD cases were not taken into account.[15]

In conclusion, in this prospective study, baseline adiposity increases the risk of developing FD-like lipid phenotype in ε2ε2 subjects from the general population. BMI, waist circumference and presence of non-TG MetS at baseline were associated with development of FD-like lipid phenotype during follow-up. These results stress the importance of a healthy body weight to lower the risk of development of dyslipidemia – likely FD – in these subjects.

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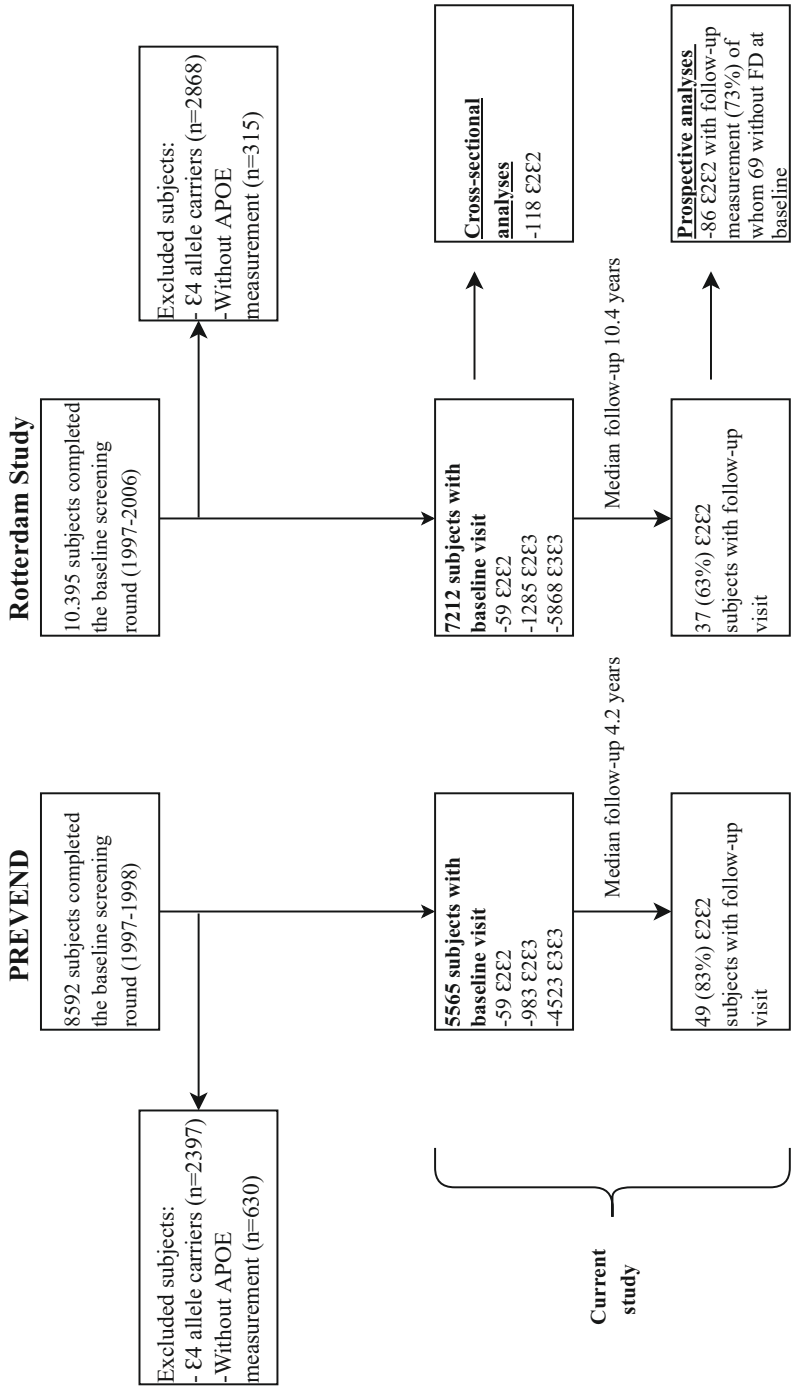
Supplementary Material



Supplementary Figure 1. Flowchart of PREVEND and Rotterdam Study subjects included or excluded

Supplementary Table 1. Baseline characteristics of both cohorts

	PREVEND **n=8592**	**Rotterdam Study** **n=10.395**
Male sex (n)	2735 (49%)	3100 (43%)
Age (years)	50 ± 13	66 ± 10
Weight (kg)	78 ± 14	77 ± 16
Body mass index (kg/m^2)	26.2 ± 4.3	27.3 ± 4.3
Systolic blood pressure (mmHg)	129 ± 20	140 ± 21
Diastolic blood pressure (mmHg)	74 ± 10	79 ± 12
Waist circumference (cm)	89 ± 13	94 ± 12
Current smoking (n)	1904 (34%)	1385 (19%)
Alcohol consumption (n)	4155 (75%)	4779 (66%)
Coronary heart disease (n)	260 (5%)	506 (7%)
Stroke (n)	55 (1%)	250 (3%)
Diabetes mellitus type 2 (n)	220 (4%)	878 (12%)
Metabolic syndrome (n)	1072 (19%)	2799 (39%)
Non-TG metabolic syndrome (n)[a]	1359 (24%)	3217 (45%)
Lipid lowering medication (n)	238 (4%)	1125 (16%)
Antihypertensives (n)	826 (15%)	2433 (34%)
Total cholesterol (mmol/L)	5.60 ± 1.11	5.67 ± 1.01
HDL-cholesterol (mmol/L)	1.34 ± 0.41	1.41 ± 0.41
Non-HDL-cholesterol (mmol/L)	4.26 ± 1.20	4.26 ± 1.01
Triglycerides (mmol/L)[b]	1.15 (0.84 - 1.68)	1.31 (0.99 - 1.79)
Apolipoprotein B (g/L)	1.02 ± 0.30	-
HsCRP (mg/L)[b]	1.40 (0.62 - 3.24)	1.72 (0.70 - 3.70)
Creatinine (umol/L)[b]	82 (73 - 92)	76 (67 - 87)

[a.] Adaptation of original criterion for MetS by replacing the criterion of elevated TG for elevated high sensitivity C-reactive protein (hsCRP) (≥2 mg/L).

[b.] Median with interquartile range.

Abbreviations: *APOE* = Apolipoprotein E; TG = triglycerides; HDL = high-density lipoprotein; non-HDL = non-high-density lipoprotein; HsCRP = high sensitivity C-reactive protein.

Supplementary Table 2. Baseline characteristics in ε2ε2 subjects with and without FD-like lipid phenotype at baseline

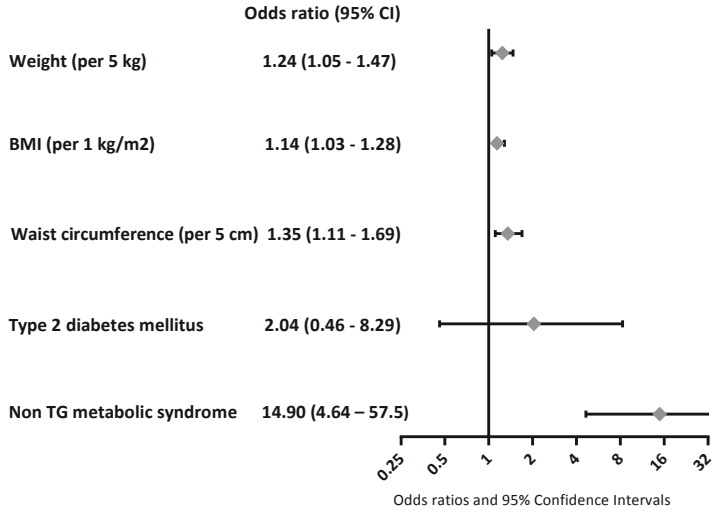
	No FD-like lipid phenotype at baseline (n=95)	**FD-like lipid phenotype at baseline (n=23)**
Male sex (n)	38 (40%)	16 (70%)
Age (years)	57.8 ± 14.6	61.2 ± 13.6
Weight (kg)	75.6 ± 16.2	87.1 ± 10.9
BMI (kg/m^2)	26.2 ± 4.7	28.7 ± 4.1
Waist circumference (cm)	89.7 ± 14.2	101.2 ± 9.3
Diabetes Mellitus type 2 (n)	8 (8%)	4 (17%)
Metabolic syndrome (n)	24 (25%)	18 (78%)
Non-TG metabolic syndrome[a] (n)	25 (26%)	19 (83%)
Use of lipid lowering medication (n)	0 (0%)	10 (43%)
Total cholesterol (mmol/L)	4.99 ± 1.35	6.24 ± 2.46
Non-HDL-cholesterol (mmol/L)	3.51 ± 1.41	5.21 ± 2.44
Triglycerides (mmol/L)[b]	1.40 (1.00 - 2.00)	3.29 (2.70 - 3.65)

[a.] Adaptation of original criterion for MetS by replacing the criterion of elevated TG for elevated high sensitivity C-reactive protein (hsCRP) (≥2 mg/L).
[b.] Median with interquartile range.
Abbreviations: BMI = Body Mass Index; TG = triglycerides; non-HDL = non-high-density lipoprotein.

Supplementary Table 3A. Logistic regression analyses showing association between individual non-TG MetS criteria and presence of FD-like lipid phenotype in ε2ε2 subjects at baseline (n=118)

	OR (95% CI)
Glucose (≥5.6 mmol/L)	7.26 (2.41-24.50)*
SBP (≥130 mmHg)	5.00 (1.48-23.20)*
DBP (≥85 mmHg)	1.86 (0.64-5.26)
Waist circumference (>102 cm for men and >88 cm for women)	5.51 (1.94 – 17.20)*
HDL-C (≤1.01 for men and ≤1.10 for women)	12.80 (3.98 – 49.40)*
hsCRP ≥2.0	1.80 (0.68 – 4.82)

Models were adjusted for age + sex + cohort.
*P <0.05



Supplementary Figure 2. Logistic regression analyses showing association between baseline characteristics and presence of FD-like lipid phenotype at baseline in ε2ε2 subjects

Models were adjusted for age + sex + cohort

Supplementary Table 3B. Logistic regression analyses showing association between individual non-TG MetS criteria and development of FD-like lipid phenotype in ε2ε2 subjects between baseline and follow-up (n=69)

	OR (95% CI)
Glucose (≥5.6 mmol/L)	5.79 (1.10 - 33.80)*
SBP (≥130 mmHg)	3.04 (0.73 - 16.00)
DBP (≥85 mmHg)	3.01 (0.58 - 15.70)
Waist circumference (>102 cm for men and >88 cm for women)	1.20 (0.23 - 5.07)
HDL-C (≤1.01 for men and ≤1.10 for women)	14.80 (3.18 - 90.72)*
hsCRP ≥2.0	1.40 (0.32 - 5.43)

Models were adjusted for age + sex + cohort.

*P <0.05

Abbreviations: SBP = systolic blood pressure; DBP = diastolic blood pressure; HDL = high-density lipoprotein; HsCRP = high sensitivity C-reactive protein

Supplementary Table 4. Baseline characteristics of subjects with and without follow-up measurement

	ε2ε2 subjects with follow-up measurement **n=86**	**ε2ε2 subjects without follow-up measurement** **n=32**
Male sex (n)	39 (45%)	15 (47%)
Age (years)	55 ± 13	68 ± 15
Weight (kg)	78 ± 16	77 ± 15
Body mass index (kg/m2)	26.5 ± 4.6	27.2 ± 5.0
Systolic blood pressure (mmHg)	134 ± 21	141 ± 23
Diastolic blood pressure (mmHg)	77 ± 12	74 ± 9
Waist circumference (cm)	91 ± 15	95 ± 12
Current smoking (n)	24 (28%)	10 (31%)
Alcohol consumption (n)	59 (69%)	21 (66%)
Coronary heart disease (n)	4 (5%)	2 (6%)
Stroke (n)	2 (2%)	1 (3%)
Diabetes mellitus type 2 (n)	5 (6%)	7 (22%)
Metabolic syndrome (n)	30 (35%)	11 (34%)
Lipid lowering medication (n)	6 (7%)	4 (13%)
Antihypertensives (n)	19 (22%)	7 (22%)
Total cholesterol (mmol/L)	5.32 ± 1.88	5.01 ± 0.99
HDL-cholesterol (mmol/L)	1.42 ± 0.43	1.33 ± 0.26
Non-HDL-cholesterol (mmol/L)	3.90 ± 2.00	3.68 ± 0.97
Triglycerides (mmol/L)[a]	1.51 (1.02 – 2.22)	1.58 (1.25 – 2.32)
hsCRP (mg/L)[a]	1.2 (0.7 – 2.5)	2.2 (0.7 – 4.0)
Creatinine (umol/L)[a]	79 (70 – 88)	78 (71 – 92)

[a.] Median with interquartile range.

Abbreviations: *APOE* = Apolipoprotein E; TG = triglycerides; HDL = high-density lipoprotein; non-HDL = non-high-density lipoprotein; HsCRP = high sensitivity C-reactive protein.

Supplementary Table 5. Baseline table stratified for *APOE* genotype (ε2ε2, ε2ε3 or ε3ε3)

***APOE* genotype**	**ε2ε2**	**ε2ε3**	**ε3ε3**
	n=118	**n=2268**	**n=10.391**
Male gender, n (%)	54 (46%)	987 (44%)	4794 (46%)
Age (years)	58 ± 14	59 ± 14	58 ± 14
Body mass index (kg/m^2)	26.7 ± 4.7	27.0 ± 4.5	26.8 ± 4.2
Systolic blood pressure (mmHg)	136 ± 21	136 ± 22	135 ± 21
Diastolic blood pressure (mmHg)	77 ± 11	77 ± 11	77 ± 11
Waist circumference (cm)	92 ± 14	92 ± 13	92 ± 13
Weight (kg)	77.9 ± 15.9	78.1 ± 16.1	77.7 ± 15.8
Current smoking, n (%)	34 (29%)	574 (25%)	2681 (26%)
Alcohol use, n (%)	80 (68%)	1556 (69%)	7298 (70%)
Coronary heart disease, n (%)	6 (5%)	126 (6%)	634 (6%)
Stroke, n (%)	3 (3%)	60 (3%)	242 (2%)
Diabetes mellitus type 2, n (%)	12 (10%)	190 (8%)	896 (9%)
Metabolic syndrome, n (%)	42 (36%)	756 (33%)	3194 (31%)
Non TG Metabolic syndrome, n (%)	44 (37%)	841 (37%)	3691 (36%)
Lipid lowering medication, n (%)	10 (8%)	186 (8%)	1167 (11%)
Antihypertensives, n (%)	26 (22%)	598 (26%)	2635 (25%)
Total cholesterol (mmol/L)	5.2 ± 1.7	5.4 ± 1.0	5.7 ± 1.1
HDL-cholesterol (mmol/L)	1.4 ± 0.4	1.4 ± 0.4	1.4 ± 0.4
Non-HDL-cholesterol (mmol/L)	3.8 ± 1.8	4.0 ± 1.1	4.3 ± 1.1
Triglycerides (mmol/L)[a]	1.6 (1.1 - 2.3)	1.3 (1.0 - 1.8)	1.2 (0.9 - 1.7)
HsCRP (mg/L)[a]	1.5 (0.7 - 2.7)	1.7 (0.7 - 3.8)	1.6 (0.7 - 3.5)
Creatinin (umol/L)[a]	79 (70 - 89)	78 (69 - 88)	79 (69 - 90)

[a.] Median with interquartile range.

Abbreviations: *APOE* = Apolipoprotein E; TG = triglycerides; HDL = high-density lipoprotein; non-HDL = non-high-density lipoprotein; HsCRP = high sensitivity C-reactive protein

Supplementary Table 6. Baseline characteristics in ε2ε3 subjects who did and did not develop FD-like lipid phenotype between baseline and follow-up

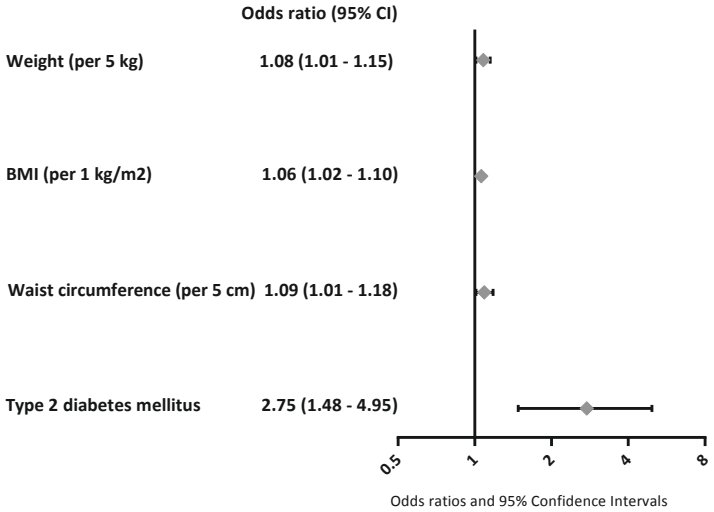
	No FD-like lipid phenotype during follow-up	**FD-like lipid phenotype during follow-up**
	n=1183	**n=146**
Male sex (n)	478 (40%)	81 (55%)
Age (years)[a]	56 (46 - 63)	62 (55 - 68)
Weight (kg)[a]	75.5 (67.4 - 85.5)	80.5 (73.0 - 90.6)
BMI (kg/m^2)[a]	25.9 (23.6 - 28.7)	27.4 (25.2 - 30.4)
Waist circumference (cm)[a]	88.0 (80.0 - 98.0)	95.1 (87.0 - 102.0)
Diabetes mellitus type 2 (n)	43 (4%)	18 (12%)
Metabolic syndrome (n)	200 (17%)	48 (33%)
Use of lipid lowering medication (n)	0 (0%)	0 (0%)
Total cholesterol (mmol/L)[a]	5.20 (4.60 - 5.80)	5.99 (5.17 - 6.74)
Non-HDL-cholesterol (mmol/L)[a]	3.71 (3.06 - 4.37)	4.58 (3.85 - 5.37)
Triglycerides (mmol/L)[a]	1.16 (0.86 - 1.56)	1.62 (1.19 - 2.29)

[a.] Median with interquartile range.
Abbreviations: BMI = Body Mass Index; non-HDL = non-high-density lipoprotein.

Supplementary Table 7. Change in baseline characteristics in ε2ε3 subjects and development of FD-like lipid phenotype between baseline and follow-up

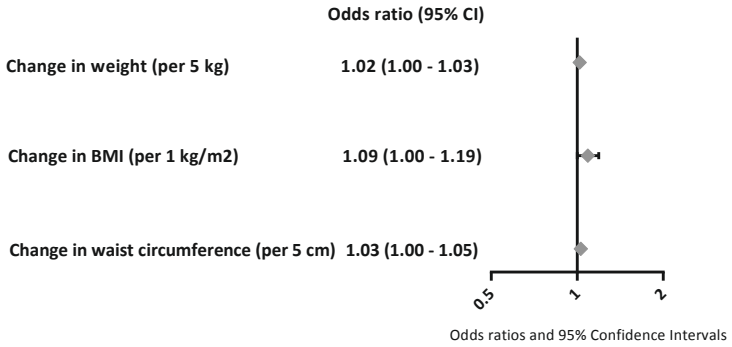
	No FD-like lipid phenotype during follow-up	**FD-like lipid phenotype during follow-up**
	n=1183	**n=146**
Change in weight (kg)[a]	1.0 (-2.0 - 3.9)	1.0 (-2.0 - 4.9)
Change in BMI (kg/m^2)[a]	0 (-0.6 - 1.4)	1.0 (-0.7 - 2.0)
Change in waist circumference (cm)[a]	2.7 (-1.8 - 6.5)	2.4 (-1.1 - 6.1)
Change in diabetes mellitus type 2 status (n)	38 (3%)	17 (12%)
Change in metabolic syndrome status (n)	75 (6 %)	9 (6%)
Change in use of lipid lowering medication (n)	0 (0%)	121 (83%)
Change in total cholesterol (mmol/L)[a]	0.02 (-0.44 - 0.40)	-1.44 (-2.08 - -0.60)
Change in non-HDL-cholesterol (mmol/L)[a]	0.06 (-0.41 - 0.48)	-1.50 (-2.20 - -0.61)
Change in triglycerides (mmol/L)[a]	0 (-0.25 - 0.25)	-0.10 (-0.52 - 0.47)

[a.] Median with interquartile range.
Abbreviations: BMI = Body Mass Index; TG = triglycerides; non-HDL = non-high-density lipoprotein



Supplementary Figure 3. Logistic regression analyses showing association between baseline characteristics and development of FD-like lipid phenotype between baseline and follow-up in ε2ε3 subjects

Models were adjusted for age + sex + cohort



Supplementary Figure 4. Logistic regression analyses showing association between change in baseline characteristics and development of FD-like lipid phenotype between baseline and follow-up in ε2ε3 subjects

Models were adjusted for age + sex + cohort + baseline value

Chapter 7

Establishing the relationship between Familial Dysbetalipoproteinemia and genetic variants in the *APOE* gene

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Clinical Genetics;102(4):253-261

Abstract

Familial Dysbetalipoproteinemia (FD) is the second most common monogenic dyslipidemia and is associated with a very high cardiovascular risk due to cholesterol-enriched remnant lipoproteins. FD is usually caused by a recessively inherited variant in the *APOE* gene (ε2ε2), but variants with dominant inheritance have also been described. The typical dysbetalipoproteinemia phenotype has a delayed onset and requires a metabolic hit. Therefore, the diagnosis of FD should be made by demonstrating both the genotype and dysbetalipoproteinemia phenotype. Next Generation Sequencing is becoming more widely available and can reveal variants in the *APOE* gene for which the relation with FD is unknown or uncertain. In this paper two approaches are presented to ascertain the relationship of a new variant in the *APOE* gene with FD. The comprehensive approach consists of determining the pathogenicity of the variant and its causal relationship with FD by confirming a dysbetalipoproteinemia phenotype, and performing *in vitro* functional tests and, optionally, *in vivo* postprandial clearance studies. When this is not feasible, a second, pragmatic approach within reach of clinical practice can be followed for individual patients to make decisions on treatment, follow-up, and family counseling.

Introduction

Familial Dysbetalipoproteinemia (FD) is the second most common monogenic dyslipidemia, with an estimated prevalence of 1 in 1000 to 1 in 2500 individuals.[1] It is characterized by a mixed hyperlipidemia (i.e. increased plasma cholesterol and triglycerides (TG)), although it can also present as predominant hypertriglyceridemia or hypercholesterolemia. The lipid abnormalities in FD are caused by cholesterol-enriched remnant lipoprotein accumulation; and associated with an increased risk of premature atherosclerotic cardiovascular disease (ASCVD). The classical diagnosis of FD requires the presence of a specific lipoprotein phenotype obtained by ultracentrifugation,[2] as well as pathogenic variants in the *APOE* gene that predispose to FD. Because ultracentrifugation is often not available in clinical practice, approaches using apolipoprotein B (apoB) can be used to establish a dysbetalipoproteinemia phenotype. In most cases (90%) the genetic basis of FD is homozygosity for the ε2 allele (ε2ε2 genotype). The other 10% of cases consist of other variants, of which 23 have been described (*Supplementary Table 1*).[3-5] Rarely, hepatic lipase deficiency is responsible for a similar dysbetalipoproteinemia phenotype.[6] Generally, only 10-15% of people with an ε2ε2 genotype develop the specific dysbetalipoproteinemia phenotype later in life, involving additional metabolic stress, usually obesity, insulin resistance or diabetes mellitus.[7,8] FD has a genetic background and is therefore hereditary, but in most cases it is a recessive disorder, with a low penetrance. So although FD is a genetic disease, the disorder does not usually run in the family and is therefore not 'familial'. When FD is suspected, genetic testing should be performed to confirm the diagnosis. Many laboratories can perform *APOE* genotyping for the common isoforms in the *APOE* gene (ε2, ε3 or ε4). When ε2 homozygosity is ruled out, the next step is Next Generation Sequencing (NGS) to identify other variants in the *APOE* gene.

It can however, be difficult to translate the results of NGS to clinical practice, for example when NGS reveals a variant in the *APOE* gene that has not been described before in a patient with a dysbetalipoproteinemia phenotype. The question arises: is the variant causally related to the observed lipid abnormalities? Furthermore, it is not uncommon that a new variant in the *APOE* gene is detected without an initial clinical suspicion of FD. In this case the question is whether there is a dysbetalipoproteinemia phenotype in the patient, and if so, if the variant is causally related to the observed lipid abnormalities. In this article, we discuss two approaches to establish whether a new *APOE* variant is causally related to FD. The first is a comprehensive approach that consists of determining the pathogenicity of the variant and its causal relationship with FD by confirming the dysbetalipoproteinemia phenotype; and by performing

in vitro functional tests and, optionally, *in vivo* postprandial clearance studies. When this approach is not feasible, a second, pragmatic approach within reach of clinical practice is suggested, that can be followed for individual patients to make decisions on treatment, follow-up, and family counseling.

Diagnosing FD

Before the two approaches will be outlined, a brief introduction to FD and the *APOE* gene will be provided in this section. The dysbetalipoproteinemia phenotype of FD, also known as hyperlipoproteinemia type III or remnant removal disease, is characterized by the accumulation of cholesterol-enriched remnant lipoproteins, usually reflected in a mixed hyperlipidemia. In general, men develop FD in young adulthood and women after menopause.[9] Although very rare, finding an orange palmar crease xanthoma on physical examination of the patient, is considered pathognomonic.[10] FD confers a very high risk of premature ASCVD, and timely and adequate lipid-lowering treatment is important to lower ASCVD risk.[11,12] Furthermore, when TGs are >10 mmol/L, these patients are also at risk for pancreatitis. Diagnosis of FD results in a clear treatment strategy of dietary lipid restriction along with prescription of statins and fibrates. Non-high density lipoprotein cholesterol (non-HDL-C) rather than low-density lipoprotein cholesterol (LDL-C) is used as treatment goal to ensure best control of atherogenic lipoproteins.[13] In addition, risk calculators to estimate 10-year ASCVD risk are not applicable in genetic lipid disorders, including FD, as they underestimate the true ASCVD risk.

A formal diagnosis of FD requires the demonstration of the dysbetalipoproteinemia phenotype *and* an *APOE* genotype that is shown to be causally related to FD (i.e. the ε2ε2 genotype or any of the rare variants described in *Supplementary Table 1*). Making a formal diagnosis of FD is important for several reasons. First, not all pathogenic variants in *APOE* are causally related to FD, even when patients present with hyperlipidemia. Variants in *APOE* have been associated with LDL hypercholesterolemia resembling Familial Hypercholesterolemia (FH),[14,15] hypertriglyceridemia[3] or lipoprotein glomerulopathy.[16] Other pathogenic variants in *APOE* are linked to neurological dysfunction or Alzheimer's disease, age-related macular degeneration[17] or sea blue histiocytosis.[18] Second, not all patients with a pathogenic variant for FD develop the dysbetalipoproteinemia phenotype (incomplete penetrance). This is best illustrated by the ε2ε2 genotype. Only 10-15% of subjects with this genotype develop the dysbetalipoproteinemia phenotype although functional tests have demonstrated that all apoE2 protein binds with less than 2% to the LDL-R

compared to the apoE3 protein.[9,19] Thus, despite apoE2 being pathogenic, not all patients carrying it will have (or get) the disease.[1,7] Third, it was demonstrated that only a minority (38%) of patients with an ultracentrifugally proven dysbetalipoproteinemia phenotype, has the ε2ε2 genotype and the remainder are presumed to have a multifactorial dysbetalipoproteinemia phenotype.[20] This is relevant because, in that study, patients that had a dysbetalipoproteinemia phenotype and an ε2ε2 genotype had an 11-fold increased risk of peripheral artery disease compared to those with the dysbetalipoproteinemia phenotype without the ε2ε2 genotype.[20] For these three reasons it is important to determine the presence of a specific dysbetalipoproteinemia phenotype and genotype, when making a FD diagnosis.

The dysbetalipoproteinemia phenotype

The dysbetalipoproteinemia phenotype cannot be detected with the standard investigations for dyslipidemia alone. Standard investigations comprise total cholesterol (TC), HDL-C, TG and LDL-C. In FD standard investigations will often result in a non-specific mixed hyperlipidemia. The reference standards for determining the dysbetalipoproteinemia phenotype are ultracentrifugation and polyacrylamide gradient gel electrophoresis (PGGE), although the specific dysbetalipoproteinemia pattern is also recognized by paper-, cellulose acetate- or agarose electrophoresis.[21] In addition, although the broad beta band on agarose gel electrophoresis was found to be highly specific for dysbetalipoproteinemia it had low sensitivity compared with polyacrylamide gradient gel electrophoresis.[22] The dysbetalipoproteinemia phenotype is defined by ultracentrifugation as an increased ratio of cholesterol to TG within VLDL (>0.42 by mass or >0.97 by molar measurements) or increased VLDL-C/total plasma TG ratio (>0.30 or >0.69 by mass or molar measurements respectively; and respectively >0.25 and >0.57 ratios are suggestive/borderline).[2,23] With PGGE a dysbetalipoproteinemia phenotype displays lipid staining in the intermediate-density lipoprotein (IDL) and/or smaller VLDL range, with little or no LDL.[22] When these methods are not available, the measurement of apoB is recommended to distinguish FD from other causes of mixed dyslipidemia such as Familial Combined Hyperlipidemia (FCHL).[24-27] Several approaches to establish a dysbetalipoproteinemia phenotype based on apoB have been developed. Compared to ultracentrifugation, the sensitivity of these approaches ranges from 89% to 97% and the specificity ranges from 95% to 97%. The diagnostic approach with the best diagnostic properties is the non-HDL-C/apoB ratio, with a cut-off of >4.91 mmol/g (sensitivity 96.8% (95% CI 89.0–99.6) and specificity 95.0% (95% CI 93.8–96.0). All diagnostic methods for the dysbetalipoproteinemia phenotype are summarized in *Table 1*.

Table 1. Cut-offs and diagnostic properties of laboratory tests to establish an FD lipoprotein phenotype

Laboratory test	Cut-off
Ultracentrifugation (reference standard)	*VLDL-C/VLDL-TG:*
	>0.97 (molar ratio) >0.42 (mass ratio)
	VLDL-C/total TG:
	>0.69 (molar ratio) >0.30 (mass ratio)
	Suggestive:
	>0.57 (molar ratio) >0.25 (mass ratio)
PGGE (qualitative)	Increased IDL and/or VLDL and no detectable LDL
PGGE (quantitative)	Videodensitometric analysis of the ratio of area under the curve > 0.5 for IDL-LDL
Non-HDL-C/apoB ratio	>4.91 mmol/g
	>3.69 mmol/g
ApoB/TC ratio	<0.15g/mmol
ApoB, TC and TG levels	3-step-algorithm:
	1) TG >75th percentile
	2) TC/apoB ratio ≥6.2 mmol/g
	3) TG/apoB ratio<10.0 mmol/g

Analysis of genetic variants in the *APOE* gene

Pathogenic variants in the *APOE* gene that have been shown to have a causal relationship with the dysbetalipoproteinemia phenotype are listed in *Supplementary Table 1*. Pathogenicity in general is the process in which a genetic variant leads to translation of a dysfunctional protein with pathogenic mechanistic properties.

As mentioned before approximately 10% of FD patients have other variants than ε2ε2 in *APOE*, and those variants are often inherited in a dominant mode.[4] Some variants inherit in a co-dominant fashion, meaning that the isoform of the other allele determines the outcome: if the other allele is ε2, the condition will resemble ε2 homozygosity. When a new variant is detected by NGS, the variant is classified on general genetic principles rather than specific mechanistic studies that would determine a causal relationship between gene and disease. Classification is based on the guidelines by the American College of Medical genetics and genomics (ACMG).[28] These are general guidelines, and therefore not specific for the *APOE* gene and not aimed at identifying FD. In brief, variants are placed in 5 classes: 'benign' (class 1), 'likely benign' (class 2), 'uncertain significance' (class 3), 'likely pathogenic' (class 4) or 'pathogenic' (class 5). The classification of pathogenicity

	Sensitivity (compared to ultracentrifugation)	Specificity (compared to ultracentrifugation)	Reference
	-	-	2
	-	-	22
	89%	100%	
	96.8% (95%CI 89.0-99.6)	95.0% (95%CI 93.8-96.0)	25
	94.8% (95%CI 90.0-97.7)	66.1% (95%CI 64.7-67.6)	26
	89% (95%CI 78 - 96)	97% (95%CI 94-98)	24
	AUC-ROC of combination 0.988		27

is based on several levels of evidence ranging from very strong to supportive. There are many types of evidence that can be used to determine pathogenicity, the details of which are outside the scope of this article. Examples of strong evidence are *in vitro* and *in vivo* functional studies or an increased prevalence of the variant in affected subjects, compared to controls. An example of moderate evidence is that the variant is the variant being in a functional domain of a protein. Examples of supporting evidence are the presence of a highly specific phenotype and *in silico* predictions. *In silico* predictions are based on the probable impact of amino acid substitutions on the structure and function of a protein (based on the degree of evolutionary conservation of the wild type amino acid and the 3D structure of the new protein).[28,29]

Approaches to establish a causal relation between a new *APOE* variant and FD

When NGS reveals a variant in *APOE* of which the causal relationship with FD is unknown we suggest two approaches. The comprehensive approach consists of determining the pathogenicity of the variant and its causal relationship with FD by confirming a

dysbetalipoproteinemia phenotype using reference methods, and performing *in vitro* functional tests and, optionally, *in vivo* postprandial clearance studies. We strongly recommend that when the comprehensive method is used for a new variant to establish or exclude a causal relationship with FD, the results of this research should be published in peer reviewed journals for use in clinical practice. However, this approach requires resources, infrastructure, specific expertise and time. Therefore, a pragmatic approach is suggested which describes how to make clinical decisions by combining presence of the dysbetalipoproteinemia phenotype with the (preliminary) degree of pathogenicity of the variant.

Comprehensive approach

The comprehensive approach consists of three parts: 1. determining pathogenicity; 2. determining a causal relation with FD; and 3. determining a dysbetalipoproteinemia phenotype in several, unrelated patients with the same variant (*Figure 1*). All three steps are necessary to make a definite FD diagnosis, although point 2 can be part of point 1, as will be explained later.

The first step is to determine the pathogenicity of the variant, using the ACMG guidelines as was described in the previous paragraph.

Step two of the comprehensive approach is determining the causal relationship of the variant with FD. This should be done by establishing impaired LDL-R and/or HSPG-R binding of remnant lipoproteins by *in vitro* functional hepatic receptor binding studies. Delayed postprandial remnant clearance with *in vivo* functional tests can be used to confirm the causal relationship with FD. An example of a postprandial remnant clearance study can be to evaluate the effect of an oral fat load (e.g. with fresh cream) and to assess retinyl palmitate levels up to 12 or even 24 hours after ingestion of the oral fat load, and to compare the response with healthy subjects. Inclusion of retinyl palmitate to the oral fat load enables tracking chylomicrons and their remnants.[30] *In vitro and in vivo* functional tests can, but do not have to be part of the determination of pathogenicity in step one. Geneticists are free to decide which levels of evidence from the ACMG guidelines they use to determine the pathogenicity of a variant. Although in most cases functional tests are likely to be part of the pathogenicity assessment, this is not essential if other criteria provide sufficient evidence for the pathogenicity of the variant. The third step in the comprehensive approach is to determine whether the variant is associated with the dysbetalipoproteinemia phenotype in several, unrelated patients

with the same variant in *APOE* using the reference standards. It should be noted here that, at least theoretically, subjects carrying an *APOE* variant that is causally related to FD may not (yet) have developed the specific dysbetalipoproteinemia phenotype. That is the reason we recommend using several patients for establishing the dysbetalipoproteinemia phenotype. When a variant has been shown to be pathogenic and to lead to impaired receptor binding of the ApoE protein, it can still be classified as FD-causing, even when not all patients carrying the variant express the dysbetalipoproteinemia phenotype. However, when the patients are under sufficient metabolic stress (e.g. metabolic syndrome, diabetes mellitus, or post-menopausal state in women), and still lack the specific phenotype, a definite relationship with FD cannot be determined and careful monitoring of the lipoprotein profiles is warranted.

Two examples of how to use the comprehensive approach are provided in *Table 2* and *Table 3*. The first example describes the apoE-Leiden (p.Glu165-Gly171dup) variant in the *APOE* gene. In this example there are five arguments for pathogenicity (according to the ACMG guidelines): one strong, two moderate and two supporting. These criteria are sufficient to classify the variant as pathogenic (class 5). Furthermore, the causal relationship with FD was established with functional *in vitro* tests showing decreased LDL-R binding of the apoE-Leiden protein. In addition, the specific dysbetalipoproteinemia phenotype was demonstrated in several unrelated patients that carried this variant, using ultracentrifugation (the reference standard). A causal relationship between this *APOE* variant and FD is thus verified.

The second example describes the p.Leu72Pro variant in the *APOE* gene. This variant does not affect the part of the ApoE protein that is critical for the clearance of remnant lipoproteins, but does typically disrupt protein structure. The likely pathogenic (class 4) status of the variant was established with one strong and two supporting arguments according to the ACMG guidelines. Binding of this apoE protein to the LDL-R was, however, normal and postprandial remnant clearance was not impaired. None of the patients had a specific dysbetalipoproteinemia phenotype determined by ultracentrifugation. A causal relationship of this variant of apoE with FD was thus excluded. This example shows that a putative pathogenic variant in *APOE* is not always causally related to FD, although the variant may still be related to dyslipidemia or other disorders.

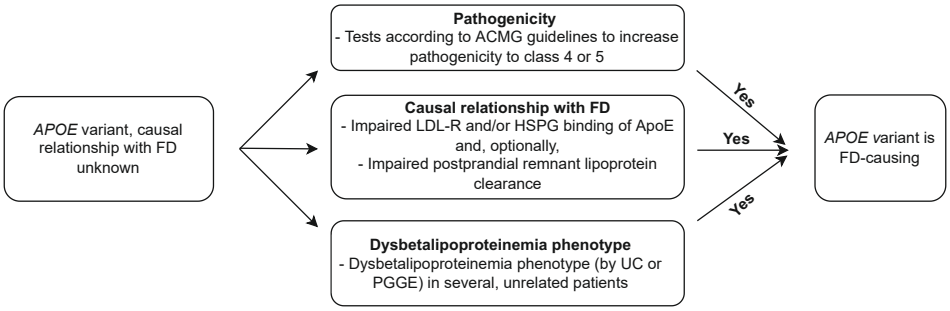

Figure 1. Comprehensive evaluation of an *APOE* variant for causal relationship for FD

When the causal relationship with FD of a variant in the *APOE* gene is unknown, attempts should be made to evaluate this. The assessment should following 3 steps. The first step is determining pathogenicity of this variant according to the ACMG guidelines; the second step is determining a causal relation with FD by *in vitro* functional studies (impaired LDL-R and/or HSPG binding of apoE) and, optionally, *in vivo* functional studies (impaired postprandial lipoprotein clearance). The third step is demonstration of a dysbetalipoproteinemia phenotype in several, unrelated patients with the same variant.

Class 4 variant = likely pathogenic variant, class 5 variant = pathogenic variant.

Abbreviations: ACMG = American College of Medical Genetics and Genomics. FD = Familial Dysbetalipoproteinemia. ApoE = Apolipoprotein E, PGGE = polyacrylamide gradient gel electrophoresis, UC = ultracentrifugation, LDL-R = low-density lipoprotein receptor = HSPG = heparan sulphate proteoglycan

Table 2. ApoE-Leiden (p.Glu165-Gly171dup) variant in *APOE* gene

Phenotype assessment		**Comment and explanation**
Ultracentrifugation		Several studies showed presence of beta-VLDL and VLDL-C/plasma TG >0.69 mmol/L in several unrelated heterozygotes.
Pathogenicity assessment according to ACMG guidelines		
Criterion	***Weight***	
Functional tests	Strong	*In vitro:* LDL-R binding is 11-25%, HSPG binding is 5% compared to apoE3 protein.
Location in gene	Moderate	Location 165-171 is not in functional domain (but variant influences the functional domain).
Protein length changes as a result of inframe insertions	Moderate	ApoE-Leiden consists of tandem repeat.
Patients phenotype (highly) specific for a disease	Supporting	FD lipoprotein phenotype confirmed in subjects evaluated in several studies.
Cosegregation with disease in multiple affected family members	Supporting	In one kindred 100% segregation of genotype and phenotype.
Conclusion	**1) FD lipoprotein phenotype? Yes** **2) (Likely) pathogenic? Yes**	**Variant is FD-causing**
	1 strong criterion, 2 moderate criteria and 2 supporting criteria for pathogenicity met, resulting in class 5 (pathogenic).	

Based on previous publications.[19,32,33]

Table 3. p.Leu72Pro variant in *APOE* gene

Phenotype assessment		**Comment and explanation**
Ultracentrifugation		In homozygotes: None VLDL-C/VLDL-TG molar ratio >0.97 or VLDL-C/plasma TG molar ratio >0.69. In 60 heterozygotes: No specific hyperlipoproteinemia phenotype.
Pathogenicity assessment according to ACMG guidelines		
Criterion	***Weight***	
Functional tests	Strong	*In vitro:* Excluded a binding defect to LDL-R. *In vivo:* Excluded accumulation of remnants
Compare prevalence variant in controls/cases (OR ≥ 5.0) or CI of OR does not include 1.0.	Strong	OR for CAD 3.1 (95% CI 1.20 – 8.0) in carriers relative to non-carriers.
Location in gene	Moderate	Location 72 is not in functional domain.
Absent from controls	Moderate	Prevalence of p.Leu72Pro in European (non-Finnish) population: 0.34%
Patients phenotype (highly) specific for a disease	Supporting	All 4 homozygotes suffered from various forms of hyperlipoproteinemia and had 3 different types of hypertriglyceridemia.
Cosegregation with disease in multiple affected family members	Supporting	Heritability and cosegregation of genotype and phenotype were studied in 7 study participants and 56 of their relatives. Genotype and phenotype were congruent in all families.
Multiple lines of computational evidence of a deleterious effect	Supporting	In silico predictions on Gnomad. Polyphen: possibly damaging, SIFT: tolerated.
Conclusion	**1) FD lipoprotein phenotype? No** **2) (Likely) pathogenic? Yes**	**Variant is likely pathogenic according to ACMG guidelines, but does not cause FD. However, this variant can increase risk for atherosclerosis by other (dyslipidemia) mechanisms.**
	1 strong criterion, 2 supporting criteria	

Based on previous publication about the p.Leu72Pro variant and website of Gnomad.[34,35]

Pragmatic approach

Healthcare providers could be faced with a situation in which a *APOE* variant is found in a patient, but definitive information on the relationship between this variant and FD is not (yet) available. To provide some guidance in these situations, the following pragmatic approach is suggested for individual patients (*Table 4*).

When a patient presents with hyperlipidemia and FD is suspected, apoB-based diagnostic methods should be used to establish a dysbetalipoproteinemia phenotype (or, if available, one of the reference standards) (*Table 1*). Second, the preliminary classification of the pathogenicity of the variant should be taken into account. This classification should be provided by the genetic laboratory that performed the NGS.

When a patient has a variant that is classified as (likely) pathogenic (class 4/5) and the patient has a dysbetalipoproteinemia phenotype according to an apoB-based diagnostic strategy such as the non-HDL-C/apoB ratio, the patient can be classified as having presumptive FD. In this case the patient can be treated as FD, but a definite diagnosis can only be made by following the comprehensive approach. When a patient has a class 3 (unknown significance) variant and the dysbetalipoproteinemia phenotype is present, the patient can be diagnosed as having probable FD and can be treated accordingly.

When a variant is (likely) pathogenic (class 4/5) and the dysbetalipoproteinemia phenotype is *not* present, there are three possibilities to consider. First, the variant may not be causal for FD (e.g. the p.Leu72Pro variant). Second, the variant causes FD, but due to delayed penetrance, has not come to expression yet. This can be the case when a variant is found in cascade screening. A third reason for the absence of the dysbetalipoproteinemia phenotype, could (theoretically) be the limited specificity of the apoB algorithm.

When the variant is classified as class 3 and the dysbetalipoproteinemia phenotype is *not* present, the diagnostic label of FD should not be used until the pathogenicity of the variant is clear from (functional) studies or the dysbetalipoproteinemia phenotype supervenes.

Although the causal relationship with FD can only be determined by specialized laboratories using data of several, unrelated patients, as described in the comprehensive approach, it is possible for individual health care providers to shed some light on the potential relation between the *APOE* variant and FD in the individual patient. This can for example be useful when a variant is classified as class 3 (unknown significance).

First, *in silico* predictions can be used. Several in silico prediction software programs can be found on www.gnomad.broadinstitue.org. However, multiple *in silico* prediction tools sometimes provide inconsistent results for the same variants, so results should be interpreted with caution. Second, the location of the variant on the gene can be considered. The LDL-R binding domain of apoE is the most vulnerable region and is located in the fourth helix, at position 180-194 (NM_001302688.1; *Supplementary Material*),[31] so when a variant is located there, the variant is more likely to be pathogenic. When using these methods it is important to note that they can never by themselves provide definite information on the causal relationship between a genetic variant and FD. Furthermore, treatment decisions are made based on the presence of a dysbetalipoproteinemia phenotype, and these strategies (*in silico* predictions or gene location) can only be supportive in this regard.

Table 4. Pragmatic approach to diagnose FD in an individual patient

Pathogenicity \ **Phenotype**		**Non-HDL-C/apoB ratio >4.91 mmol/g (or if available: ultracentrifugation or PGGE)**	
		Yes	**No**
(Likely) pathogenic (class 4/5)	**Yes**	*Presumptive FD (treat as FD)*	*Unknown* • Variant is not causally associated with FD • Variant may eventually lead to FD under sufficient metabolic stress
	No	*Possibly FD (treat as FD)*	*Exclude FD* • Monitor updates on pathogenicity classification and lipoprotein phenotype of patient

Discussion and conclusion

FD is a complex disorder with a very specific dysbetalipoproteinemia phenotype, a delayed penetrance, and a heterogeneous genetic basis. Not all pathogenic variants in the *APOE* gene are causally related to FD, and not all patients with a genetic predisposition to FD develop the dysbetalipoproteinemia phenotype (incomplete penetrance). The diagnosis of FD can therefore only be made by demonstration of both the specific dysbetalipoproteinemia phenotype and a specific causal *APOE* genotype.

In this paper two strategies are proposed to establish whether a variant in *APOE* causes FD. The first approach requires comprehensive investigation which is only feasible at specialized laboratories which should collect information in several unrelated patients

with the same variant. The second, pragmatic approach is aimed at clinical practice. This approach requires the addition of apoB to demonstrate the dysbetalipoproteinemia phenotype (although with less confidence).

Currently, the ACMG guidelines standardize the classification and reporting of the pathogenicity of all new genetic variants, irrespective of the gene or the disease. When a (likely) pathogenic variant in *APOE* is automatically classified as FD causing, without determining a causal relationship, this might lead to misdiagnosis of patients.

Cooperation between physicians and laboratories is encouraged to investigate clusters of patients with the same variant. A registry of new variants in *APOE*, that includes lipid profiles of patients, will enhance linking novel genetic variants to FD. Such information should be published according to ClinVar (a public database for clinical laboratories, researchers, expert panels, and others to share their interpretations of variants along with their evidence) and ClinGen regulations.

The main limitation of this article is that the recommendations are based on expert opinion. This article was written to address a current need for guidance in the interpretation of the relationship between new variants in the *APOE* gene and FD in clinical practice, but further studies to substantiate these approaches are warranted.

To conclude, FD is an important cause of mixed hyperlipidemia that is highly atherogenic and whose diagnosis consists of a specific phenotype and genotype. To evaluate whether a new *APOE* variant is causally related to FD is challenging. In this paper we present two approaches that can be followed. The comprehensive approach consists of determining the pathogenicity of the variant and establishing a causal relation with FD in several unrelated patients with the same variant with more detailed lipoprotein characterization and functional studies. The pragmatic strategy was developed for clinical practice and can be followed for individual patients to make decisions on treatment, follow-up, and family counseling.

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Supplementary Material

Nomenclature of *APOE* gene

For the nomenclature of a gene, the most recent guidelines advise the use of the longest transcript of a gene.[28] The transcript of *APOE* (NM_001302688.1) therefore currently consists of 343 amino acids. The nomenclature of the *APOE* gene has changed over the years. Initially, to determine the position of a variant the signal peptide (i.e. the first 18 amino acids of apoE) was excluded and the mature protein served as reference. In this convention the ε2 allele differed from the ε3 allele at position 158. Later, when the signal peptide was included, the nucleotide change in the ε2 allele was located at position 176 (158+18). The newest nomenclature of *APOE* adds 44 amino acids compared to the initial nomenclature. Therefore, the ε2 allele is currently located at position 202 (158+44). This means that variants with different names in the literature (p.Arg158Cys, p.Arg176Cys and p.Arg202Cys) all refer to the same variant. In this paper the newest nomenclature is used, therefore we transformed older annotations from previous literature to the newest nomenclature.

Supplementary Table 1. Established *APOE* variants with a causal relationship with FD

Number	Name of variant NM_001302688.1, (most recent annotation)	Name of variant NM_000041.4, (previous annotation -26)	Name of variant (previous annotation -44)
1.	p.Thr31*	p.Thr5*	p.Thr-13*
2.	p.Glu57Lys;p.Arg189Cys	p.Glu31Lys;p.Arg163Cys	p.Glu13Lys;p.Arg145Cys
3.	p.Trp64*	p.Trp38*	p.Trp20*
4.	p.Gly75Valfs*29	p.Gly49Valfs*29	p. Gly31Valfs*29
5.	p.139Lys/p. Glu140insGly*50/51	p.Lys113/p. Glu114insGly*50/51	p.Lys95/p. Glu96insGly*50/51
6.	p.Glu165_Gly171dup	p.Glu139_Gly145dup	p.Glu121_Gly127dup
7.	p.Gly171Asp	p.Gly145Asp	p.Gly127Asp
8.	p.Arg180Cys	p.Arg154Cys	p.Arg136Cys
9.	p.Arg180His	p.Arg154His	p.Arg136His
10.	p.Arg180Ser	p.Arg154Ser	p.Arg136Ser
11.	p.Arg180fs*96	p.Arg154fs*96	Arg136fs*96
12.	p.Arg186Cys	p.Arg160Cys	p.Arg142Cys
13.	p.Arg186Ser	p.Arg160Ser	p.Arg142Ser
14.	p.Arg186Leu	p.Arg160Leu	p.Arg142Leu
15.	p.Arg189Cys	p.Arg163Cys	p.Arg145Cys

	Common name	Details	Ref
		Located on ε4 allele; Nonsense variant (leads to truncated apoE protein); Precipitates to FD possibly only in combination withε2 allele or other FD precipitating factors (reduced penetrance)	36
	Philadelphia	Allele unknown; expression of FD possible in combination with ε3 or ε4 allele; Dominant mode of inheritance with reduced penetrance (factors including age influence expression)	37,38
		Located on ε3 allele; Nonsense variant (leads to truncated apoE protein); Precipitates to FD possibly only in combination with ε2 allele or other FD precipitating factors (reduced penetrance)	39
		Allele unknown; Frameshift variant leading to premature stop codon (nonsense variant) and truncated apoE protein; Reduced penetrance (expression in combination with other dominant *APOE* variant or age)	40
	Groningen	Located on ε3 allele; Frameshift variant leading to premature stop codon (nonsense variant) and truncated apoE protein; Precipitates to FD possibly only in combination with ε2 allele or other FD precipitating factors	41
	Leiden	Located on ε3 allele; Expression of FD possible in combination with ε3 or ε4 allele; Dominant mode of inheritance with reduced penetrance (factors including age and BMI influence expression)	32,33
		Located on ε2 allele; Precipitates to FD possibly only in combination with ε2 allele or other FD precipitating factors	40,42,43
	Heidelberg	Allele unknown; Precipitates to FD possibly only in combination with other FD precipitating factors	44,45
	Montreal	Allele unknown; Effect other allele on expression FD not clear	4,46
	Christchurch	Located on ε3 allele; Precipitates to FD possibly only in combination with ε2 allele, partial expression in combination with ε4 allele; This variant was also associated with FCHL and hypertriglyceridemia	47-49
		Allele unknown; Leads to truncated apoE protein; Precipitates to FD only in combination with ε2 allele	50
		Located on ε4 allele; Expression of FD possible in combination with ε3 or ε4 allele; Dominant mode of inheritance	51-53
	Nagoya	Variant found in only one patient; In this patient the other allele was ε2, possibly required for expression of FD	54
		Allele unknown; Expression of FD possible in combination with ε3 allele	55
		Allele unknown; Expression of FD possible in combination with ε3 allele. Dominant mode of inheritance with reduced penetrance (factors including age and sex influence expression)	56

Supplementary Table 1. Continued

Number	Name of variant NM_001302688.1, (most recent annotation)	Name of variant NM_000041.4, (previous annotation -26)	Name of variant (previous annotation -44)
16.	p.Arg189His	p.Arg163His	p.Arg145His
17.	p.Lys190Gln	p.Lys164Gln	p.Lys146Gln
18.	p.Lys190Glu	p.Lys164Glu	p.Lys146Glu
19.	p.Lys190Asn;p.Arg191Trp	p.Lys164Asn;p.Arg165Trp	p.Lys146Asn;p.Arg147Trp
20.	p.Gln231Glu	p.Gln205Glu	p.Gln187Glu
21.	p.Ala253Glyfs*20	p.Ala227Glyfs*20	p.Ala209Glyfs*20
22.	p.Trp254*	p.Trp228*	p.Trp210*
23.	p.Glu288Lys, p.Glu289Lys	p.Glu270Lys,p.Glu271Lys	p.Glu244Lys, p.Glu245Lys

*stopcodon

	Common name	Details	Ref
	Kochi	Allele unknown; This variant is also associated with mild hypertriglyceridemia; Effect other allele on expression FD not clear, although it seems with reduced penetrance	**57**
		Allele unknown; Dominant mode of inheritance, with a high penetrance	58-61
	Harrisburg	Allele unknown; Expression of FD possible in combination with ε3 allele. Dominant mode of inheritance	62-65
	Hammersmith	Located on ε3 allele; Expression of FD possible in combination with ε3 allele. Dominant mode of inheritance	66
	Toranomon	Located on ε3 allele; Precipitates to FD possibly only in combination with ε2 allele	67
		Allele unknown; Nonsense variant (leads to truncated apoE protein); Dominant mode of inheritance with reduced penetrance (factors including age influence expression)	68
	Washington	Allele unknown; Nonsense variant (leads to truncated apoE protein); Index patient was homozygous for this variant and had complete apoE deficiency	69
	Suita	Allele unknown; Associated with several types of dyslipidemia, including FD (with incomplete penetrance); Effect other allele on expression FD not clear	70

Chapter 8

Low-density lipoprotein cholesterol and non-high-density lipoprotein cholesterol measurement in Familial Dysbetalipoproteinemia

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In revision

Abstract

Aim: The aim of this study was to compare LDL-C concentrations using the Friedewald formula, the Martin-Hopkins formula, a direct assay and polyacrylamide gradient gel electrophoresis (PGGE) to the reference standard density gradient ultracentrifugation in patients with Familial Dysbetalipoproteinemia (FD) patients. We also compared non-HDL-cholesterol concentrations by two methods.

Methods: For this study data from 28 patients with genetically confirmed FD from the placebo arm of the EVOLVE-FD trial were used. Four different methods for determining LDL-C were compared with ultracentrifugation. Non-HDL-C was measured with standard assays and compared to ultracentrifugation. Correlation coefficients and Bland-Altman plots were used to compare the methods.

Results: Mean age of the 28 FD patients was 62 ± 9 years, 43% were female and 93% had an ε2ε2 genotype. LDL-C determined by Friedewald (R^2=0.62, p=<0.01), Martin-Hopkins (R^2=0.50, p=0.01) and the direct assay (R^2=0.41, p=0.03) correlated with density gradient ultracentrifugation. However, Bland-Altman plots showed considerable over- or underestimation by the four methods compared to ultracentrifugation. Non-HDL-C showed good correlation and agreement.

Conclusion: In patients with FD, all four methods investigated over- or underestimated LDL-C concentrations compared with ultracentrifugation. In contrast, standard non-HDL-C assays performed well, emphasizing the use of non-HDL-C as treatment goal in FD.

Introduction

In clinical practice low-density lipoprotein-cholesterol (LDL-C) is calculated using the Friedewald formula based on measurement of total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C) and triglycerides (TG). The Friedewald formula assumes a fixed ratio of cholesterol to TG in the very-low-density lipoprotein (VLDL) fraction: LDL-C = TC minus HDL-C minus TG/2.2 (in mmol/L) or TG/5 (in mg/dL).[1] The original publication of the Friedewald formula, noted three exceptions to its use: non-fasting values, TG >4.52 mmol/L and Familial Dysbetalipoproteinemia (FD). FD is the second most common monogenic lipid disorder, after Familial Hypercholesterolemia (FH).[2] The hallmarks of FD are cholesterol-enriched VLDL together with raised remnant lipoproteins and low LDL-C concentrations. As a result of the cholesterol enrichment of remnants, the fixed ratio of cholesterol to TG in VLDL in Friedewald is invalid in FD. However, this exception to the Friedewald formula is often not appreciated in clinical practice, where LDL-C is still used as treatment goal or risk predictor in FD patients by some physicians. An alternative for the Friedewald formula is the Martin-Hopkins formula, which replaces the fixed ratio by an adjustable factor based on individual non-HDL-C and TG levels (LDL-C = TC minus HDL-C minus TG/adjustable factor).[3,4] However, the Martin-Hopkins formula is also not recommended in the setting of severe hypertriglyceridemia (>4.52 mmol/L).[4] When standard formulas are not applicable, guidelines recommend direct (homogeneous) LDL-C assays.[5] These direct assays have shown problems with accuracy and standardization in patients with type 2 diabetes mellitus (T2DM) and metabolic syndrome.[6] It is not known how well direct assays perform in patients with FD. Another possibility to measure LDL-C concentrations is polyacrylamide gradient gel electrophoresis (PGGE), that separates lipoproteins based on size and stains neutral lipids (i.e. cholesterol and TG).[7] It is not known how well PGGE performs in patients with FD. The reference standard for determining LDL-C is ultracentrifugation. In FD the treatment goal is non-HDL-C.[8] Non-HDL-C is calculated as TC minus HDL-C, with TC and HDL-C measured using standard assays. The performance of non-HDL-C compared to ultracentrifugation in FD is not known. The aim of this study was to compare LDL-C concentrations using the Friedewald formula, the Martin-Hopkins formula, a direct assay and PGGE to ultracentrifugation in FD patients. Furthermore, we compared non-HDL-C concentrations measured by standard assays to ultracentrifugation.

Methods

Study design and patients

For this study data from 28 patients with genetically confirmed FD from the placebo arm of the EVOLVE-FD (Effects of EVOLocumab VErsus placebo added to standard lipid-lowering therapy on fasting and post fat load lipids in patients with Familial Dysbetalipoproteinemia) trial were used. The design and rationale of the EVOLVE-FD study were previously described.[9] In short, this was a multicenter, randomized, placebo-controlled, double-blind, crossover study (*Supplementary Figure 1*). The study investigated the effect of evolocumab 140 mg on top of standard lipid-lowering medication compared with placebo. A FD genotype (an ε2ε2 genotype or a pathogenic dominant *APOE* variant associated with a FD phenotype) confirmed by genotyping or isoelectric focusing was required for participation. A complete list of in- and exclusion criteria was previously described.[9] During the study patients received an oral fat load that consisted of unsweetened fresh cream. Venous blood samples were collected before and up to 8 hours after the oral fat load. The study was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice. The study was approved by the Medical Ethics Review Committee of the UMC Utrecht and each patient provided written informed consent. The EVOLVE-FD study was registered at www.clinicaltrials.gov (NCT03811223).

Laboratory measurements

Density gradient ultracentrifugation

Density gradient ultracentrifugation[10] was performed by the laboratory of Vascular Medicine at the Erasmus University Medical Center, Rotterdam, the Netherlands. This method was used to measure the cholesterol content in the chylomicron, VLDL, IDL, LDL and HDL fractions. A detailed description of the procedure is provided in the *Supplementary Methods*.

Total cholesterol, HDL-cholesterol and triglycerides

All three were measured with an Atellica CH Analyzer (Siemens Healthcare Diagnostics). These analyses were performed at the Laboratory Department of the UMC Utrecht according to standard procedures.

Friedewald formula and Martin-Hopkins formula

The Friedewald and Martin-Hopkins formulas were used to calculate LDL-C based on TC, HDL-C and TG levels. LDL-C concentrations (in mmol/L) based on the Friedewald formula, were calculated as follows: TC minus HDL-C minus TG/2.2.[1] LDL-C concentrations based on the Martin-Hopkins formula were calculated as follows: TC minus HDL-C minus TG/an adjustable factor. This factor was selected from a previously published table based on the patient's non-HDL-C and TG values in mmol/L.[11]

Homogeneous direct assay
Homogeneous LDL-C was measured with an enzymatic colorimetric test (Human, Wiesbaden, Germany) and performed at the Laboratory Department of the UMC Utrecht. This assay combined two steps; the first step removed chylomicrons, VLDL and HDL. The second step determined LDL-C by enzymatic reactions, employing specific surfactants for LDL.

Polyacrylamide gradient gel electrophoresis
The analyses of non-denaturing polyacrylamide gradient gels were performed by the laboratory of Chemical Pathology at the University of Cape Town, South Africa. The preparation of PGGE was previously described.[7] Details with regard to this procedure are provided in the *Supplementary Methods*.

Non-HDL-cholesterol
Non-HDL-C was calculated as total cholesterol minus HDL-cholesterol. TC and HDL-C were measured with standard assays and compared to ultracentrifugation with non-HDL-C defined as cholesterol levels in the chylomicron, VLDL, IDL and LDL fractions.

Data analyses
Paired *t*-tests were used to evaluate the differences between the four methods and gradient density ultracentrifugation. Furthermore, differences between the four methods and ultracentrifugation were analyzed and Pearson correlation coefficients were used to determine their correlation. Linear regression analysis were used to fit regression lines in the correlation plots. The correlation and differences were stratified by TG levels. A TG concentration <1.7 mmol/L was defined as normotriglyceridemia, TG <4.52 mmol/L is often used as the cut-off for using the Friedewald and Martin-Hopkins formula and TG <9 mmol/L was the maximum concentration for the total study population (based on the exclusion criteria of the study). Bland-Altman plots were used to visually assess the agreement between the investigated methods and ultracentrifugation. Similar analyses were performed for non-HDL-C by comparing standard assays and ultracentrifugation.

Several sensitivity analyses were performed. First, fasting and non-fasting LDL-C and non-HDL-C concentrations up to eight hours after the oral fat load were compared. Second, we stratified LDL-C concentrations according to high and low lipoprotein (a) (Lp(a)) levels. This was performed for all methods, except for PGGE, because the LDL fraction on PGGE does not contain Lp(a). High Lp(a) levels were defined as the 80th percentile (>50 mg/dL) in accordance with previous literature, since above this threshold cardiovascular disease (CVD) risk is increased.[12] Third, we stratified LDL-C concentrations

for (types of) lipid-lowering medication. There were no missing values for standard laboratory, ultracentrifugation or PGGE samples. All analyses were performed with R statistical software (Version 3.5.1; R foundation for Statistical Computing, Vienna, Austria). All *p*-values were two-tailed, with statistical significance set at 0.05.

Results

Baseline characteristics

The baseline characteristics of the 28 FD patients are presented in *Table 1*. The mean age was 62 ± 9 years and 12 patients (43%) were female. Overall, 25% had CVD and 32% had T2DM. Twenty-six patients (93%) used lipid-lowering therapy; most patients used a combination of a statin and ezetimibe (29%) or a statin and a fibrate (29%). In addition, 25% patients used a high-intensity statin. At baseline, mean total cholesterol was 4.9 ± 1.9 mmol/L, median TG 2.8 (IQR 1.8–3.5) mmol/L and mean HDL-C was 1.3 ± 0.4 mmol/L. *Supplementary Figure 2* shows the distribution of fasting TG across the study population.

LDL-C concentrations according to different diagnostic methods

With density gradient ultracentrifugation the mean LDL-C concentration was 0.6 ± 0.3 mmol/L. With the Friedewald formula, the LDL-C concentration was significantly higher with 2.1 ± 1.2 mmol/L (p<0.001). The LDL-C concentration calculated with the Martin-Hopkins formula was 2.6 ± 1.1 (p<0.001 compared to ultracentrifugation). The LDL-C concentration measured by a direct assay was 1.8 ± 0.8 mmol/L (p<0.001). Lastly, the LDL-C concentration measured with PGGE, was 0.07 ± 0.05 mmol/L, which was significantly lower compared to ultracentrifugation (p<0.001) (*Figure 1*).

Differences

Friedewald, Martin-Hopkins and the direct assay all overestimated mean LDL-C by on average at least 1 mmol/L compared to ultracentrifugation. In contrast, PGGE underestimated mean LDL-C concentration by approximately 0.5 mmol/L on average. Including only patients with TG <4.52 mmol/L (n=22) did not change the results. When including only patients with normal TG (<1.7 mmol/L) (n=4) there were fewer outliers, but there was still an overestimation of LDL-C concentrations by Friedewald, Martin-Hopkins and the direct assay and an underestimation by PGGE (*Supplementary Figure 3*).

Table 1. Baseline characteristics

	Patients n=28
Age (years)	62 ± 9
Female sex (n,%)	12 (43)
APOE genotype (n,%)	
• ε2ε2	26 (93)
• Dominant *APOE* variant	3 (11)
Cardiovascular disease (n,%)	7 (25)
Diabetes mellitus type 2 (n,%)	9 (32)
Hypertension (n,%)	22 (79)
Metabolic syndrome (n,%)	21 (75)
Lipid-lowering treatment (n,%)	26 (93)
• Statin only	6 (21)
• Ezetimibe only	2 (7)
• Fibrate only	1 (4)
• Statin + ezetimibe	8 (29)
• Statin + fibrate	8 (29)
• Statin + ezetimibe + fibrate	1 (4)
High intensity statin (n,%)	7 (25)
Current smoking (n,%)	1 (4)
Body mass index (kg/m^2)	29.5 ± 3.6
Laboratory measurements	
• Total cholesterol (mmol/L)	4.9 ± 1.9
• Triglyceridesa (mmol/L)	2.8 (1.8 – 3.5)
• Non-HDL-cholesterol (mmol/L)	3.6 ± 1.7
• HDL-cholesterol (mmol/L)	1.3 ± 0.4
• Apolipoprotein B (g/L)	0.8 ± 0.2
• Lipoprotein (a)[a] (mg/dL)	8.2 (3.3 – 31.2)

Twenty-six patients had an ε2ε2 genotype (93%), two patients had a dominant variant in *APOE* and one patient had an ε2ε2 genotype and a dominant variant in *APOE* (n=3, 11%).
Data shown as mean with standard deviation (SD) or number (n) with percentage (%) unless stated otherwise.
[a] median with interquartile range.

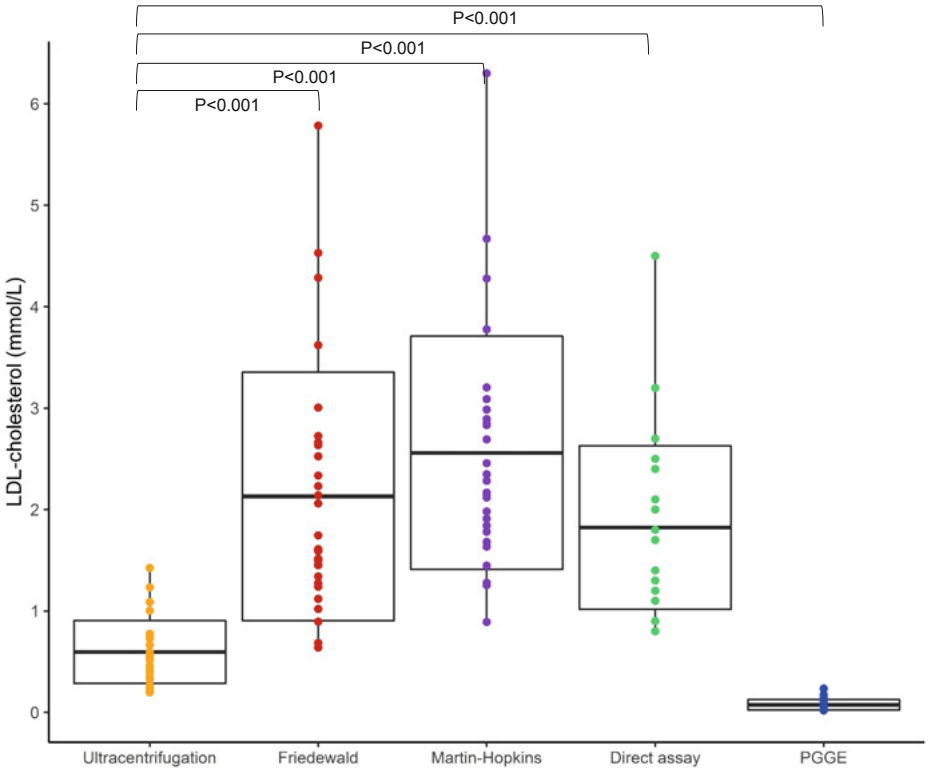

Figure 1. LDL-C concentration in patients with FD (n=28)

Box represents mean with standard deviation

Correlation and agreement

The Friedewald (R^2=0.62, *p*=<0.01), Martin-Hopkins (R^2=0.50, *p*=0.01), and direct assay (R^2=0.41, *p*=0.03) were significantly correlated with density gradient ultracentrifugation, and PGGE was not (R^2=0.18, *p*=0.37) (*Figure 2*). To evaluate agreement, the difference between the four diagnostic methods and ultracentrifugation (defined as LDL-C bias) was plotted against their mean in the Bland-Altman plots (*Figure 3*). All four methods over- or, in case of PGGE, underestimated LDL-C compared with ultracentrifugation. This difference depended on the mean value between the two measurements, reflecting proportional bias, indicating there was no systematic under- or overestimation for any of the methods compared to ultracentrifugation.

Non-HDL-cholesterol

Mean non-HDL-C was 3.6 ± 1.4 mmol/L and 3.5 ± 1.4 mmol/L (*p*=0.43) measured with standard assays and UC respectively (*Figure 4*). Stratification by TG levels did not change the results (*Supplementary Figure 4*). Non-HDL-C measured with standard

assays and ultracentrifugation showed good correlation (R^2=0.81, p=<0.001) and agreement, without over- or underestimation or proportional bias in the Bland-Altman plots (*Figure 5A* and *5B*).

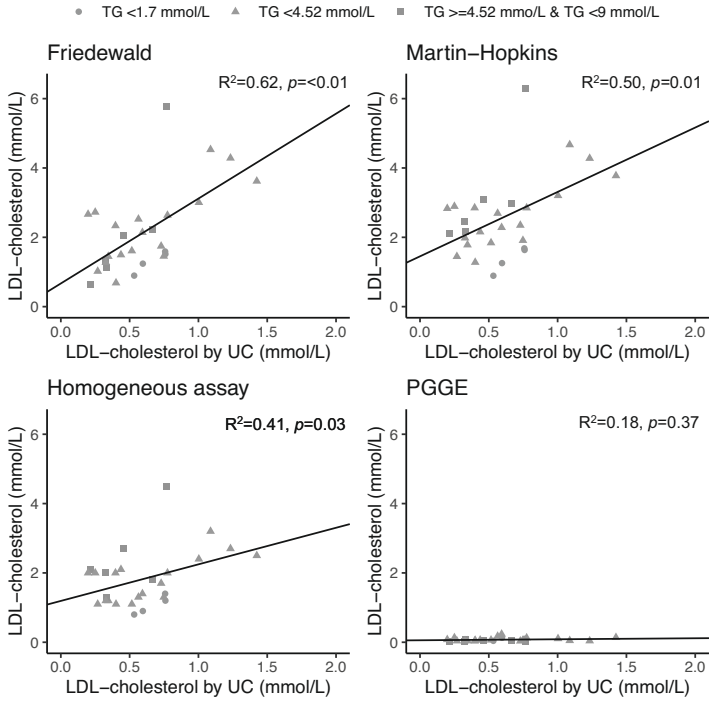

Figure 2. Correlation between diagnostic methods and ultracentrifugation

Scatter plots with regression lines and correlation coefficients (R^2), stratified for triglyceride levels.

PGGE = polyacrylamide gradient gel electrophoresis, TG = triglycerides, UC = ultracentrifugation, R^2 = correlation coefficient, LDL= low-density lipoprotein

8

Sensitivity analyses

LDL-C values measured with ultracentrifugation were the same after an oral fat load compared to the fasting values. The direct assay and PGGE also show very stable LDL-C concentrations before and after the oral fat load, while the LDL-C concentrations calculated with the Friedewald and Martin-Hopkins formulas decreased after an oral fat load, due to increasing TG concentrations (*Supplementary Figure 5A*). Non-HDL-C concentrations measured with standard assays and ultracentrifugation were the same before and after an oral fat load (*Supplementary Figure 5B*). Median Lp(a) concentrations for this study population were 8.2 (IQR 3.3-31.2) mg/dL. Three patients had Lp(a) concentrations >50 mg/dL. The distribution of Lp(a) is provided

in *Supplementary Figure 6*. The Friedewald, Martin-Hopkins and direct assay overestimated LDL-C concentrations compared with ultracentrifugation, independent of Lp(a) levels. However, the overestimation was less remarkable in patients with high Lp(a) concentrations (*Supplementary Table 1*). Non-HDL-C measurement with standard assays was independent of Lp(a) concentrations.

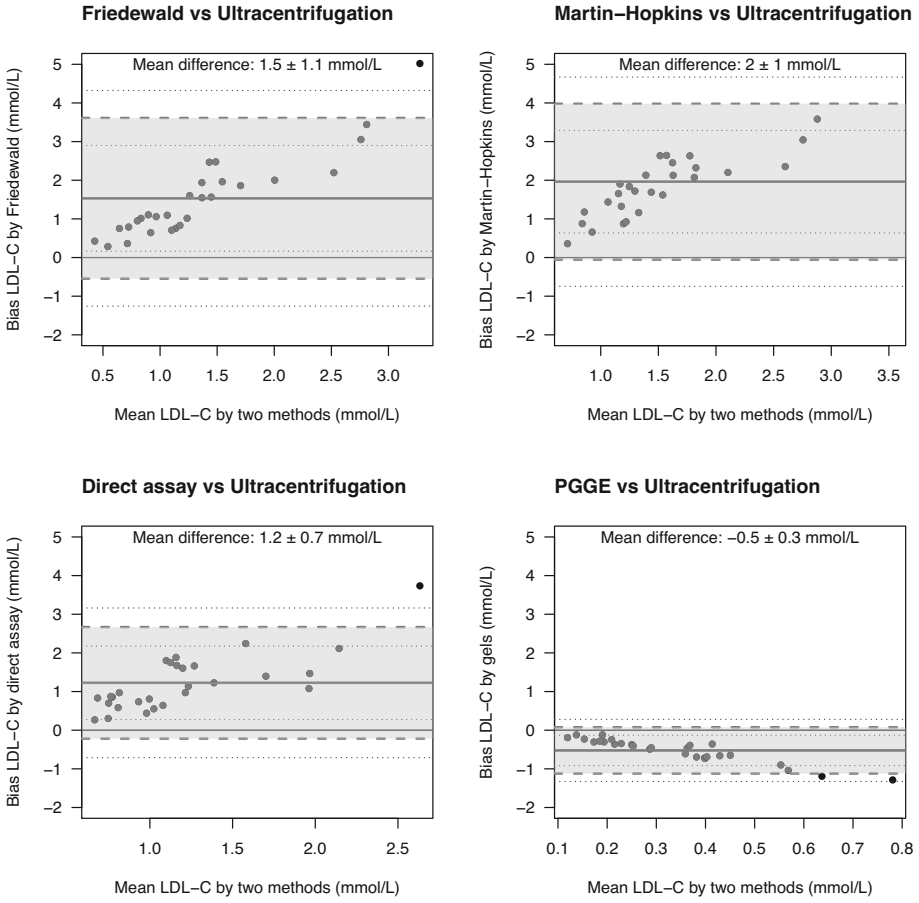

Figure 3. Agreement between diagnostic methods and ultracentrifugation (Bland-Altman plots)

Bland-Altman plots showing four diagnostic methods versus ultracentrifugation to measure LDL-C in patients with FD. The blue line is the mean difference between the two methods. The upper and lower limits of agreement (red dashed lines) are the mean difference ± 1.96 × standard deviation. Mean LDL-C (x-axis) is calculated per patient by adding the LDL-C values from both methods and dividing by 2. The LDL-bias (y-axis) is calculated per patient by subtracting the LDL-C value of the diagnostic method from the LDL-C value measured by ultracentrifugation

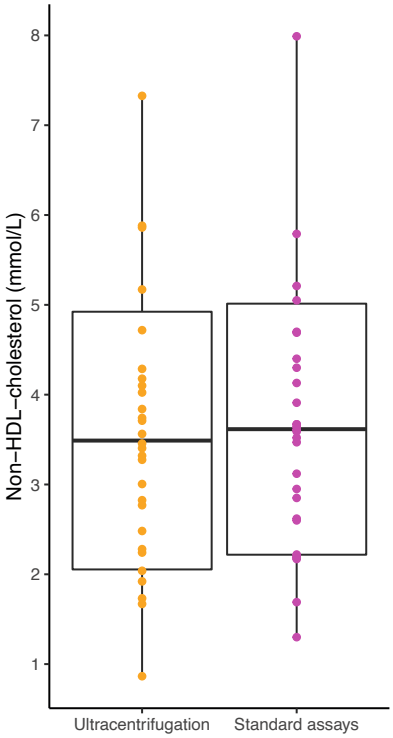

Figure 4. Non-HDL-C concentration in patients with FD (n=28)
Box represents mean with standard deviation

The Friedewald, Martin-Hopkins and direct assay overestimated LDL-C concentrations compared with ultracentrifugation in all types of lipid-lowering medication, whereas PGGE underestimated LDL-C concentrations in all types of lipid-lowering medication. However, the over- or underestimation was less extreme when patients were using evolocumab. For non-HDL-C there were no important differences with regard to lipid-lowering medication (*Supplementary Table 2*).

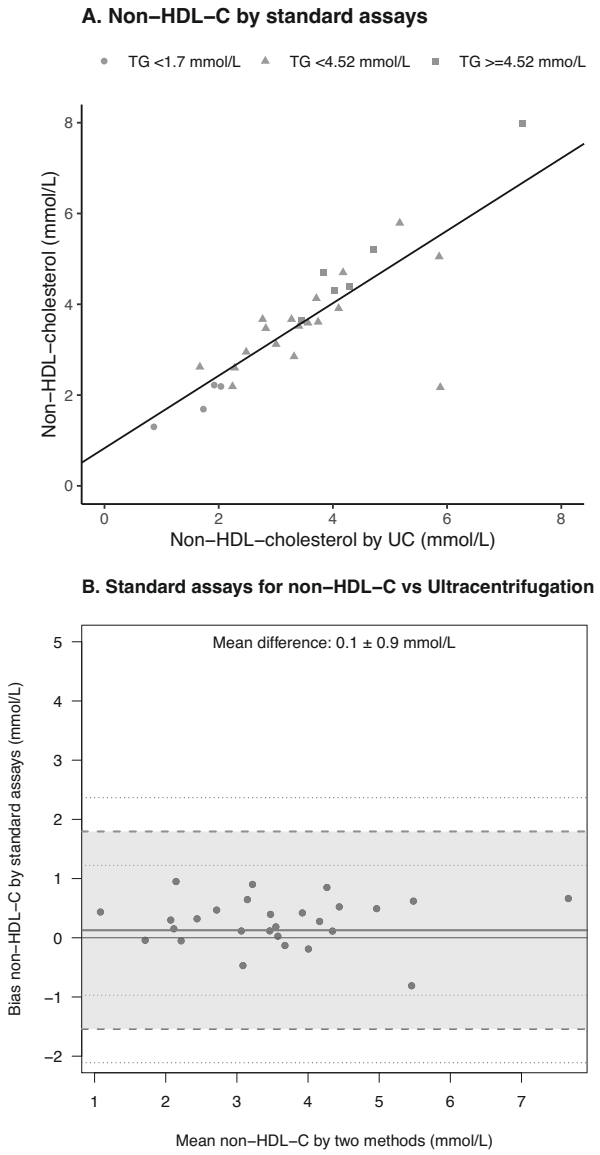

Figure 5. Correlation and agreement of non-HDL-cholesterol

A. Scatter plot with regression line and correlation coefficient of non-HDL-C (measured with standard assays for total cholesterol and HDL-cholesterol) versus non-HDL-C measured by ultracentrifugation (defined as cholesterol content in the chylomicron, VLDL, IDL and LDL fraction), stratified for triglyceride levels.

B. Bland-Altman showing non-HDL-C measured with direct assay versus non-HDL-C measured by ultracentrifugation. The blue line is the mean difference. The upper and lower limits of agreement (red dashed lines) are the mean difference ± 1.96 × standard deviation.

UC = ultracentrifugation, non-HDL-C = non-HDL-cholesterol.

Discussion

In patients with FD the four diagnostic methods under investigation (i.e. Friedewald formula, Martin-Hopkins formula, direct homogenous assay and PGGE) over- or underestimated LDL-C concentrations compared to density gradient ultracentrifugation. The results showed that neither the Friedewald formula nor the Martin-Hopkins formula nor the direct homogeneous assay can be used in patients with FD. To the contrary, non-HDL-C measured with standard assays performed well compared to density gradient ultracentrifugation, underscoring the importance of using non-HDL-C instead of LDL-C as treatment goal in FD.

The use of LDL-C is not recommended in FD, for several reasons. First, as was shown in this study, LDL-C in FD cannot be estimated reliably by formulas or measured in routine clinical laboratories. Second, although LDL-C is usually low or absent in FD patients, they have a very high CVD risk. Therefore, LDL-C is not a reliable marker to estimate risk nor an appropriate treatment goal in FD. There are two mechanisms that contribute to the relatively low plasma concentrations of LDL in FD patients. First, it is a consequence of the impaired lipolysis from the VLDL delipidation cascade to LDL, as apolipoprotein E2 (apoE2) displaces apolipoprotein C2, the cofactor of lipoprotein lipase, and the action of hepatic lipase on remnants is impaired by apoE2, by mechanisms yet unknown.[13] Second, the very low binding affinity of the apoE2 protein to the low-density lipoprotein-receptor (LDL-R) leads to a reduced influx of remnants into the liver, which leads to an upregulation of LDL-R, resulting in a greater internalization of LDL, which requires apoB100 for uptake by the LDL-R.[13,14]

For this study density gradient ultracentrifugation was used while the reference method to measure LDL-C recommended by the Center for Disease Control is beta-quantification. The difference between these two methods is very small. For beta-quantification the chylomicron, VLDL and IDL fractions are cut out, after which LDL is precipitated and cholesterol in this fraction is measured, whereas in density gradient ultracentrifugation the tube is fractionated and the fractions HDL, LDL, IDL and VLDL are pooled separately. Subsequently, cholesterol in these fractions is determined. The latter method requires more steps and is therefore more prone to error. The problem with both methods is that they can include cholesterol in remnants and Lp(a) in the LDL-C fraction. This can happen when the cut-off between remnants and LDL is not clear, which is often the case in FD. Therefore, the validity of ultracentrifugation as the reference standard in FD needs consideration because it can overestimate 'true' LDL-C concentrations in FD.

PGGE, which separates lipoproteins based on size, might therefore be a better estimation of 'true' LDL-C in FD then ultracentrifugation. The results of the present study are consistent with previous findings from a study in 64 patients with ultracentrifugally proven FD of whom 43% had no detectable LDL on PGGE. In patients that were untreated (n=39) this was 72%.[7] Although LDL-C concentrations derived with PGGE were low in our study, the LDL-C measurement may still be an overestimation since PGGE measures neutral lipid and LDL particles can be TG-enriched in FD.[15] Although PGGE is not available in clinical practice, it could be an attractive alternative to ultracentrifugation because of its lower costs and possible higher accuracy in FD. However, whether PGGE is a more suitable measurement of LDL-C in FD is very difficult to test due to lack of a suitable reference standard.

Although it has been known since 1972 that in FD the Friedewald formula underestimates VLDL-C and subsequently overestimates LDL-C, many laboratories today still report LDL-C concentrations in patients with FD and physicians use it to estimate cardiovascular risk and as treatment goal. The Martin-Hopkins formula was developed in 2013 for patients with low LDL-C and/or (mildly) increased TG.[5] Although low LDL-C and increased TG are also found in dysbetalipoproteinemia, the present study showed that the Martin-Hopkins formula resulted in overestimation of LDL-C concentrations compared to density gradient ultracentrifugation in FD patients. The Martin-Hopkins formula was not validated in patients with TG >4.52 mmol/L but we found the same overestimation in FD patients irrespective of TG concentration. These results suggest that the VLDL-C to VLDL-TG ratio changes differently in FD than is assumed by the Martin-Hopkins formula or that other mechanisms play a role in estimating LDL-C in FD. The latter is supported by the original publication of the Martin-Hopkins formula[4] that showed that one-third of the variance in the VLDL-C to VLDL-TG ratio is not explained by the standard lipid profile. Of the total validation dataset of the Martin-Hopkins formula, which included 1.35 million people, 446 were found to have a dysbetalipoproteinemia phenotype (based on ultracentrifugation (defined as VLDL-C/TG ratio >0.3, TG>130 mg/dL, and LDL-C<90th percentile), but not genetically confirmed). They found that the largest discordance of all types of hyperlipoproteinemia was found in a dysbetalipoproteinemia phenotype and therefore acknowledge limitations of the use of the Martin-Hopkins formula in the setting of FD.[4] It is good to note that the performance of the Martin-Hopkins formula in FD patients was only tested against a direct LDL-C assay and not ultracentrifugation. One other study used the Martin-Hopkins formula to estimate LDL-C concentrations in a cohort with FD patients (with TG levels <4.5 mmol/L), and found (depending on the definition of FD used) median LDL-C concentrations between 2.6 (2.0–3.5) and 3.6 (2.6 – 4.5) mmol/L, which is in line with the mean LDL-C values we found using this formula.[16] The results were not compared to ultracentrifugation. The EAS/EFLM guidelines endorse that the Martin-Hopkins formula is preferred to the Friedewald

formula in patients with TG levels between 2.0 and 4.5 mmol/L,[5] but the present study demonstrates that neither Friedewald nor Martin-Hopkins is applicable in FD patients, including FD patients with TG <4.52 mmol/L.

Direct chemical assays are often used to measure LDL-C when standard formulas are not applicable. Although it is recommended by the EAS/EFLM guidelines that direct assays for LDL-C should be used when TG levels are >4.52 mmol/L (which is the limit of use of Friedewald and Martin-Hopkins formulas), they acknowledge that direct assays do not necessarily yield accurate measurements of LDL-C in every patient.[5] In clinical practice, a direct LDL-C assay is often used to measure LDL-C in patients with FD but the 'lipoprotein specific' surfactant might not discriminate perfectly between LDL and remnant lipoproteins.[17] This is also the reason why direct assays have limited accuracy in patients with high TG and mixed dyslipidemia (which often includes remnant lipoproteins).[5,18,19] In line with the findings in the present study, two studies evaluated different direct LDL-C assays in 348 patients with and without several types of dyslipidemia, including 6 patients with FD. Both studies showed that LDL-C concentrations were overestimated with most direct assays compared with beta quantification in FD patients.[17,18] Taken together, all results suggest that direct assays should not be used to measure LDL-C in FD.

Non-HDL-C calculated based on standard assays of TC and HDL-C showed good correlation and agreement compared to ultracentrifugation and confirmed that non-HDL-C can be reliably measured in FD. Non-HDL-C is therefore the lipid measurement of choice to use as treatment goal in FD.

The strengths of this study include the well-characterized and relatively large FD population, the systematic measurement of LDL-C and non-HDL-C with different laboratory methods, with extensive measurement of postprandial lipids in patients on different (combinations of) lipid-lowering medication. Some limitations should be considered. Firstly, only one homogeneous assay for the direct measurement of LDL-C was evaluated, while the results might be highly dependent on the manufacturer.[17] Although there is no evidence that other direct assays would perform very differently compared to the homogeneous LDL-C assay evaluated in this study, the results are should be applied to other direct assays with care. Second, patient samples were analyzed after different freezing periods. The direct assay was usually analyzed within 24 hours, while ultracentrifugation and PGGE were analyzed after variable intervals of up to three months. Although freezing could have variable influence on the different lipoprotein classes[20], it is not known whether this happened in our samples and if so, whether this influenced the results.

All four methods to determine LDL-C in patients with FD investigated here, either over- or underestimated LDL-C concentrations compared with density gradient ultracentrifugation. In FD patients the Friedewald formula, Martin-Hopkins formula and the direct homogeneous assay should not be used. Although PGGE underestimated LDL-C values compared to ultracentrifugation, it might reflect 'true' LDL-C in FD better. In contrast, non-HDL-C performed well, emphasizing the use of non-HDL-C as treatment goal in FD instead of LDL-C.

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Supplementary material

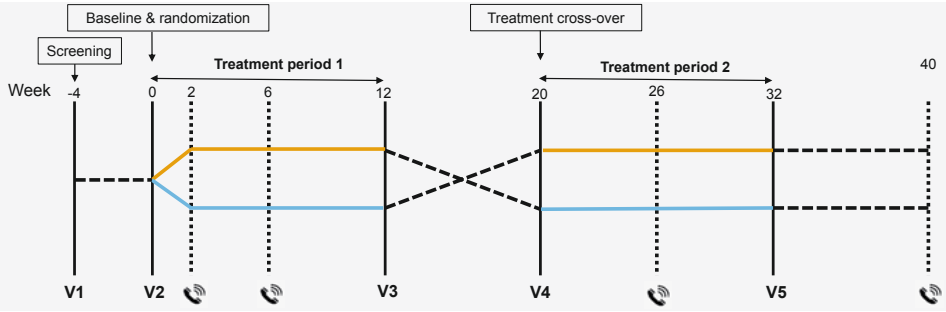
Supplementary Methods

Density gradient ultracentrifugation

KBr (0.35 g/mL plasma) was added to plasma to obtain a density of 1.26 g/mL. Of this plasma 1 mL was placed in an ultracentrifuge tube, followed by 1.9 ml of KBr solutions (of 1.21, 1.10, 1.063, 1.04 and 1.02 g/mL) in physiological salt and 1 mL of water. Samples were centrifuged for 30 minutes at 20°C at 207.000 g using a SW41 rotor in an Optima XPN-80 ultracentrifuge (Beckman Instruments, Indianapolis, IN, USA). After this chylomicrons were isolated from the top 1 mL of the tube and this volume was replaced with 1 mL water before further centrifugation at 207.000 g for 18 hours at 4 °C, using the same rotor and centrifuge. After centrifugation the fractions of 250 microL were eluted from the bottom of the tube. LDL was recovered in fractions with densities ranging from 1.04 to 1.063 g/mL. Cholesterol was measured using a Selectra E Analyzer (DDS Diagnostic system, Istanbul, Turkey).

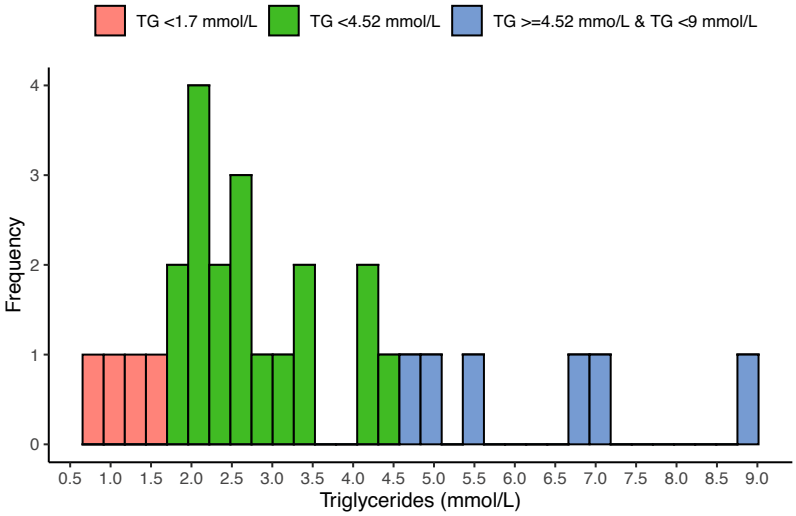
Polyacrylamide gradient gel electrophoresis

Neutral lipids (cholesterol and TG) were prestained with Sudan Black. The gels were calibrated with lipoprotein fractions (VLDL1, VLDL2, IDL, and LDL) isolated by density gradient ultracentrifugation from controls. Gels were placed in the photographic chamber of a Bio-Rad Gel Doc EZ Imager (Bio-Rad Laboratories, Johannesburg, South Africa) and images were captured by the mounted video camera. The image was digitized for further analysis of the lanes by video densitometry in the free-imaging processing software ImageJ.[20] Subsequently, the density plots were analyzed with R statistical software (Version 3.5.1; R foundation for Statistical Computing, Vienna, Austria). The migration range (in inches) of the lipoprotein fractions was standardized and expressed in terms of the retardation factor (Rf), for which the beginning of the separation gel was taken as zero and the end of small dense LDL as one. The cut-offs of the markers were determined automatically for each gel, and placed at the point where the relative intensity of the next marker was higher than the previous one. The area under the curve (AUC) for the LDL fraction was calculated. It was assumed that all neutral lipids in LDL consisted of cholesterol and therefore it could be used to estimate LDL-C levels. To estimate the absolute cholesterol concentration, the AUC of the lanes were compared with ultracentrifugally prepared LDL of several predefined LDL-C concentrations.

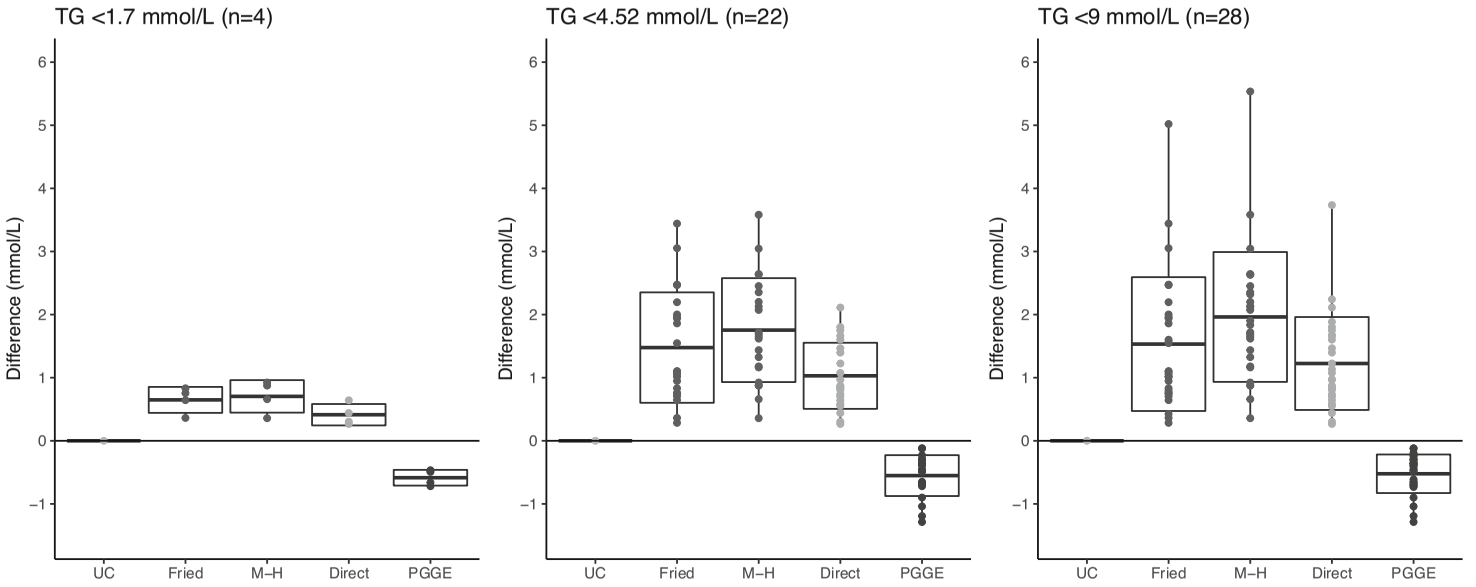


Supplementary Figure 1. Study design EVOLVE-FD

At visit 2, 3, 4 and 5 an oral fat load was given and during 8 hours blood was drawn. Patients were randomized to treatment order, meaning that all patients used both evolocumab and placebo during the study. At week 2, 6, 26 and 40 there were phone calls to assess adherence to study medication, the injecting procedure and potential adverse events. Depending on randomization order, patients received evolocumab (orange) and placebo (blue) in the first or second treatment period.



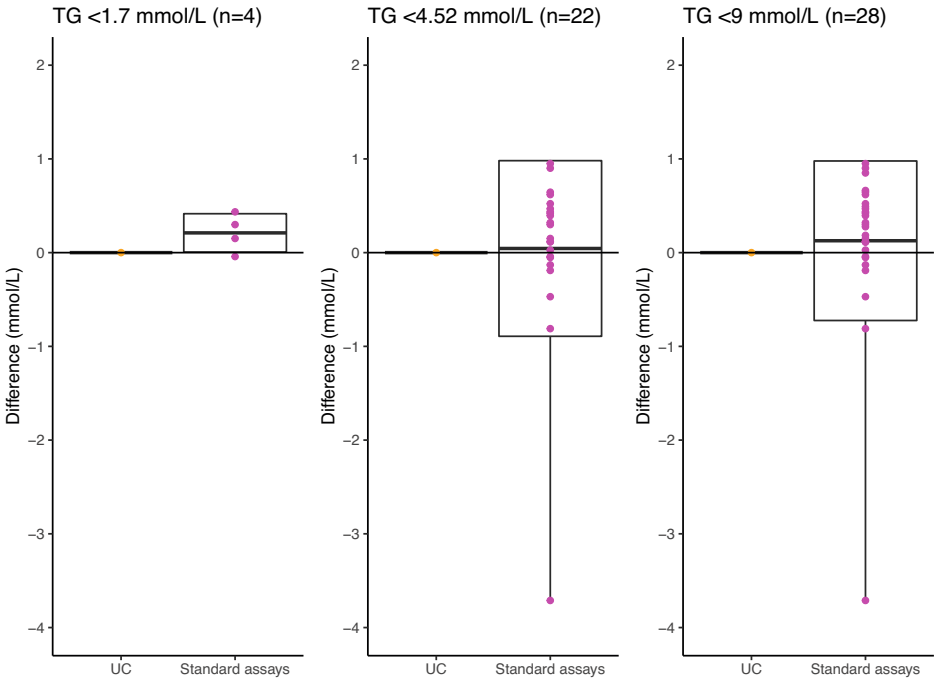
Supplementary Figure 2. Distribution of fasting triglyceride levels



Supplementary Figure 3. Difference in LDL-C concentration between diagnostic methods and ultracentrifugation

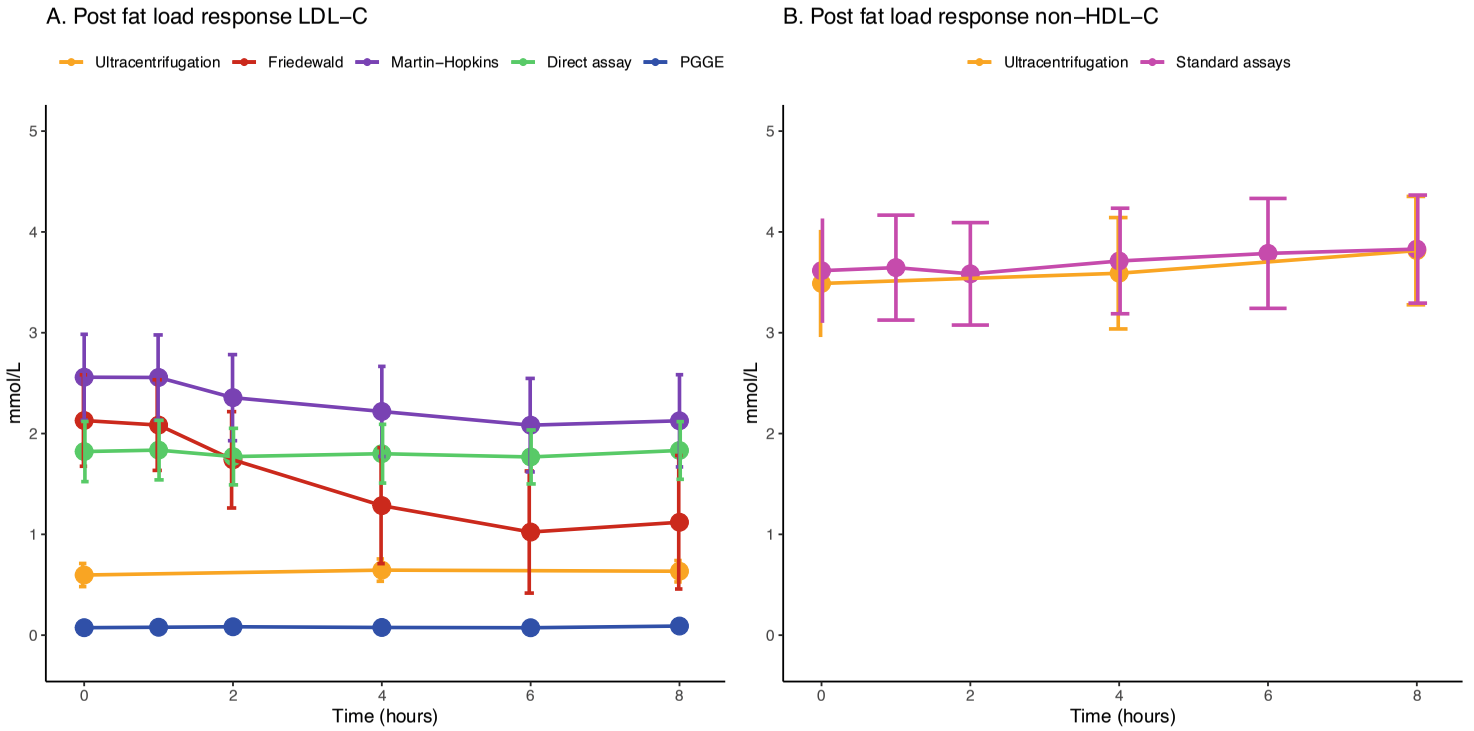
Box represents mean with standard deviation.

UC = density gradient ultracentrifugation, Fried= Friedewald formula, M-H = Martin-Hopkins formula, Direct = direct assay, PGGE = Polyacrylamide gradient gel electrophoresis. TG= triglycerides.

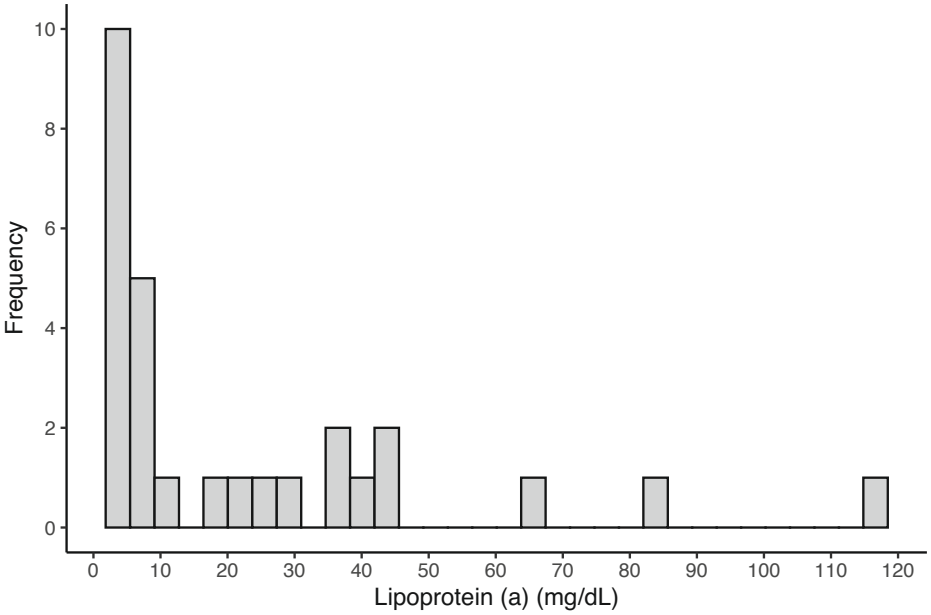


Supplementary Figure 4. Difference in non-HDL-C concentration between standard assays and ultracentrifugation

UC = ultracentrifugation, TG = triglycerides



Supplementary Figure 5. Fasting and post fat load LDL-C and non-HDL-C concentrations



Supplementary Figure 6. Distribution of Lp(a) levels

Supplementary Table 1. Difference in LDL-C and non-HDL-C concentration, stratified for Lp(a) levels

Diagnostic method compared to UC	**Total n=28**	**Lp(a) levels <50 mg/dL n=25**	**Lp(a) levels >50 mg/dL n=3**
LDL-C*			
Friedewald			
Mean difference (mmol/L)	1.5 ± 1.1	1.5 ± 1.0	1.4 ± 1.4
Martin-Hopkins			
Mean difference (mmol/L)	2.0 ± 1.0	2.0 ± 1.0	1.4 ± 1.4
Direct assay			
Mean difference (mmol/L)	1.2 ± 0.7	1.3 ± 0.7	0.8 ± 0.6
Non-HDL-C			
Standard assays vs UC			
Mean difference (mmol/L)	0.1 ± 0.9	0.0 ± 0.9	-0.03 ± 0.7

*These analyses were not performed for PGGE, because the LDL fraction on PGGE does not contain Lp(a).

Supplementary Table 2. Difference in LDL-C and non-HDL-C concentration, stratified for type of lipid-lowering medication

Diagnostic method compared to UC	Total	Any lipid-lowering	Statins	Fibrates	Eze-timibe	Evolo-cumab
	n=28	n=26	n=23	n=10	n=11	n=28
LDL-C						
Friedewald						
Mean difference (mmol/L)	1.5 ± 1.1	1.4 ± 1.0	1.3 ± 1.0	1.8 ± 1.3	1.2 ± 0.9	0.4 ± 0.4
Martin-Hopkins						
Mean difference (mmol/L)	2.0 ± 1.0	1.9 ± 1.0	1.8 ± 1.0	2.2 ± 1.3	1.7 ± 0.9	0.7 ± 0.5
Direct assay						
Mean difference (mmol/L)	1.2 ± 0.7	1.2 ± 0.8	1.1 ± 0.8	1.2 ± 0.9	1.2 ± 0.7	0.4 ± 0.2
PGGE						
Mean difference (mmol/L)	-0.5 ± 0.3	-0.5 ± 0.3	-0.5 ± 0.3	-0.6 ± 0.3	-0.5 ± 0.3	-0.2 ± 0.1
Non-HDL-C						
Standard assays						
Mean difference (mmol/L)	0.1 ± 0.9	0.2 ± 0.9	0.1 ± 0.9	0.3 ± 0.3	0.04 ± 1.3	0.1 ± 0.3

When a patient is part of a specific subgroup of lipid-lowering therapy this does not reflect monotherapy. Therefore, several combinations of lipid-lowering therapy could be possible (Table 1). In addition, for the evolocumab (n=28) group, the evolocumab arm of the trial was used. This treatment was added to standard lipid-lowering therapy, thus does not reflect monotherapy with evolocumab. This means that 26 out of 28 patients were on concurrent lipid-lowering therapy (23 used a statin, 10 used a fibrate and 11 used ezetimibe).

Chapter 9

Effect of evolocumab on fasting and post fat load lipids and lipoproteins in Familial Dysbetalipoproteinemia

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In revision

Abstract

Background: Familial Dysbetalipoproteinemia (FD) is the second most common monogenic lipid disorder (prevalence 1 in 1000-2500), characterized by postprandial remnant accumulation and associated with increased cardiovascular disease (CVD) risk. Many FD patients do not achieve non-HDL-C treatment goals, indicating the medical need for additional lipid-lowering treatment options.

Objectives: To evaluate the effect of the PCSK9 monoclonal antibody evolocumab added to standard lipid-lowering therapy on fasting and post fat load lipids and lipoproteins in patients with FD.

Methods: A randomized placebo-controlled double-blind crossover trial comparing evolocumab (140 mg subcutaneous every 2 weeks) with placebo during two 12-week treatment periods. At the start and end of each treatment period patients received an oral fat load. The primary endpoint was the 8-hour post fat load non-HDL-C area under the curve (AUC). Secondary endpoints included fasting and post fat load lipids and lipoproteins.

Results: In total, 28 patients completed the study. Mean age was 62 ± 9 years and 93% had an E2E2 genotype. Evolocumab reduced the 8-hour post fat load non-HDL-C AUC with 49% (95%CI 42-55) and triglyceride AUC with 20% (95%CI 10-29). Other fasting and post fat load lipids and lipoproteins including apolipoprotein B and remnant cholesterol were also significantly reduced by evolocumab, except for HDL-C.

Conclusions: Evolocumab added to standard lipid-lowering therapy significantly reduced fasting and post fat load non-HDL-C and other atherogenic lipids and lipoproteins in FD patients. The clinically significant decrease in lipids and lipoproteins can be expected to translate into a reduction in CVD risk in these high-risk patients.

Introduction

Familial Dysbetalipoproteinemia (FD), also kown as 'remnant removal disease', is the second most common monogenic lipid disorder after heterozygous Familial Hypercholesterolemia (heFH), with an estimated prevalence of 1 in 1000 to 1 in 2500 individuals.[1] FD is characterized by accumulation of triglyceride rich lipoproteins (TRLs), especially in the postprandial phase. TRLs are atherogenic and causally related to cardiovascular disease (CVD), therefore FD patients have a very high risk of premature CVD.[1,2] Specific genetic variants in the *APOE* gene lead to a greatly reduced affinity of apolipoprotein E (apoE) for hepatic clearance receptors. In combination with a second metabolic hit (in most cases obesity and/or insulin resistance), this can lead to the typical cholesterol-enriched remnant lipid phenotype seen in FD.[3] In clinical practice, FD is often not recognized and therefore underdiagnosed. It should be suspected when a patient presents with a mixed dyslipidemia combined with relatively low apolipoprotein B (apoB) levels, and when genotyping demonstrates a specific *APOE* variant (in most cases homozygosity for ε2 allele), the diagnosis of FD is confirmed.[1]

Remnant accumulation in FD is particularly pronounced during the postprandial phase, with considerably increased and prolonged postprandial remnant lipoprotein concentrations which is associated with a very high risk of CVD.[4,5] Accumulation of TRLs is reflected in increased non-high-density lipoprotein cholesterol (non-HDL-C) levels that consist of cholesterol in all atherogenic lipoproteins such as chylomicrons, very-low-density lipoproteins (VLDL), their remnants and low-density lipoprotein (LDL). In FD patients, LDL and LDL-cholesterol (LDL-C) levels are generally low or even absent,[6,7] and thus do not adequately reflect CVD risk. Therefore, treatment goals for patients with FD are based on non-HDL-C levels. Dietary advice is recommended for all FD patients, as patients with FD generally respond well to dietary changes.[8] Furthermore, medical treatment with statins, and optionally fibrates, are recommended to achieve non-HDL-C treatment goals. In clinical practice 60% of FD patients do not achieve non-HDL-C treatment goals, indicating the need for more intensive lipid-lowering treatment.[2]

Proprotein convertase subtilisin/kexin type 9 (PCSK9) monoclonal antibodies (mAbs) neutralize circulating PCSK9 and thereby prevent degradation of the LDL-receptor (LDL-R). PCSK9 mAbs proved to lower LDL-C by 50-60%[9,10] and reduce CVD risk with 20% in high-risk patients.[11,12] In patients with type 2 diabetes mellitus (T2DM) PCSK9 mAbs effectively lower postprandial TRLs by approximately 30-40%.[13-16] The present study was designed to investigate whether the effect of PCSK9 mAbs would be similar in FD patients, since they generally have low LDL-C levels and dysfunctional apoE that does not bind to the LDL-R. The aim of the EVOLVE-FD (Effects of EVOLocumab VErsus placebo added to standard lipid-lowering therapy on fasting and post fat load lipids

in patients with Familial Dysbetalipoproteinemia) study was to evaluate the effect of evolocumab 140 mg every 2 weeks added to standard lipid-lowering therapy on fasting and post fat load lipids and lipoproteins in patients with FD.

Methods

The EVOLVE-FD trial was an investigator-initiated study conducted at four University Medical Centers (University Medical Center Utrecht, Erasmus MC University Medical Center Rotterdam, Amsterdam University Medical Center, Radboud University Medical Center Nijmegen) in the Netherlands. The study was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice. The study was approved by the Medical Ethics Review Committee of the UMC Utrecht and by the competent authority of the Netherlands. All patients provided written informed consent before study procedures were initiated.

Patients

Patients diagnosed with FD between 18 and 80 years of age, were eligible for study participation. A FD genotype (an *ε2ε2* genotype or a pathogenic dominant *APOE* variant known to associate with a FD phenotype) confirmed by genotyping or isoelectric focusing was required. FD lipid phenotype was defined as either apoB/total cholesterol (TC) ratio <0.39 mg/dL [<0.15 mmol/g][17], TC >193 mg/dL [>5 mmol/L] and triglycerides (TG) >266 mg/dL [>3 mmol/L][18], or non-HDL-C/apoB >1.43 mg/dL [>3.69 mmol/g][19], with or without lipid-lowering medication. If patients were using lipid-lowering medication the dose must have been stable for at least three months and non-HDL-C levels had to be >62 mg/dL [>1.6 mmol/L]. A complete list of in- and exclusion criteria is available in the *Supplementary methods*.

Study design and study drug

EVOLVE-FD was a multicenter, double-blind, placebo-controlled, crossover study (*Figure 1*). Patients received subcutaneous auto-injectors of evolocumab 140 mg or auto-injectors with matching placebo every 2 weeks during 2 periods of 12 weeks in a random order (both provided by Amgen, Breda, the Netherlands). Between the 2 treatment periods the washout period of 8 weeks without study medication to prevent carryover effects. This duration was chosen because the estimated half-life of evolocumab is 11-17 days.[20] After the second 12-week treatment period there was a run-out period of 8 weeks to assess any potential adverse events. Randomization for treatment order was based on computer generated randomization with variable block size, stratified for participating center. Patients and staffs were blinded for treatment and outcome measures throughout the study.

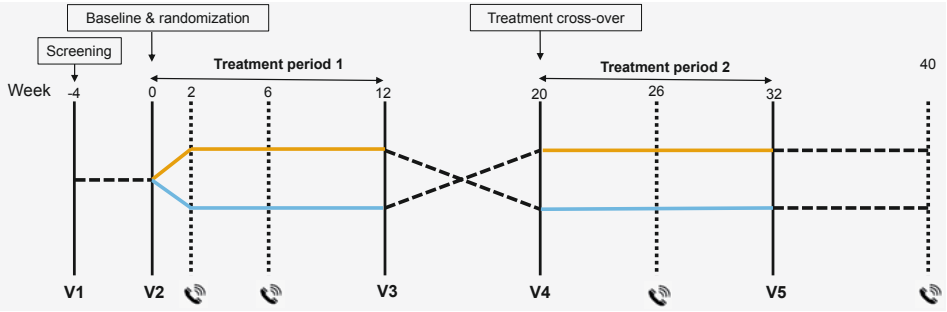

Figure 1. Cross-over study design

At visit 2, 3, 4 and 5 an oral fat load was given and during 8 hours blood was drawn. Patients were randomized to treatment order, meaning that all patients used both evolocumab and placebo during the study. At week 2, 6, 26 and 40 there were phone calls to assess adherence to study medication, the injecting procedure and potential adverse events. Depending on randomization order, patients received evolocumab (orange) and placebo (blue) in the first or second treatment period.

Study procedures, oral fat load and data collection

At every visit, each patient underwent a standardized protocol including measurements of anthropometric characteristics, blood pressure and pulse. Use of medication, consumption of alcohol, smoking and physical activity were recorded. Potential adverse events were recorded and physical examination was performed. Patients were instructed not to change their diet, alcohol use, physical activity or dose and type of standard lipid-lowering medication during the study. At the start and at the end of both 12-week treatment periods, patients visited the hospital after a ≥12 hour overnight fast and received an oral fat load. The oral fat load consisted of unsweetened fresh cream (Albert Heijn, Zaandam, the Netherlands) with a fat content of 35% (mass/volume). Cream was administered at a dose of 110 g of fat per square meter of body surface area, with a maximum of 500 mL and ingested within a 10-minute time period. Cream was chosen to challenge the metabolic system with an extreme intake of fat and because it is a standardized product. Before and 1, 2, 4, 6 and 8 hours after the oral fat load venous blood samples were collected. During these 8 hours patients were not allowed to eat or to drink (except water). Further methods including definitions and (laboratory) measurements are provided in the *Supplementary methods*.

Primary and secondary study endpoints

The primary study endpoint was the difference in the 8 hours post fat load area under the curve (AUC) in non-HDL-C after treatment of 12 weeks evolocumab compared to placebo. The AUC reflects the total exposure up to 8 hours after an oral fat load. Secondary endpoints were fasting and post fat load levels (8 hours post fat load AUC and 8 hour post fat load incremental AUC (iAUC) of non-HDL-C, TC, TG, apoB,

HDL-C, VLDL-C, remnant cholesterol (remnant-C), and fasting lipoprotein(a) (Lp(a)). The samples were analyzed in a central clinical laboratory practicing quality control for these analyses. VLDL-C and remnant-C were measured with ultracentrifugation. A detailed outline of all laboratory techniques is provided in the *Supplementary methods*. AUC was calculated with the trapezoidal rule. The iAUC was calculated after adjustment for fasting lipid levels by subtracting eight (hours)*(value at time point 0) from the AUC (*Supplementary Figure 1*). Also, baseline lipid concentrations were taken into account. In these analyses the differences in change from baseline after treatment with evolocumab and placebo were compared.

The proportion of patients who achieved their non-HDL-C treatment goals was assessed. The non-HDL-C treatment goals in FD are defined as <131 mg/dL [<3.4 mmol/L] for FD patients without CVD and <100 mg/dL [<2.6 mmol/L] for FD patients with established CVD or T2DM, according to European guidelines for patients with increased triglycerides.[21]

The safety of evolocumab was assessed through adverse event reporting and safety laboratory measurements. Adverse events for placebo and evolocumab were reported over a 20 week period, including the 12 week treatment period and the subsequent 8 week wash-out period.

Power calculation

The sample size was based on an expected reduction of 8 hours post fat load AUC non-HDL-C by evolocumab of 25% compared to placebo, which was based on a previous meta-analysis that showed a 56.1% reduction in fasting non-HDL-C by evolocumab.[22] Based on the working mechanism of evolocumab, this finding was expected to consist largely of LDL-C reduction. Patients with FD have no or little LDL-C[6,7] and therefore a conservative, but clinically relevant, 25% reduction in non-HDL-C was chosen. With a power of 85% and an alpha of 5%, 74 evaluable subjects were needed in a parallel study. For a crossover design this sample size could be reduced by 65% due to within-person controls ((1-rho)/2, with rho 0.3).[23] Therefore the required sample size for the study was 74*0.35=26 subjects that completed the study.

Data analyses

The 8 hours post fat load lipids and lipoproteins were expressed as AUC and iAUC. Absolute and percentage difference between two treatment arms were calculated and, to obtain robust confidence intervals (CIs) with corresponding *p*-values, CIs were computed by bootstrapping (1000 samples with replacement). Carryover and period effects were assessed with an independent samples t-test. No carryover (p=0.65) or

period effects (p=0.13) were observed. All clinical variables at baseline were complete, except for waist circumference (n=5). All lab variables were complete except for one apoB measurement at a single post fat load time point. Missing values were imputed with last observation carried forward. P <0.05 was considered statistically significant. All analyses were performed with RStudio, version 3.5.1.

Results

Patient disposition

Thirty-six patients were screened, and 31 patients were randomized. Reasons for screening failure were severe dyslipidemia requiring initiation of lipid-lowering treatment first and not having an *APOE* genotype that was associated with FD. During follow-up, there was 1 withdrawal of informed consent and 2 dropouts, because they did not complete all (post fat load) measurements to assess the primary endpoint, making 28 patients eligible for the analyses (*Figure 2*). Details on reasons for screening failure, withdrawal and dropout are shown in *Supplementary Table 1* and baseline characteristics of patients who withdrew consent or dropped out are shown in *Supplementary Table 2*. There were no clinically relevant differences at baseline between the patients in- and excluded in the efficacy analysis.

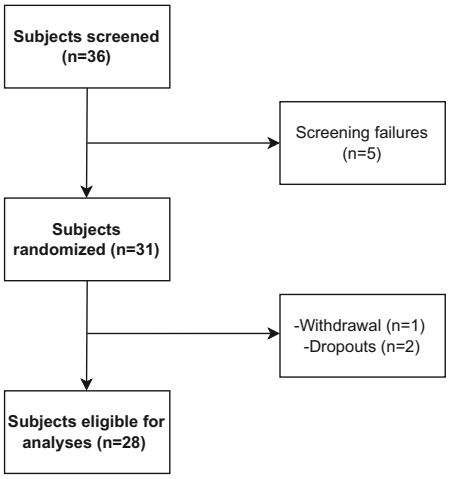

Figure 2. Patient disposition

In total, 31 patients were randomized. There was 1 withdrawal and there were 2 dropouts, resulting in 28 patients eligible for analyses. Detailed information on reasons for screening failure, withdrawal and dropout, as well as baseline information for the 3 randomized patients who did not finish the study are provided in the Supplementary Table 1 and 2.

Baseline characteristics

The mean age of the 28 FD patients who completed the study was 62 ± 9 years and 43% were women (*Table 1*). The majority (93%) of the patients had an ε2ε2 genotype, two patients had a pathogenic dominant variant in their *APOE* gene known to be associated with FD (apoE3-Leiden and p.Arg180His and one patient had an ε2ε2 genotype in combination with a dominant variant in *APOE* (p.Gly171Asp). Twenty-five percent of patients had a history of CVD, 32% had T2DM and 75% fulfilled the criteria for metabolic syndrome using the NCEP ATP III criteria at baseline.[24] Almost all (93%) patients used lipid-lowering medication, mostly a combination of a statin and ezetimibe (29%) or a statin and a fibrate (29%). Two patients were not taking lipid-lowering medication, one had mild dyslipidemia and was not taking lipid-lowering medication yet, another patient preferred to use red yeast rice only. This patient stopped using red yeast rice prior and during the study. High-intensity statins were used by 25% of the study population. Mean baseline non-HDL-C was 139 ± 66 mg/dL [3.6 ± 1.7 mmol/L] and mean baseline triglycerides were 275 ± 168 [3.1 ± 1.9 mmol/L]. The baseline table stratified for treatment group is provided in *Supplementary Table 3*.

Table 1. Baseline characteristics

	Patients n=28
Age (years)	62 ± 9
Female sex	12 (43)
APOE genotype	
• *ε2ε2*	26 (93)
• Dominant *APOE* variant	3 (11)
Cardiovascular disease	7 (25)
• Coronary heart disease	2 (7)
• Peripheral vascular disease	1 (4)
• Cerebrovascular disease	3 (11)
• Abdominal aortic aneurysm	1 (4)
Diabetes mellitus type 2	9 (32)
Hypertension	22 (79)
Metabolic Syndrome	21 (75)
Family history of premature CVD	7 (25)
Lipid-lowering treatment	26 (93)
• Statin only	6 (21)
• Ezetimibe only	2 (7)

Table 1. Continued

	Patients n=28
• Fibrate only	1 (4)
• Statin + ezetimibe	8 (29)
• Statin + fibrate	8 (29)
• Statin + ezetimibe + fibrate	1 (4)
High intensity statin	7 (25)
Current smoking	1 (4)
Body mass index (kg/m^2)	29.5 ± 3.6
Waist circumference (cm)	107 ± 11
Systolic blood pressure (mmHg)	141 ± 15
Diastolic blood pressure (mmHg)	85 ± 8
Laboratory measurements	
• Total cholesterol (mg/dL [mmol/L])	189 ± 73 [4.9 ± 1.9]
• Triglycerides (mg/dL [mmol/L])	275 ± 168 [3.1 ± 1.9]
• Non-HDL-cholesterol (mg/dL [mmol/L])	139 ± 66 [3.6 ± 1.7]
• HDL-cholesterol (mg/dL [mmol/L])	50 ± 15 [1.3 ± 0.4]
• Apolipoprotein B (mg/dL [g/L])	80 ± 20 [0.8 ± 0.2]
• Lipoprotein (a)* (mg/dL [mg/L])	8.2 (3.3 – 31.2) [82 (33 - 312)]
• Glucose (mg/dL [mmol/L])	110 ± 27 [6.1 ± 1.5]

Data are shown as n (%), mean ± standard deviation, or when not-normally distributed as median (interquartile range), indicated by *
Abbreviations: CVD = cardiovascular disease

Fasting lipids and lipoproteins

Compared with placebo the mean absolute reduction in fasting non-HDL-C levels after 12 weeks evolocumab was 75 ± 44 mg/dL [1.9 ± 1.1 mmol/L], corresponding to a 51% (95%CI 43–57) relative reduction. With the exception of HDL-C, compared with placebo all fasting lipids and lipoproteins were significantly reduced after 12 weeks treatment with evolocumab. Compared with placebo the absolute reduction in fasting triglyceride levels after 12 weeks evolocumab was 96 ± 140 mg/dL [1.1 ± 1.6 mmol/L], corresponding to a 27% (95%CI 17 – 36) relative reduction. The mean relative reduction in fasting apoB was 48% (95%CI 42–53), in fasting VLDL-C 42% (95%CI 29–53) and in fasting remnant-C 44% (95%CI 30–55). Also, compared to placebo the median absolute reduction in fasting Lp(a) levels after treatment with evolocumab was 3.4 (IQR 0.1 – 13) mg/dL, corresponding to a 35% (95%CI 16–42) relative reduction (*Table 2* and *Figure*

3). The results were similar when taking the baseline measurements into account by comparing the difference in *change* in fasting lipids and lipoproteins from baseline (*Supplementary Table 4* and *Supplementary Figure 2*).

Table 2. Effect of 12 weeks evolocumab compared to placebo on fasting lipids

		After placebo	After evolocumab	Difference	% Difference (95%CI)	P-value
Non-HDL-C	mg/dL	140 ± 54	65 ± 26	-75 ± 44	-51 (-57 – -43)	<0.001
	mmol/L	3.6 ± 1.4	1.7 ± 0.7	-1.9 ± 1.1		
Triglycerides	mg/dL	293 ± 173	197 ± 110	-96 ± 140	-27 (-36 – -17)	<0.001
	mmol/L	3.3 ± 2.0	2.2 ± 1.2	-1.1 ± 1.6		
Total cholesterol	mg/dL	189 ± 57	112 ± 32	-77 ± 46	-39 (-45 – -32)	<0.001
	mmol/L	4.9 ± 1.5	2.9 ± 0.8	-2.0 ± 1.2		
apoB	mg/dL	77 ± 19	40 ± 14	-37 ± 17	-48 (-53 – -42)	<0.001
	g/L	0.8 ± 0.2	0.4 ± 0.1	-0.4 ± 0.2		
VLDL-C	mg/dL	66 ± 36	32 ± 18	-34 ± 34	-42 (-53 – -29)	<0.001
	mmol/L	1.7 ± 0.9	0.8 ± 0.5	-0.9 ± 0.9		
Remnant-C	mg/dL	21 ± 11	10 ± 5	-12 ± 10	-44 (-55 – -30)	<0.001
	mmol/L	0.5 ± 0.3	0.3 ± 0.1	-0.3 ± 0.3		
HDL-C	mg/dL	50 ± 14	47 ± 14	-2.7 ± 7.4	-4.3 (-10 – 3.0)	0.20
	mmol/L	1.3 ± 0.4	1.2 ± 0.4	-0.1 ± 0.2		
Lp(a)*	mg/dL	7.2 (3.1 – 35)	3.7 (3.0 – 22)	-3.4 (-13 – -0.1)	-35 (-42 – -16)	<0.001
	mg/L	72 (31 – 353)	37 (30 – 216)	-34 (-132 – -1)		

Values are mean ± standard deviation. * Shown as medians (interquartile range) and percentage difference shown as median (95% confidence interval).
Abbreviations: ApoB = apolipoprotein B, AUC = area under the curve, HDL-C = high-density lipoprotein-cholesterol, Lp(a) = Lipoprotein, Non-HDL-C = high-density lipoprotein-cholesterol, remnant-C = remnant-cholesterol, VLDL-C = very-low-density lipoprotein-cholesterol

Post fat load lipids and lipoproteins

Compared with placebo the mean absolute reduction in 8 hour post fat load non-HDL-C levels after 12 weeks evolocumab was 590 ± 352 mg/dL.8h [15.3 ± 9.1 mmol/L.8h], corresponding to a 49% (95%CI 42–55) relative reduction (*Figure 3* and *Supplementary Figure 4*). Compared to placebo the mean percentage reduction in 8 hour post fat load triglyceride levels after evolocumab was 20% (95%CI 10–29). Also, the mean reduction in 8 hour post fat load apoB levels was 47% (95%CI 41–53). Eight hour post fat load levels of the other lipids and lipoproteins, including VLDL-C (45% (95%CI 32–55) and

remnant-C (49% (95%CI 38–59), were significantly reduced, except for HDL-C (3.4% (95%CI -8.5–2.1)) (*Figure 3* and *Table 3*).

There were no differences between evolocumab and placebo in the iAUC (postprandial rise) during the 8 hours after the oral fat load for any of the lipids and lipoproteins (*Supplementary Table 5*). The 8 hour post fat load results were similar when taking baseline measurements into account by comparing the difference in *change* from baseline (*Supplementary Tables 6* and *7* and *Supplementary Figures 2* and *3*). The individual responses for non-HDL-C and triglycerides after evolocumab and placebo are provided in *Supplementary Figure 5*.

Table 3. Effect of 12 weeks evolocumab compared to placebo on 8 hour post fat load lipids

		AUC after placebo	AUC after evolocumab	Difference	% Difference (95%CI)	P-value
Non-HDL-C	mg/dL.8h	1145 ± 438	555 ± 215	-590 ± 352	-49 (-55 – -42)	<0.001
	mmol/L.8h	29.7 ± 11.3	14.4 ± 5.6	-15.3 ± 9.1		
Triglycerides	mg/dL.8h	3579 ± 1878	2623 ± 1209	-956 ± 1428	-20 (-29 – -10)	<0.001
	mmol/L.8h	40.4 ± 21.2	29.6 ± 13.6	-10.8 ± 16.1		
Total cholesterol	mg/dL.8h	1531 ± 470	926 ± 260	-605 ± 362	-38 (-43 – -31)	<0.001
	mmol/L.8h	39.7 ± 12.2	24.0 ± 6.7	-15.7 ± 9.4		
apoB	mg/dL.8h	629 ± 157	329 ± 130	-296 ± 133	-47 (-53 – -41)	<0.001
	g/L.8h	6.3 ± 1.6	3.3 ± 1.3	-3.0 ± 1.3		
VLDL-C	mg/dL.8h	537 ± 296	249 ± 129	-288 ± 273	-45 (-55 – -32)	<0.001
	mmol/L.8h	13.9 ± 7.7	6.5 ± 3.3	-7.5 ± 7.1		
Remnant-C	mg/dL.8h	156 ± 83	70 ± 36	-87 ± 69	-49 (-59 – -38)	<0.001
	mmol/L.8h	4.0 ± 2.1	1.8 ± 0.9	-2.2 ± 1.8		
HDL-C	mg/dL.8h	386 ± 111	371 ± 111	-15 ± 48	-3 (-9 – 2)	0.21
	mmol/L.8h	10.0 ± 2.9	9.6 ± 2.9	-0.4 ± 1.3		

Values are mean ± standard deviation

Abbreviations: ApoB = apolipoprotein B, AUC = area under the curve, HDL-C = high-density lipoprotein-cholesterol, Non-HDL-C = high-density lipoprotein-cholesterol, remnant-C = remnant-cholesterol, VLDL-C = very-low-density lipoprotein-cholesterol

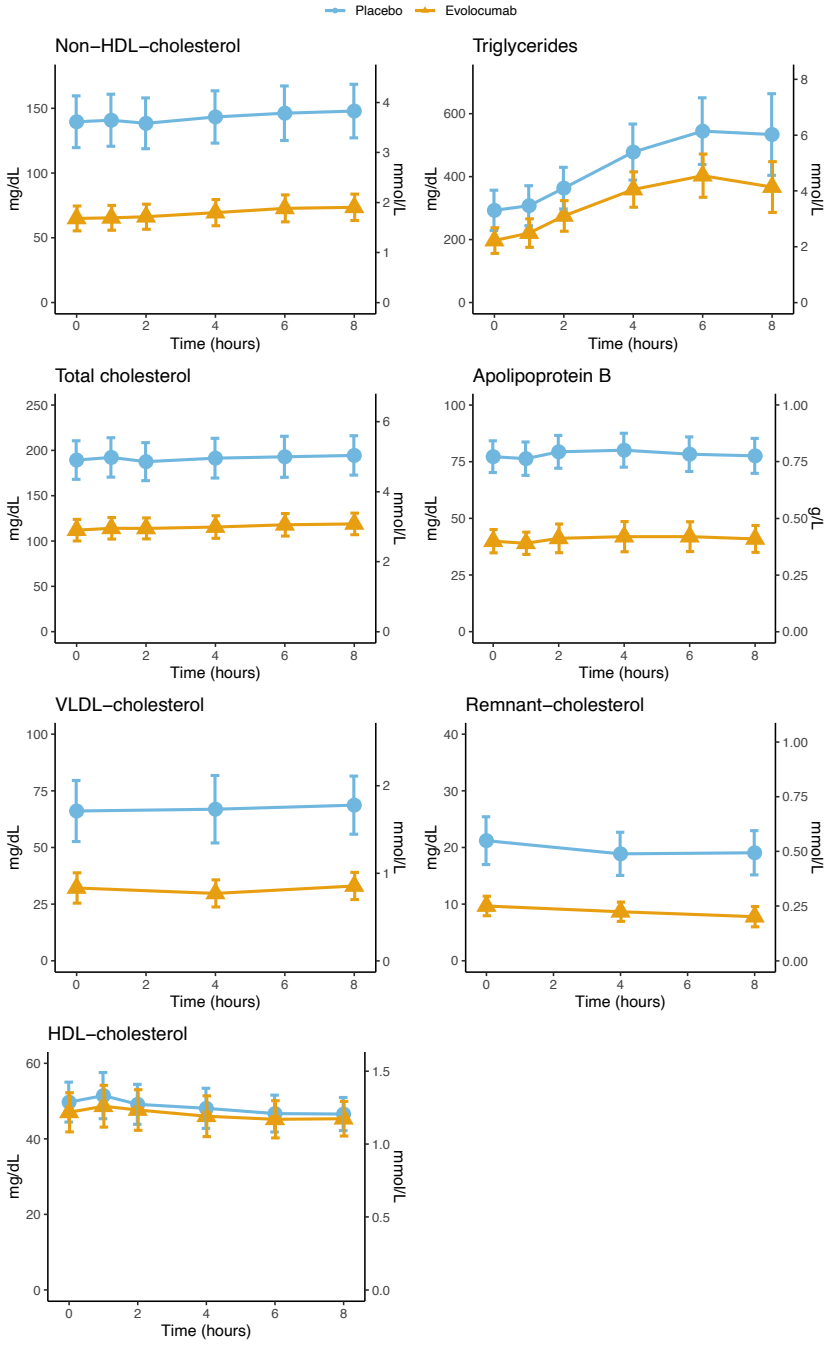

Figure 3. Effect of evolocumab and placebo on fasting and post fat load lipids and lipoproteins

Fasting and 8-hour post fat load lipid and lipoprotein levels after an oral fat load, after treatment with evolocumab (orange) or placebo (blue).

Non-HDL-C treatment goals

After 12 weeks treatment with evolocumab added to regular lipid-lowering treatment 89% of patients achieved their non-HDL-C treatment goal (<131 mg/dL [<3.4 mmol/L] or <100 mg/dL [<2.6 mmol/L]) compared with 36% after placebo. Moreover, 54% of the patients achieved a >50% reduction in non-HDL-C after 12 weeks treatment with evolocumab compared to none after placebo (*Figure 4*).

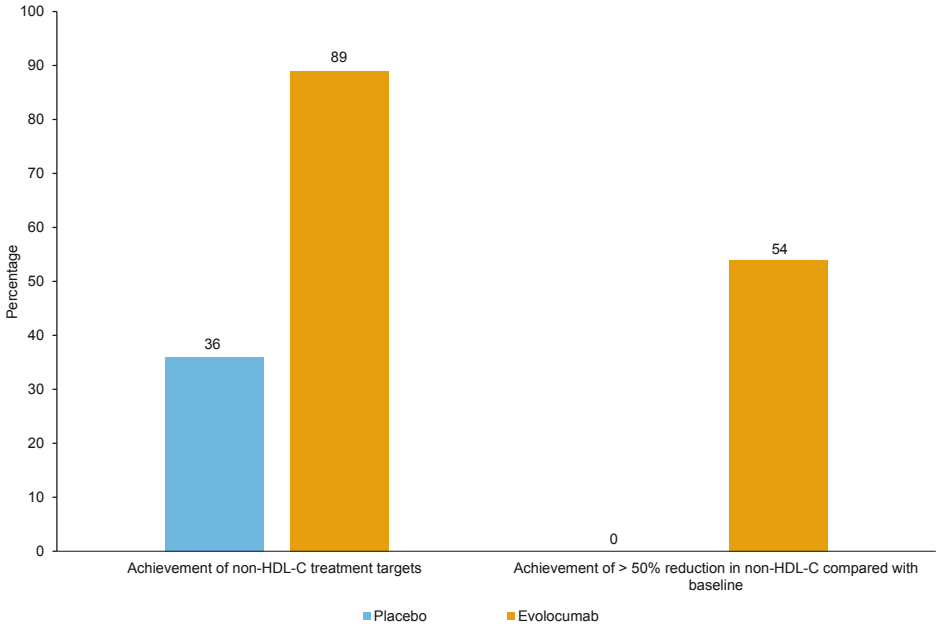

Figure 4. Achievement of treatment goals after evolocumab compared with placebo

Achievement of treatment goals (non-HDL-C levels of <131 mg/dL [<3.4 mmol/L] or <100 mg/dL [<2.6 mmol/L] when CVD or T2DM at baseline) after treatment with evolocumab, compared with placebo.

Abbreviations: Non-HDL-C = non-high-density lipoprotein cholesterol.

Adverse events

In total, 75 adverse events occurred in 20 of the 31 patients that were randomized and received ≥1 dose of the study drug (evolocumab or placebo). In total 30 adverse events (in 13 patients) during treatment with placebo and 45 (in 17 patients) during treatment with evolocumab were reported. Most adverse events were mild and temporary. One adverse event classified as definitely related to study drug and concerned a reaction at the injection site. In general, most common adverse events were gastro-intestinal complaints, muscle complaints and COVID-19 infection. An overview of (serious) adverse events is provided in *Table 4* and *Supplementary Table 8*.

Table 4. Overview of adverse events

	During treatment with placebo	During treatment with evolocumab
	Number of subjects (total AEs)	Number of subjects (total AEs)
Adverse events		
Any	17 (30)	13 (45)
Most common adverse events		
Nausea	3 (5)	4 (5)
Myalgia	3 (4)	3 (4)
Diarrhea	2 (3)	4 (4)
COVID-19	1 (1)	4 (4)
Arthralgia	1 (1)	2 (3)
Related to study drug		
Injection site reaction	0 (0)	1 (1)
Serious		
Any	0 (0)	4 (7)*

Values are n.

*In total seven SAEs occurred in four patients. The first patient was admitted to the hospital due to complications after an elective colonoscopy (1). The second patient was admitted due to complications of an elective cholecystectomy (2). A few days later the patient was readmitted after a bile leak after the cholecystectomy (3). The third patient was admitted to the hospital due to complication of a COVID-19 infection (4). The fourth patient was hospitalized for a coronary artery bypass graft surgery and aorta valve replacement (5). A few days later this patient was readmitted because of intermittent atrial fibrillation (6) and heart failure (7).

Discussion

In this randomized clinical trial in patients with genetically established FD, 12 weeks treatment with evolocumab compared to placebo, added to regular care, resulted in significant and clinically relevant reductions in fasting and post fat load atherogenic lipids and lipoproteins. However, there was relatively little impact on the post fat load increase in triglycerides. Almost all (89%) patients achieved their non-HDL-C treatment goals after treatment with evolocumab. Evolocumab was safe and well tolerated.

The main working mechanism of PCSK9 mAbs is increasing the number of LDL-R on the hepatocyte surface through inhibition of PCSK9. In FD the affinity of TRLs for the LDL-R is severely decreased (<2%) and FD patients usually have no or little LDL due to impaired lipolysis of VLDL remnants, which is thought to require functional apoE.[6,7] Furthermore, in patients with homozygous FH (hoFH) without residual LDL-R activity

(defined as <2% residual activity for the LDL-R), PCSK9 mAbs have not been observed to have an effect on lipid levels. Therefore, it was unknown whether PCSK9 mAbs could play a role in reducing fasting and postprandial atherogenic remnant lipoproteins in FD patients. However, in a small observational non-randomized and unblinded study a reduction of 42% in fasting non-HDL-C and 44% in VLDL-C after PCSK9 mAbs for 12 weeks was observed in 3 patients with FD.[25] This is line with our findings that shows that, although the mechanisms still need to be elucidated, PCSK9 mAbs are able to lower atherogenic lipids and lipoproteins in FD.

Several other studies investigated the effects of PCSK9 mAbs in populations with mixed dyslipidemias from other causes, such as T2DM.[13-16] Although patients with T2DM have an intact binding of apoE to the LDL-R, their lipoprotein phenotypes are somewhat similar to FD. An exploratory analysis in 57 patients with T2DM showed that 3 hour post fat load non-HDL-C levels after a mixed-meal were reduced with 43% compared to baseline after treatment with PCSK9 mAbs.[13] Fasting non-HDL-C was significantly reduced with 46-56% in that study. In addition, that and other studies in patients with T2DM[13-16], showed strikingly identical reductions in other lipid and lipoprotein fractions after treatment with PCSK9 mAbs (including TC, TG, apoB and VLDL-C) as seen in the present study in FD patients. This observation supports the idea that the effects of PCSK9 mAbs on TRLs may be, at least partly, independent of the binding of apoE to the LDL-R, because while apoE of FD patients binds the LDL-R with severely reduced affinity, FD patients show similar reductions in their lipids and lipoproteins after PCSK9 mAbs treatment as T2DM patients whose TRLs bind the LDL-R with much greater affinity.[16]

Patients with FD have impaired lipolysis of TRLs, leading to increased and prolonged plasma concentrations of remnant lipoproteins in the postprandial phase.[5] In the present study, evolocumab reduced all atherogenic lipids and lipoproteins in the postprandial phase (AUC). It was found that this reduction was based on a reduction in fasting and post fat load lipids and lipoproteins rather than an increase in postprandial clearance (iAUC). In other words, this study suggests that in FD, a disorder characterized by impaired clearance of TRLs, PCSK9 mAbs do not affect the postprandial uptake of CMs, lipolysis or the postprandial clearance. This study indicates that PCSK9 mAbs lead to lower post fat load levels as a result of a lower metabolic equilibrium of lipid metabolism. Postprandial lipemia is associated with a very high risk of CVD in non-FD patients and several prospective studies showed that elevated non-fasting TG plasma concentrations (as a marker for increased remnant-cholesterol) are associated with a 17-fold increase in the risk of myocardial infarction in women and 5-fold increase in men. Non-fasting TG concentrations

are also associated with an increased risk for ischemic stroke and early death.[4,26] Therefore, the findings of this study are of particular interest in patients with FD, because the significant reductions in post fat load lipids can be expected to translate into a reduction in CVD risk.

How PCSK9 mAbs reduce fasting and post fat load lipid and lipoproteins in FD patients is not known. Several hypotheses could be considered. First, a substantial increase in the number of LDL-Rs could have a lipid and lipoprotein lowering effect, even in the case of TRLs that have a severely reduced affinity for the LDL-R. In line with this, in our study there were two patients with a dominant variant in the *APOE* gene (with a higher affinity of apoE to the LDL-R receptor compared to the *ε2ε2* genotype).[27] Both patients had an above average response to PCSK9 inhibition (*Supplementary Figure 3*), supporting the concept that upregulation of LDL-R contributes to reductions in fasting and post fat load lipids and lipoproteins in FD.

Second, TRLs are not exclusively cleared by the LDL-R. An important LDL-R independent system for clearance of TRLs is the low-affinity, high-capacity heparin sulfate proteoglycan (HSPG) system.[28] One could hypothesize that PCSK9 mAbs not only inhibit PCSK9, but also other members of the proprotein convertase (PC) family (such as furin, PCSK5, PCSK6 or PCSK7). These other PCs stimulate the cleavage of angiopoietin-like protein (ANGPTL) 3, 4 and/or 8,[29] thereby inhibiting the activity of lipoprotein lipase and endothelial lipase. Inhibition of these pathways by PCSK9 mAbs, can lead to increased lipolysis and remodeling of TRLs, resulting in smaller remnants that are more rapidly and efficiently cleared by the HSPG system. However, a major effect of PCSK9 mAbs on ANGPTL3/4 activity is unlikely in view of the absence of an LDL-C lowering effect in HoFH; a hallmark of ANGPTL3 inhibition.[30]

Third, it is possible that PCSK9 affects other hepatic clearance receptors. There are several indications that PCSK9 mAbs upregulate receptors related to the LDL-R, including the VLDL-R and apoE2 receptor; However, neither receptors are located in the liver and it is thought that they play a limited role in the clearance of TRLs and are less sensitive to the effect of PCSK9 inhibition.[31] Although LDL-R-related protein 1 (LRP1) does play a significant role in TRL clearance, studies in vitro and mice have been shown that this receptor is not degraded by PCSK9 overexpression.[32,33] Stable isotope studies with labeled TRLs could provide further insight into the mechanism of action of PCSK9 mAbs.

In the present study 89% of patients attained their non-HDL-C treatment goals after treatment with evolocumab. After placebo this was 36%. This is in line with the 40% that was found in an observational cross-sectional study in 305 FD patients in the pre-PCSK9 era.[2]

In this study it was found that evolocumab had a good safety profile (*Supplementary Table 9*). FD patients often use a combination of lipid-lowering medication such as statins, fibrates and ezetimibe. In the present study the rate of adverse events by PCSK9-mAbs on top of these lipid-lowering medications was similar as reported in a meta-analysis assessing the safety of PCSK9 mAbs in patients with dyslipidemia or CVD.[34]

Strengths of the study include the largest group of FD patients ever investigated in a randomized trial, the well characterized population, the crossover trial design, the fact that fasting and post fat load lipids and lipoproteins were studied in a single study and that evolocumab was studied on top of a background of treatment with different (combinations of) lipid-lowering medication.

A potential limitation of this study is the measurement of lipid levels up to 8 hours after the oral fat load. This might still not be sufficient to cover the complete post fat load response in patients with FD. Measuring the response up to 24 hours after an oral fat load would have provided more complete information on the late phase of post fat load clearance. However, we decided that the additional patient burden of a 24 hour fast did not outweigh the extra information this measurement would provide. Second, only 29% of the patients were using a combination of a statin and a fibrate at baseline; while 82% used a statin (alone or in combination). The European Society of Cardiology (ESC) guidelines advise a statin or, if the lipoprotein phenotype is dominated by high TGs, a fibrate and state that often a combination of statin and fibrate may be needed.[21] Therefore, there currently is not a 'optimal' treatment strategy against which the PCSK9 mAb treatment could have been compared. Furthermore, there is no interaction of PCSK9 mAbs with statin therapy at baseline. In other words, PCSK9 mAbs lower atherogenic lipid levels to the same extent with or without statins.[35] This is not known for fibrates, but fibrates have limited effects on non-HDL-C levels. Therefore, it is assumed that the results of this study can be generalized to all FD patients, regardless of their background lipid lowering medication. Third, although there was a very consistent but variable reduction of lipids and lipoproteins in study participants, it cannot be ruled out that dietary changes or illnesses during the study have influenced lipids and lipoprotein levels as FD patients are very sensitive to any changes in diet or weight. Of the five patients that got COVID-19 during the study, three patients used evolocumab and no patients used placebo (two patients had COVID-19 during wash-out or follow-up). Although this might have led to an overestimation of the effect of evolocumab, a comparison of the results of these three patients showed even slightly higher non-HDL-C plasma levels when compared with the other patients (*Supplementary Table 10*). Fourth, not all patients achieved their pre-randomization

baseline lipid values prior to start of the second treatment period when using PCSK9 in the first period (*Supplementary Figure 6*). Although this could theoretically be due to lingering effects of PCSK9 mAbs administered in the first treatment period, there were no cross-over effects (p=0.65) and mean lipid values at start of the second treatment period were not lower in patients who first received PCSK9 mAb and then placebo (*Supplementary Figure 4*).

To conclude, evolocumab added to standard lipid-lowering therapy significantly reduced fasting and post fat load non-HDL-C and other atherogenic lipids and lipoproteins in FD patients. The large decrease in fasting and post fat load lipids and lipoproteins can be expected to translate into a reduction in CVD risk in these high-risk patients.

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Disclosures

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Supplementary material

Supplementary methods

Inclusion criteria

1. Subjects previously diagnosed with Familial Dysbetalipoproteinemia; defined as
   * Known ε2ε2 genotype or known dominant *APOE* mutation genotype (confirmed by genotyping or isoelectric focusing) and
   * Proven phenotype of familial dysbetalipoproteinemia (defined as an ApoB/TC ratio <0.39 mg/dL [<0.15 mmol/g][1] or TC >193 mg/dL [5 mmol/L] and TG >266 mg/dL [3 mmol/L][2] or non-HDL-C/ApoB >1.43 mg/dL [>3.69 mmol/g][3]; with or without use of lipid lowering medication.
2. If using any lipid lowering treatment: dose must have been stable for at least three months with non-HDL-C levels >62 mg/dL [1.6 mmol/L].
3. >18 or <80 years old (on the day of signing informed consent).
4. Women were postmenopausal and not receiving systemic cyclic estrogen hormone agonist/antagonist therapy to prevent external effects due to estrogen on lipoprotein metabolism. Postmenopausal status was defined as: *no menses for ≥3 years or; *no menses for ≥1 year but <3 years and confirmed by FSH levels elevated into the postmenopausal range (15-150 IU/L).
5. Willingness to maintain a stable diet for the duration of the study.
6. Understanding the study procedures, alternative treatments available, and risks involved with the study and voluntarily agreement to participate by giving written informed consent.

Exclusion criteria

1. Intolerance, known allergy or hypersensitivity to evolocumab (or other PCSK9 monoclonal antibodies), latex or any of the components of the medication.
2. Exposure or prior exposure (< 1 year before screening) and no discontinuation with PCSK9-inhibitor mAbs due to side effects) to evolocumab or another PCSK9-inhibitor mAb at screening visit.
3. Unable or unwilling to drink an oral fat load.
4. Premenopausal women.
5. Uncontrolled diabetes (defined as HbA1c >8.5% [69 mmol/mol] at screening visit.
6. BMI >40 kg/m^2 at screening visit.
7. Uncontrolled blood pressure with systolic blood pressure >180 mmHg or diastolic blood pressure >110 mmHg at screening visit.
8. Increased hepatic enzymes, defined as alanine transaminase (ALAT) or aspartate transaminase (ASAT) >3 times the upper limit of normal (ULN), or

active liver disease defined as nonalcoholic steatohepatitis (NASH), cirrhosis or Child Pugh B and C, or history of chronic active hepatitis B or C; subjects with documented resolution after treatment are permitted.
9. Impaired renal function, defined by an estimated glomerular filtration rate (eGFR) <30 ml/min/1.73m2, and/or need of renal placement therapy or other clinically significant renal disease.
10. (Sub)clinical hypothyroidism defined as TSH >5.0 mIU/L or (sub)clinical hyperthyroidism defined as TSH <0.35 mIU/L at screening visit.
11. Increased levels of creatinine kinase defined as >3 times the ULN at screening visit.
12. Increased fasting levels of triglycerides defined as >887 mg/dL [10 mmol/L] at screening visit.
13. History of organ transplantation.
14. Current use or use in the past 3 months of immunosuppressive medication at screening visit.
15. Use of fish oil , red yeast rice, bempedoic acid, niacin, CETP inhibitors, lomitapide, mipomersen <6 weeks prior to the study or the use of siRNA targeting PCSK9 inhibitors <36 weeks prior to the study.
16. Active malignancy (<2 year prior to informed consent), except non-melanoma skin cancer or carcinoma in situ of the cervix.
17. Known infection with Human Immunodeficiency Virus (HIV) or AIDS.
18. Known celiac disease or other disorder associated with significant intestinal malabsorption, including short-bowel syndrome after intestinal resection or gastric bypass.
19. Known galactose-intolerance, Lapp-lactase deficiency or glucose-galactose malabsorption.
20. Alcohol use, defined as >14 alcoholic consumptions per week for women and >21 alcohol consumptions per week for men. One alcohol consumption unit was defined as follows: 350 mL beer, 150 mL wine or 45 mL alcohol for mixed drinks.
21. Participation or participation in a study with an investigational compound or device within 30 days of signing informed consent.
22. Any medical, social or physiological circumstance which interfered the study, based on judgement by the principal investigator.

Definitions and measurements

Coronary artery disease was defined as angina pectoris, myocardial infarction, coronary artery bypass graft or percutaneous intervention. Cerebrovascular disease was defined as a TIA, stroke or carotid artery intervention. Peripheral artery disease was defined as leg claudication or peripheral revascularization. An aneurysm of the aorta was self-reported and as a diameter of the abdominal aorta of ≥3 cm or an abdominal

aneurysm intervention. Family history of premature cardiovascular disease (CVD) was self-reported and defined as a first degree relative with myocardial infarction, stroke or sudden death) at age < 55 years (father or brother) or <65 years (mother or sister). Type 2 diabetes mellitus (T2DM) was defined as self-reported presence of T2DM, use of glucose-lowering medication, fasting plasma glucose levels ≥126 mg/dL [7.0 mmol/L] at baseline or HbA1c >6.5% [48 mmol/mol] at screening.[4] Hypertension was defined as self-reported presence of hypertension, use of antihypertensive medication, or hypertension at baseline (systolic blood pressure (BP) ≥140 mmHg or diastolic BP ≥90 mmHg). BP was measured 3 times at the dominant arm (arm with highest blood pressure during screening). The mean value of these 3 measurements for systolic and diastolic BP was used. High intensity statins were defined as atorvastatin ≥40 mg or rosuvastatin ≥20 mg. BMI was calculated by dividing mass (in kilograms) by height (in meters) squared. Waist circumference was measured halfway between the lower costal margin and the iliac crest when standing. Metabolic syndrome was defined using the ATP III criteria, as having at least three of the following metabolic abnormalities at baseline: Waist circumference >102 cm for males and >88 cm for females; fasting triglycerides (TG) ≥ 151 mg/dL [1.7 mmol/L]; HDL-C <40 mg/dL [1.03 mmol/L] for males and HDL-C < 50 mg/dL [1.29 mmol/L] for females; systolic BP ≥130 mmHg or diastolic BP ≥85 mmHg; fasting plasma glucose ≥ 101 mg/dL [5.6 mmol/L].[5]

Laboratory analyses

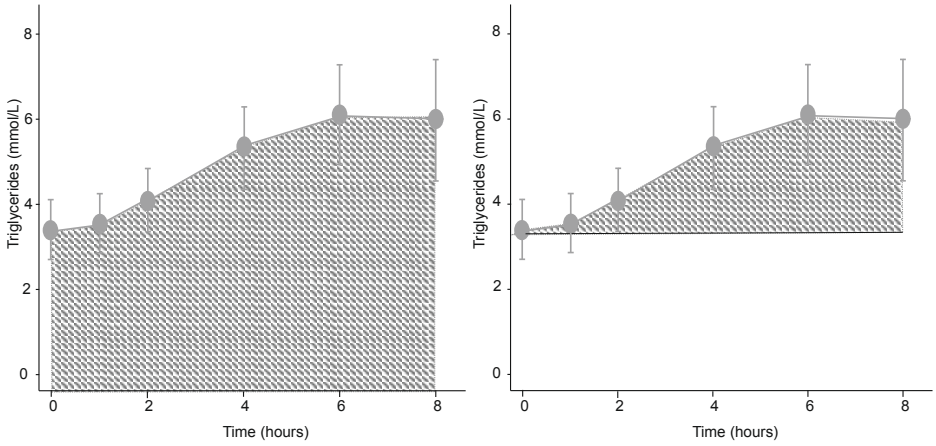
Safety, baseline and conventional lipid measurements

Laboratory samples were analyzed on coded specimens without knowledge of treatment allocation. Clinical chemistry and conventional lipids were measured at the Laboratory Department of the UMC Utrecht according to standard procedures. Creatinine, creatinine kinase, ASAT, ALAT, glucose, TSH, FSH, HDL-C, total cholesterol and TG were measured with an Atellica CH Analyzer (Siemens Healthcare Diagnostics). ApoB was measured by Abbott ARCHITECT and Lipoprotein (a) levels were measured by Attilica neph 360 (Siemens Healthcare Diagnostics). Glycated hemoglobin (HbA1c) was measured using high performance liquid chromatography on a HA-8180 analyzer (Menarini Diagnostics, Florence, Italy).

Density gradient lipoprotein measurements

Density gradient ultracentrifugation was used to measure the cholesterol content in the VLDL and IDL fractions before, 4 and 8 hours after de oral fat load. These analyses were performed by the laboratory of Vascular Medicine in Erasmus Medical Center, Rotterdam, the Netherlands. For this, KBr (0.35 g/mL plasma) was added to plasma to obtain a density of 1.26 g/ml. Of this plasma 1 mL was placed in an ultracentrifuge tube, followed by 1.9 mL of KBr solutions (of 1.21, 1.10, 1.063, 1.04 and 1.02 g/mL) in

physiological salt and 1 mL of water. Samples were centrifuged for 30 minutes at 20°C at 207.000 g using a SW41 rotor in a Optima XPN-80 ultracentrifuge (Beckman Instruments, Indianapolis, IN, USA). After this chylomicrons were isolated from the top 1 mL of the tube. Removed volume was replaced with 1 mL water and samples were centrifuged at 207.000 g for 18 hours at 4 °C, using the same rotor and centrifuge. After centrifugation the density gradient was separated from the bottom to the top in fractions of 250 microL in which total cholesterol was measured using a Selectra E (DDS Diagnostic system, Istanbul, Turkey). Lipoproteins were separated based on fraction number and total cholesterol pattern. VLDL was recovered in fractions 41 – 46 and fractions 34 – 40 were designated as IDL. Cholesterol content in both fractions was measured.



Supplementary Figure 1. Example of area under the curve (left) and incremental area under the curve (right)

Supplementary Table 1. Reasons for screening failure and dropout

Reasons for screening failure	**Number of patients**
• Severe dyslipidemia requiring initiation of lipid-lowering treatment first	2
• Not having an *APOE* genotype that was associated with FD	3
Reason for withdrawal	**Number of patients**
• Withdrawal of consent because of pain due to pre-existent peripheral artery disease	1
Reasons for dropout	**Number of patients**
No completion of all measurements to assess the primary endpoint: • One patient was not able to ingest fresh cream due to a recent cholecystectomy, therefore only fasting samples were drawn at that visit. • One patient was not feeling well after ingestion of the oral fat load, therefore 2 post fat load samples were missing at one visit.	2

Supplementary Table 2. Baseline table of dropouts and withdrawal

	Patient 1 Dropout	Patient 2 Dropout	Patient 3 Withdrawal
Age (years)	49	52	58
Female sex	no	no	yes
APOE genotype			
• ε2ε2	yes	yes	yes
• Dominant *APOE* variant	no	no	no
Cardiovascular disease			
• Coronary heart disease	no	no	no
• Peripheral vascular disease	no	no	yes
• Cerebrovascular disease	no	no	no
• Abdominal aortic aneurysm	no	no	no
Diabetes mellitus type 2	no	no	no
Hypertension	yes	yes	no
Metabolic Syndrome	yes	no	yes
Family history of premature CVD	no	no	no
Lipid lowering treatment	yes	yes	yes
• Statin only	yes	yes	no
• Ezetimibe only	no	no	no
• Fibrate only	no	no	no
• Statin + ezetimbe	no	no	no
• Statin + fibrate	no	no	no
• Statin + ezetimibe + fibrate	no	no	yes
High intensity statin	no	no	yes
Current smoking	no	no	yes
Body mass index (kg/m^2)	31.7	22.2	35.4
Waist circumference (cm)	110	83	117
Systolic blood pressure (mmHg)	166	153	133
Diastolic blood pressure (mmHg)	104	99	76
Laboratory measurements			
• Total cholesterol (mg/dL [mmol/L])	243 [6.3]	178 [4.6]	320 [8.3]
• Triglycerides (mg/dL [mmol/L])	248 [2.8]	266 [3.0]	807 [9.1]
• Non-HDL-cholesterol (mg/dL [mmol/L])	189 [4.9]	139 [3.6]	266 [6.9]
• HDL-cholesterol (mg/dL [mmol/L])	54 [1.4]	39 [1.0]	60 [1.5]
• Apolipoprotein B (mg/dL [g/L])	90 [0.9]	70 [0.7]	100 [1.0]
• Lipoprotein (a) (mg/dL [mg/L])	5.5 [55]	9.2 [92]	9.4 [94]
• Glucose (mg/dL [mmol/L])	96 [5.3]	108 [6.0]	119 [6.6]

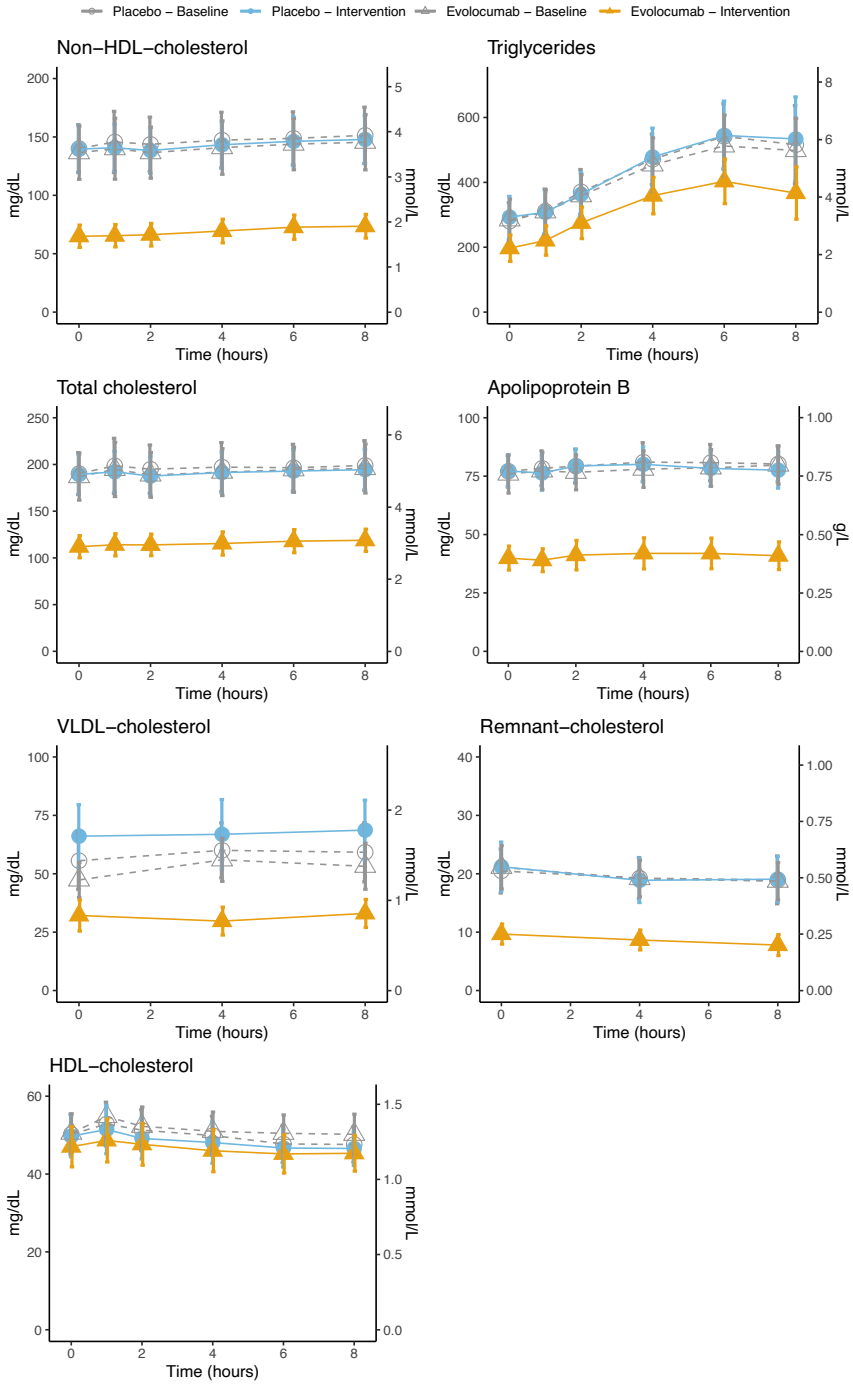
Abbreviations: CVD = cardiovascular disease

Supplementary Table 3. Baseline table stratified for treatment group

	First evolocumab, second placebo n=15	**First placebo, second evolocumab n=13**
Age (years)	49	52
Female sex	7 (47)	5 (38)
APOE genotype		
• ε2ε2	13 (87)	13 (100)
• Dominant *APOE* variant	2 (13)	1 (8)
Cardiovascular disease		
• Coronary heart disease	1 (7)	1 (8)
• Peripheral vascular disease	0 (0)	1 (8)
• Cerebrovascular disease	1 (7)	2 (15)
• Abdominal aortic aneurysm	0 (0)	1 (8)
Diabetes mellitus type 2	5 (33)	4 (31)
Hypertension	11 (73)	11 (85)
Metabolic Syndrome	11 (73)	7 (78)
Family history of premature CVD	2 (13)	5 (38)
Lipid lowering treatment	14 (93)	12 (92)
• Statin only	4 (27)	2 (15)
• Ezetimibe only	1 (7)	1 (8)
• Fibrate only	0 (0)	1 (8)
• Statin + ezetimibe	5 (33)	3 (23)
• Statin + fibrate	3 (20)	5 (38)
• Statin + ezetimibe + fibrate	1 (7)	0 (0)
High intensity statin	2 (13)	5 (38)
Current smoking	1 (7)	0 (0)
Body mass index (kg/m^2)	29.2 ± 2.9	29.9 ± 4.5
Waist circumference (cm)	106 ± 8	109 ± 17
Systolic blood pressure (mmHg)	140 ± 18	141 ± 13
Diastolic blood pressure (mmHg)	85 ± 9	84 ± 8
Laboratory measurements		
• Total cholesterol (mg/dL [mmol/L])	193 ± 81 [5.0 ± 2.1]	185 ± 62 [4.8 ± 1.6]
• Triglycerides (mg/dL [mmol/L])	302 ± 186 [3.4 ± 2.1]	248 ± 133 [2.8 ± 1.5]
• Non-HDL-cholesterol (mg/dL [mmol/L])	143 ± 77 [3.7 ± 2.0]	135 ± 54 [3.5 ± 1.4]
• HDL-cholesterol (mg/dL [mmol/L])	50 ± 15 [1.3 ± 0.4]	50 ± 15 [1.3 ± 0.4]
• Apolipoprotein B (mg/dL [g/L])	80 ± 30 [0.8 ± 0.3]	70 ± 20 [0.7 ± 0.2]
• Lipoprotein (a)[a] (mg/dL [mg/L])	7.6 (4.2 – 36.9) [76 (42 -369)]	8.8 (3.0 – 28.5) [88 (30 - 285)]
• Glucose (mg/dL [mmol/L])	115 ± 32 [6.4 ± 1.8]	105 ± 16 [5.8 ± 0.9]

Data are shown as n (%), mean ± standard deviation, or when not-normally distributed as median (interquartile range), indicated by [a]

Abbreviations: CVD = cardiovascular disease

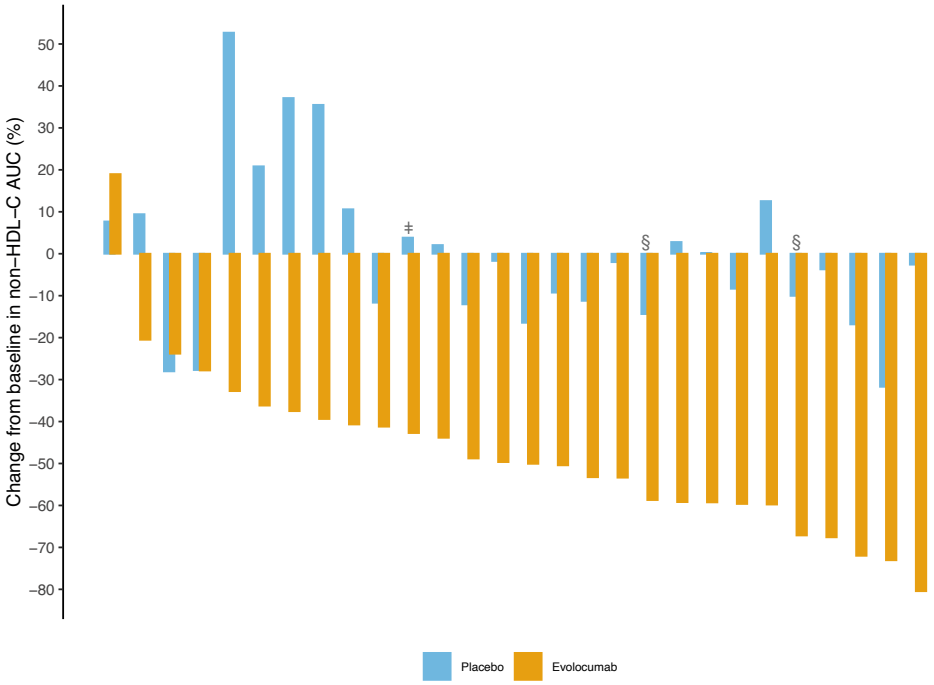


Supplementary Figure 2. Effect of evolocumab and placebo on fasting and post fat load lipids and lipoproteins with baseline measurements

Supplementary Table 4. Effect of 12 weeks evolocumab compared to placebo on change from baseline on fasting lipids

		Placebo			
		Baseline	**Placebo**	**Change**	**% change**
Non-HDL-C	mg/dL	140 ± 55	140 ± 54	-0.5 ± 28	1.3 ± 21
	mmol/L	3.6 ± 1.4	3.6 ± 1.4	0.0 ± 0.7	
Triglycerides	mg/dL	280 ± 155	293 ± 173	13 ± 110	7.9 ± 35
	mmol/L	3.2 ± 1.8	3.3 ± 2.0	0.1 ± 1.2	
Total cholesterol	mg/dL	190 ± 61	189 ± 57	-1.0 ± 31	0.9 ± 16
	mmol/L	4.9 ± 1.6	4.9 ± 1.5	0.0 ± 0.8	
ApoB	mg/dL	77 ± 18	77 ± 19	0.1 ± 9.5	0.6 ± 11.6
	g/L	0.8 ± 0.2	0.8 ± 0.2	0.0 ± 0.1	
VLDL-C	mg/dL	56 ± 33	66 ± 36	11 ± 23	30 ± 51
	mmol/L	1.4 ± 0.9	1.7 ± 0.9	0.3 ± 0.6	
Remnant-C	mg/dL	21 ± 10	21 ± 11	0.7 ± 6.8	4.8 ± 35
	mmol/L	0.5 ± 0.3	0.5 ± 0.3	0.0 ± 0.2	
HDL-C	mg/dL	50 ± 14	50 ± 14	-0.5 ± 6.2	-0.6 ± 13
	mmol/L	1.3 ± 0.4	1.3 ± 0.4	0.0 ± 0.2	
Lp(a)[a]	mg/dL	8.0 (3.0 - 30)	7.2 (3.1 - 35)	0.1 (-0.5 - 2.5)	0.7 (-4.1 -–17)
	mg/L	80 (30 - 304)	72 (31 - 353)	1 (-5 - 25)	

	Evolocumab			Difference	% Difference	*P*-value
Baseline	**Evolocumab**	**Change**	**% change**	**in change**	**in change** (95%CI)	
137 ± 62	65 ± 26	-72 ± 52	-49 ± 19	-71 ± 55	-51 (-59 - -41)	<0.001
3.5 ± 1.6	1.7 ± 0.7	-1.9 ± 1.3		-1.8 ± 1.4		
284 ± 172	197 ± 110	-87 ± 119	-24 ± 35	-100 ± 123	-32 (-48 - -17)	<0.001
3.2 ± 1.9	2.2 ± 1.2	-1.0 ± 1.3		-1.1 ± 1.4		
187 ± 68	112 ± 32	-75 ± 57	-37 ± 17	-74 ± 59	-38 (-44 - -31)	<0.001
4.8 ± 1.8	2.9 ± 0.8	-1.9 ± 1.5		-1.9 ± 1.5		
76 ± 22	40 ± 14	-36 ± 19	-46 ± 18	-36 ± 21	-47 (-53 - -39)	<0.001
0.8 ± 0.2	0.4 ± 0.1	-0.4 ± 0.2		-0.4 ± 0.2		
47 ± 20	32 ± 18	-15 ± 17	-27 ± 41	-26 ± 26	-56 (-79 - -35)	<0.001
1.2 ± 0.5	0.8 ± 0.5	-0.4 ± 0.4		-0.7 ± 0.7		
21 ± 10	9.7 ± 4.6	-11 ± 8.3	-50 ± 23	-12 ± 12	-55 (-71 - 38)	<0.001
0.5 ± 0.3	0.3 ± 0.1	-0.3 ± 0.2		-0.3 ± 0.3		
51 ± 14	47 ± 14	-3.5 ± 8.3	-5.9 ± 18	-3.0 ± 9.1	-5.3 (-13 - 2.5)	0.17
1.3 ± 0.3	1.2 ± 0.4	-0.1 ± 0.2		-0.1 ± 0.2		
8.1 (3.3 - 32)	3.7 (3.0 - 22)	-3.7 (-10 - -0.3)	-32 (-41 - -8)	-3.9 (-13 - 0)	-36 (-49 - -10)	<0.001
81 (33 - 318)	37 (30 - 216)	-37 (-102 - -3)		-39 (-126 - 0)		



Supplementary Figure 3. Individual change from baseline in non-HDL-cholesterol AUC after evolocumab and placebo

‡ patient with ε2ε2 genotype in combination with dominant variant in *APOE* gene
§ patient with heterozygous variant in *APOE* gene
Abbreviations: AUC = area under the curve, Non-HDL-cholesterol = non high-density lipoprotein cholesterol

Supplementary Table 5. Effect of 12 weeks evolocumab compared to placebo on 8 hour post fat load lipids (iAUC)

		iAUC after placebo	iAUC after evolocumab	Difference (95%CI)	*P*-value
Non-HDL-C	mg/dL.8h	28 ± 45	36 ± 28	7.3 (-8.7 – 25)	0.39
	mmol/L.8h	0.7 ± 1.2	0.9 ± 0.7	0.2 (-0.2 – 0.6)	
Triglycerides	mg/dL.8h	1236 ± 806	1048 ± 512	-188 (-454 – 35)	0.13
	mmol/L.8h	13.9 ± 9.1	11.8 ± 5.8	-2.1 (-5.1 – 0.4)	
Total cholesterol	mg/dL.8h	17 ± 56	31 ± 39	14 (-7.7 – 37)	0.20
	mmol/L.8h	0.4 ± 1.4	0.8 ± 1.0	0.4 (-0.2 – 0.9)	
ApoB	mg/dL.8h	12 ± 43	9.9 ± 49	-0.3 (-12 – 12)	0.99
	g/L.8h	0.1 ± 0.4	0.1 ± 0.5	0.0 (-0.1 – 0.1)	
VLDL-C	mg/dL.8h	8.3 ± 74	-7.9 ± 39	-16 (-46 – 17)	0.33
	mmol/L.8h	0.2 ± 1.9	-0.2 ± 1	-0.4 (-1.2 – 0.4)	
Remnant-C	mg/dL.8h	-14 ± 22	-7.8 ± 16	5.8 (-4.2 – 17)	0.28
	mmol/L.8h	-0.4 ± 0.6	-0.2 ± 0.4	0.1 (-0.1 – 0.4)	
HDL-C	mg/dL.8h	-12 ± 18	-4.9 ± 24	6.7 (-2.3 – 17)	0.28
	mmol/L.8h	-0.3 ± 0.5	-0.1 ± 0.6	0.2 (-0.1 – 0.4)	

Values are mean ± standard deviation

Abbreviations: ApoB = apolipoprotein B, AUC = area under the curve, HDL-C = high-density lipoprotein-cholesterol, Non-HDL-C = high-density lipoprotein-cholesterol, remnant-C = remnant-cholesterol, VLDL-C = very-low-density lipoprotein-cholesterol

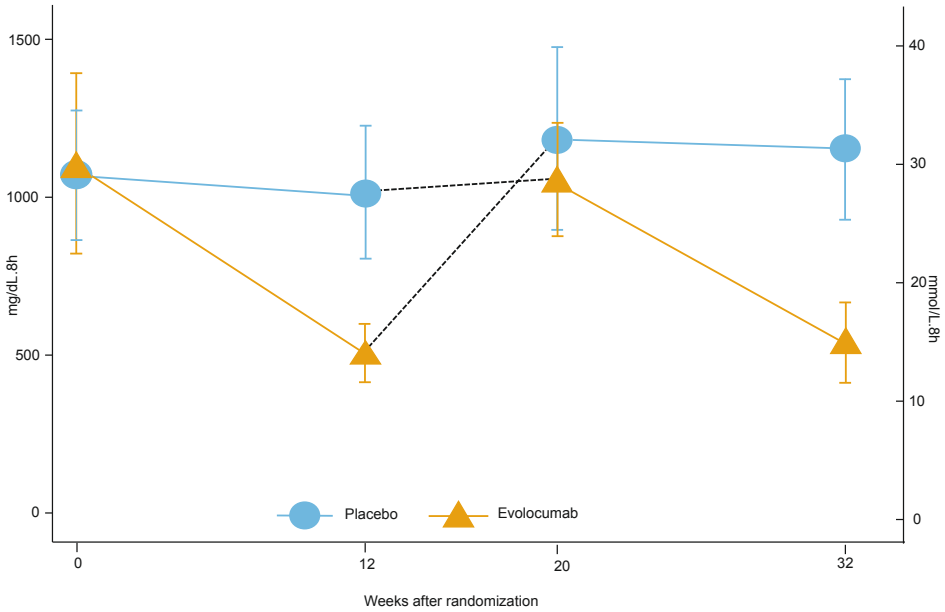
Supplementary Table 6. Effect of 12 weeks evolocumab compared to placebo on change from baseline on 8 hours post fat load lipids

		Placebo			
		AUC baseline	**AUC placebo**	**Change**	**% change**
Non-HDL-C	mg/dL.8h	1175 ± 506	1145 ± 438	-30 ± 224	-0.4 ± 20
	mmol/L.8h	30.4 ± 13.1	29.7 ± 11.3	-0.8 ± 5.8	
Triglycerides	mg/dL.8h	3589 ± 1751	3579 ± 1878	30 ± 986	2.7 ± 24
	mmol/L.8h	40.0 ± 20.4	40.4 ± 21.2	0.3 ± 11.1	
Total cholesterol	mg/dL.8h	1573 ± 5560	1531 ± 470	-41 ± 255	-0.9 ± 15
	mmol/L.8h	40.7 ± 14.5	39.7 ± 12.2	-1.1 ± 6.6	
ApoB	mg/dL.8h	640 ± 160	630 ± 157	-11 ± 70	-1.2 ± 10
	gl/L.8h	6.4 ± 1.6	6.3 ± 1.6	-0.1 ± 0.7	
VLDL-C	mg/dL.8h	470 ± 254	537 ± 296	67 ± 182	21 ± 45
	mmol/L.8h	12.2 ± 6.6	13.9 ± 7.7	1.7 ± 4.7	
Remnant-C	mg/dL.8h	156 ± 80	156 ± 83	0.2 ± 53	1.5 ± 24
	mmol/L.8h	4.0 ± 2.1	4.0 ± 2.1	0.0 ± 1.4	
HDL-C	mg/dL.8h	398 ± 107	386 ± 111	-12 ± 50	-2.7 ± 11
	mmol/L.8h	10.3 ± 2.8	10.0 ± 2.9	-0.3 ± 1.3	

	Evolocumab				Difference in change	% Difference in change (95%CI)	*P*-value
	AUC baseline	**AUC evolocumab**	**Change**	**% change**			
	1128 ± 494	555 ± 215	-573 ± 418	-48 ± 20	-543 ± 345	-47 (-55 - -38)	<0.001
	29.2 ± 12.8	14.4 ± 5.6	-14.8 ± 10.8		-14.1 ± 8.9		
	3419 ± 1704	2623 ± 1209	-796 ± 1189	-17 ± 34	-826 ± 1306	-19 (-33 - 5.1)	0.003
	38.6 ± 19.2	29.6 ± 13.6	-9.0 ± 13.4		-9.3 ± 13.7		
	1539 ± 544	926 ± 260	-613 ± 462	-37 ± 17	-572 ± 354	-36 (-41 - -30)	<0.001
	39.9 ± 14.1	24.0 ± 6.7	-15.9 ± 12		-14.8 ± 9.2		
	623 ± 166	330 ± 130	-294 ± 152	-46 ± 19	-278 ± 143	-45 (-50 - -37)	<0.001
	6.2 ± 1.7	3.3 ± 1.3	-2.9 ± 1.5		-2.8 ± 1.4		
	423 ± 179	249 ± 129	-176 ± 132	-40 ± 23	-243 ± 234	-61 (-77 - -45)	<0.001
	11.0 ± 4.6	6.5 ± 3.3	-4.6 ± 3.4		-6.3 ± 6.1		
	156 ± 69	70 ± 36	-87 ± 56	-53 ± 17	-87 ± 72	-55 (-65 - -44)	<0.001
	4.0 ± 1.8	1.8 ± 0.9	-2.2 ± 1.4		-2.3 ± 1.9		
	412 ± 106	371 ± 111	-40 ± 68	-9.1 ± 17	-29 ± 64	-6.3 (-12 - 0.1)	0.051
	10.7 ± 2.7	9.6 ± 2.9	-1.0 ± 1.8		-0.7 ± 1.7		

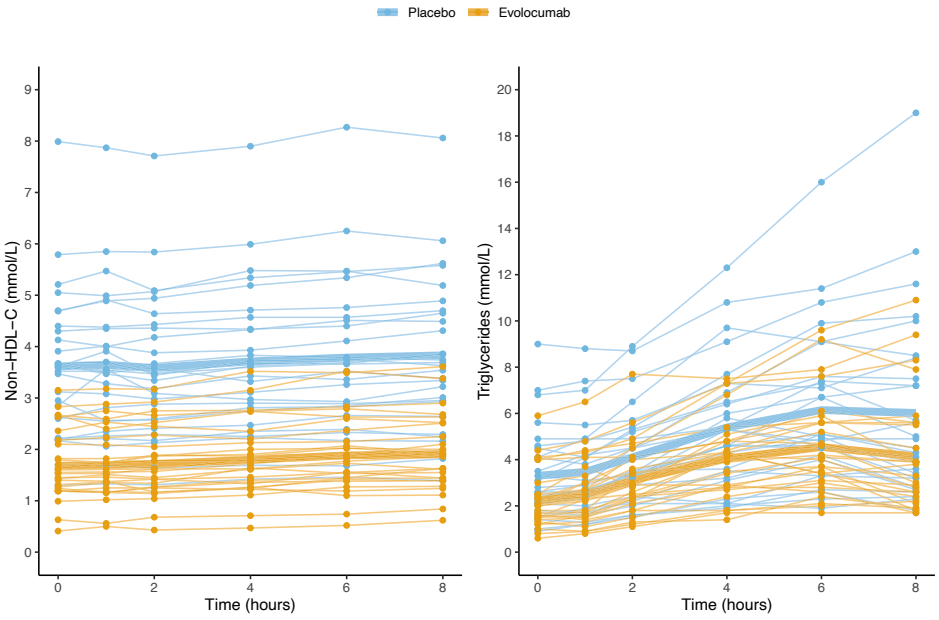
Supplementary Table 7. Effect of 12 weeks evolocumab compared to placebo on change from baseline on 8 hours post fat load lipids (iAUC)

		Placebo			Evolocumab				
		iAUC baseline	**iAUC placebo**	**Change**	**iAUC baseline**	**iAUC evolocumab**	**Change**	**Difference in change (95%CI)**	***P*-value**
Non-HDL-C	mg/dL.8h	54 ± 128	28 ± 45	-26 ± 131	36 ± 55	36 ± 28	-0.4 ± 51	26 (-16 - 83)	0.32
	mmol/L.8h	1.4 ± 3.3	0.7 ± 1.2	-0.7 ± 3.4	0.9 ± 1.4	0.9 ± 0.7	0.0 ± 1.3	0.7 (-0.4 - 2.1)	
Triglycerides	mg/dL.8h	1307 ± 665	1237 ± 806	-72 ± 712	1145 ± 753	1048 ± 512	-97 ± 538	-26 (-424 - 371)	0.91
	mmol/L.8h	14.7 ± 7.5	13.9 ± 9.1	-0.8 ± 8.0	12.9 ± 8.5	11.8 ± 5.8	-1.1 ± 6.2	-0.3 (-4.8 - 4.2)	
Total cholesterol	mg/dL.8h	50 ± 157	17 ± 56	-34 ± 163	43 ± 63	31 ± 39	-13 ± 65	21 (-32 - 90)	0.50
	mmol/L.8h	1.3 ± 4.1	0.4 ± 1.4	-0.9 ± 4.2	1.1 ± 1.6	0.8 ± 1.0	-0.3 ± 1.7	0.5 (-0.8 - 2.3)	
ApoB	mg/dL.8h	23 ± 50	12 ± 43	-13 ± 41	16 ± 67	9.9 ± 49	-5.9 ± 101	5.0 (-36 - 50)	0.83
	gl/L.8h	0.2 ± 0.5	0.1 ± 0.4	-0.1 ± 0.4	0.2 ± 0.7	0.1 ± 0.5	-0.1 ± 1.0	0.0 (-0.4 - 0.5)	
VLDL-C	mg/dL.8h	25 ± 69	8.3 ± 74	-17 ± 91	46 ± 94	-7.9 ± 39	-54 ± 110	-37 (-103 - 14)	0.22
	mmol/L.8h	0.6 ± 1.8	0.2 ± 1.9	-0.4 ± 2.3	1.2 ± 2.4	-0.2 ± 1	-1.4 ± 2.9	-1.0 (-2.7 - 0.4)	
Remnant-C	mg/dL.8h	-8 ± 21	-14 ± 26	-5.6 ± 29	-13 ± 25	-7.8 ± 16	4.7 ± 30	10 (-5.1 - 25)	0.20
	mmol/L.8h	-0.2 ± 0.5	-0.4 ± 0.6	-0.1 ± 0.7	-0.3 ± 0.6	-0.2 ± 0.4	0.1 ± 0.8	0.3 (-0.1 - 0.7)	
HDL-C	mg/dL.8h	-4.1 ± 41	-12 ± 18	-7.6 ± 42	7.4 ± 31	-4.9 ± 24	-12 ± 31	-4.7 (-23 - 13)	0.61
	mmol/L.8h	-0.1 ± 1.1	-0.3 ± 0.5	-0.2 ± 1.1	0.2 ± 0.8	-0.1 ± 0.6	-0.3 ± 0.8	-0.1 (-0.6 - 0.3)	



Supplementary Figure 4. Effect of evolocumab and placebo on non-HDL-C AUC measured at the start and end of both treatment periods

Means of non-HDL-C AUC with 95%CI; At the start and after placebo (blue) and at the start and after treatment with evolocumab (orange), with a crossover period in between (dotted black lines). Abbreviations: AUC = area under the curve, Non-HDL-C = non-high-density lipoprotein-cholesterol.



Supplementary Figure 5. Individual effect of evolocumab and placebo on fasting and post fat load non-HDL-C and triglyceride levels

Individual fasting and 8-hour post fat load non-HDL-C and triglyceride levels after an oral fat load, after treatment with evolocumab (orange) or placebo (blue).

Supplementary Table 8. Overview of Serious Adverse Events

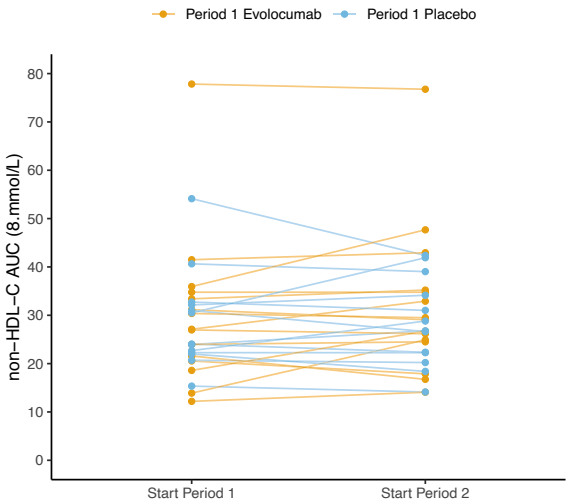
Number	Serious Adverse Event	Action taken with regard to study drug	Outcome	Relatedness
1.	Hospitalization due to colon perforation and intestinal bleeding as a result of colonoscopy for the evaluation of iron deficiency anemia based on an intestinal polyp.	None	Resolved	Not related
2	Hospitalization after complex cholecystectomy due to symptomatic gallstone disease.	None	Resolved	Not related
3.	Rehospitalization after complicated (bile leak) after cholecystectomy.	None	Resolved	Not related
4.	Hospitalization due to complications COVID-19 infection.	None	Residual effects present, treated	Not related
5.	Hospitalization after symptomatic angina pectoris for which subject received coronary artery bypass graft surgery and aorta valve replacement earlier than initially planned.	None	Resolved	Not related
6.	Rehospitalization with intermittent atrial fibrillation (SAE nr 6) and heart failure (SAE nr 7).	None	Resolved	Not related
7.	Rehospitalization with intermittent atrial fibrillation (SAE nr 6) and heart failure (SAE nr 7).	None	Resolved	Not related

Supplementary Table 9. Safety laboratory measurements of all randomized patients, except for withdrawal (n=30)

	Placebo n=30	Evolocumab n=30	*P*-value
ASAT (U/L)	26.3 ± 12.1	26.8 ± 9.9	0.79
ALAT (U/L)	31.8 ± 14.4	33.2 ± 18.1	0.57
CK (U/L)	111 (92 – 159)	123 (75 – 184)	0.97
Creatinine (mg/dL)	0.9 ± 0.2	0.9 ± 0.2	0.35
Glucose (mg/dL)	106 ± 20	110 ± 31	0.53

Supplementary Table 10. Comparison of lipid levels in patients with and without COVID-19 infection

	Patients having COVID during treatment with evolocumab n=3	Patients not having COVID during treatment with evolocumab n=27
Non-HDL-C (mmol/L)	2.0 ± 1.0	1.6 ± 0.6



Supplementary Figure 6. Non-HDL-C AUC prior to treatment period 1 and treatment period 2

Non-HDL-cholesterol area under the curve (AUC) (in 8h.mmol/L) at the start of treatment period 1 and 2

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Supplementary references

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Chapter 10

Effect of evolocumab on fasting and post fat load lipoprotein distribution and composition in Familial Dysbetalipoproteinemia

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Abstract

Background: PCSK9 monoclonal antibodies (mAbs) reduce fasting and post fat load non-HDL-cholesterol and IDL-cholesterol in Familial Dysbetalipoproteinemia (FD). However, the effect of PCSK9 mAbs on the distribution and composition of atherogenic lipoproteins in patients with FD is unknown.

Objective: To evaluate the effect of the PCSK9 mAb evolocumab added to standard lipid-lowering therapy in patients with FD on fasting and post fat load lipoprotein distribution and composition.

Methods: Randomized placebo-controlled double-blind crossover trial comparing evolocumab (140 mg subcutaneous every 2 weeks) with placebo during two 12-week treatment periods. Patients received an oral fat load at the start and end of each treatment period. (Apo)lipoproteins were measured with ultracentrifugation, polyacrylamide gels, retinyl palmitate and SDS-PAGE.

Results: Evolocumab significantly reduced particle number of all atherogenic lipoproteins, especially of IDL-apoB (-49%, 95%CI 41–59) and LDL-apoB (-58%, 95%CI 50–73). Evolocumab significantly reduced cholesterol and triglyceride (TG) levels in VLDL, IDL and LDL, but reduced cholesterol per particle relatively more than TG (VLDL-C -48% (95%CI 29–63%) and VLDL-TG -20% (95%CI (6.3–41%)). Evolocumab did not affect the post fat load response of chylomicrons.

Conclusion: Evolocumab added to standard lipid-lowering therapy in FD patients significantly reduced lipoprotein particle number, in particular the smaller and more cholesterol-rich lipoproteins (i.e. IDL and LDL). In addition, cholesterol levels were reduced more than TG levels in all lipoproteins. Evolocumab did not affect chylomicron metabolism. It seems likely that the observed effects of evolocumab are achieved by increased hepatic lipoprotein clearance, but the specific mechanism in FD patients remains to be elucidated.

Introduction

Approximately 0.7% of the general population is homozygous for the ε2 allele in the *APOE* gene.[1] Since about 10-18% of these ε2ε2 subjects develop the specific dysbetalipoproteinemia lipid phenotype,[2,3] the estimated prevalence of Familial Dysbetalipoproteinemia (FD) is 1 in 1000 individuals, making FD the second most common monogenic lipid disorder after Familial Hypercholesterolemia.[4] FD is associated with a very high risk of cardiovascular disease (CVD), due to accumulation of atherogenic cholesterol-enriched triglyceride-rich lipoproteins (TRLs).[5,6] Patients with FD can be treated with diet, statins and fibrates, but with the current treatment options the non-high-density lipoprotein-cholesterol (non-HDL-C) treatment goal is only attained in 40% of FD patients.[7] For that reason the EVOLVE-FD trial was conducted to investigate the effect of evolocumab in a crossover randomized trial in 28 FD patients. The trial showed large reductions in fasting and post fat load non-HDL-C, IDL-cholesterol and apolipoprotein B (apoB). However, it is not known what the effect of evolocumab is on the distribution and composition of lipids and apolipoproteins in the different lipoproteins. This is relevant since smaller TRLs are more atherogenic and to better understand the underlying mechanism of action of PCSK9 mAbs in FD.

Studies in healthy subjects and patients with type 2 diabetes mellitus (T2DM) demonstrated a large reduction in cholesterol levels in apoB-containing lipoproteins and a variable but more limited reduction in triglyceride (TG) levels.[8-13] A study in 13 patients with T2DM showed that evolocumab did not affect concentrations of large TG-rich lipoproteins (VLDL1 and chylomicrons), but reduced the concentration of smaller cholesterol-rich lipoproteins (VLDL2, IDL and LDL), caused by an increase in the clearance of VLDL2-apoB100 and VLDL2-TG, and also by an increase in the clearance of IDL-apoB100 and LDL-apoB100.[9] Most studies showed no effect of PCSK9 mAbs on chylomicron metabolism.[9,10,13,14]

The aim of this study was to evaluate the effect of evolocumab on top of standard lipid-lowering treatment, compared with placebo, on fasting and post fat load lipoprotein distribution and composition in FD patients.

Methods

Patients and study design

Details of in- and exclusion criteria and study design have been reported elsewhere.[15] In brief, patients between 18 and 80 years who were genetically diagnosed with FD before screening, were eligible to participate in the study. FD consisted of a specific

phenotype and -genotype. An FD lipid phenotype was defined as either apoB/total cholesterol (TC) ratio <0.15 mmol/g,[16] TC >5 mmol/L and TG >3 mmol/L[17] or non-HDL-C/apoB >3.69 mmol/g,[18] with or without lipid-lowering medication. An FD genotype was defined as an ε2ε2 genotype or a heterozygous dominant *APOE* variant known to associate with an FD phenotype, confirmed by genotyping or isoelectric focusing. When patients were using lipid-lowering medication the dose must have remained stable for at least 12 weeks. Non-HDL-C levels had to be >1.6 mmol/L and fasting triglycerides <10 mmol/L. Main exclusion criteria were uncontrolled T2DM (defined as HbA1c >69 mmol/mol), morbid obesity (BMI >40 kg/m^2), uncontrolled blood pressure (>180/110 mmHg), significant kidney or liver disease, premenopausal status, and excessive alcohol consumption (>21 units per week for men and >14 units per week for women).

The EVOLVE-FD study (Effects of EVOLocumab VErsus placebo added to standard lipid-lowering therapy on fasting and post fat load lipids in patients with Familial Dysbetalipoproteinemia) had a randomized, double-blind, placebo-controlled, crossover design (*Supplementary Figure 1*). Evolocumab 140 mg or matching placebo were administered subcutaneously every 2 weeks during two 12-week treatment periods in a random order (both supplied by Amgen, Breda, the Netherlands). There was an 8-week wash-out period between the two treatment periods.

All patients signed written informed consent. The study was approved by the Medical Ethics Review Committee of the UMC Utrecht and by the competent authority of the Netherlands. The study was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice. The EVOLVE-FD study was registered at www.clinicaltrials.gov (NCT03811223).

Oral fat load and retinyl palmitate

At the start and at the end of both treatment periods, patients visited the medical center in the morning after a ≥12 hour fast. At every visit patients underwent a standardized protocol and received an oral fat load. The oral fat load consisted of unsweetened fresh cream (Albert Heijn, Zaandam, the Netherlands) with a fat content of 35% (mass/volume). Cream was administered at a dose of 110 g of fat per m^2 of body surface area, with a maximum of 500 mL and ingested within 10 minutes. The fresh cream was mixed with 2 mL of retinyl palmitate, used to investigate exogenous lipoprotein metabolism in the post fat load phase. Venous blood samples were obtained before (at 0 hour) and 1, 2, 4, 6 and 8 hours after the oral fat load. During these eight hours, only drinking water was allowed. Other details of the study procedures and methods have been described elsewhere.[15]

Laboratory measurements

Density gradient ultracentrifugation

Before, 4 and 8 hours after the oral fat load the levels of cholesterol, TG and apoB were determined in the chylomicron, VLDL, IDL and LDL fractions isolated by density gradient ultracentrifugation.[19] Briefly, KBr (0.35 g/mL plasma) was added to plasma to achieve a density of 1.26 g/mL. One mL of plasma was placed in an ultracentrifuge tube followed by 1.9 mL of KBr solutions (of 1.21, 1.10, 1.063, 1.04 and 1.02 g/mL) in physiological saline and 1 mL of water. Samples were centrifuged for 30 minutes at 20°C at 207.000 g with an SW41 rotor in an Optima XPN-80 ultracentrifuge (Beckman Instruments, Indianapolis, IN, USA). After this, chylomicrons were isolated from the top 1 mL of the tube and this volume was replaced with 1 mL water before further centrifugation for 18 hours at 4 °C at 207.000 g, using the same rotor and centrifuge. After centrifugation fractions of 250 µL were eluted from the bottom of the tube in which cholesterol, TG and apoB were measured using a Selectra E (DDS Diagnostic system, Istanbul, Turkey). Lipoproteins were separated based on density. Chylomicrons were above fraction 46, VLDL was found in fractions 41–46. Fractions 34–40 were designated as IDL, and LDL as <34, in line with the corresponding standard density ranges in g/mL from ultracentrifugation. Density gradient ultracentrifugation was performed by the Laboratory of Vascular Medicine in Erasmus Medical Center, Rotterdam, the Netherlands.

Polyacrylamide gradient gel electrophoresis

The preparation of non-denaturing polyacrylamide gradient gels is described elsewhere.[20] In short, neutral lipids (cholesterol and TG) were prestained with Sudan Black. One gel was made for each visit, consisting of 6 lanes (at time point 0, 1, 2, 4, 6, 8 hours after the oral fat load) of the patient. The gels were calibrated with markers of ultracentrifugationally prepared VLDL1, VLDL2, IDL and LDL. Gels were placed in a photographic chamber and images were captured by a mounted video camera. The image was digitized for densitometric analysis in ImageJ.[21] After this, the lanes, which were converted to density plots in ImageJ, were analyzed with RStudio statistical software (version 3.5.1; R foundation for Statistical Computing, Vienna, Austria). The migration range (in inches) of the lipoprotein fractions was standardized and presented as a retardation factor (Rf), for which the beginning of the separation gel was defined as zero and the end of small dense LDL as one. The cut-offs of the markers were automatically defined for each gel separately, and were set when the relative intensity of the next marker was higher than that of the previous marker. The total area under the curve (AUC) for total staining and the AUC for the separate fractions (CM, VLDL1, VLDL2, IDL and LDL) were calculated. The relative AUCs of the separate fractions were compared with the total AUC and expressed as percentage of the total staining. Thus, this method did not allow quantification of lipoprotein concentrations in absolute terms,

but did allow insight into the relative distribution of neutral lipid among the different lipoprotein fractions. An example of one gel (for one visit) and its corresponding density plot is provided in *Supplementary Figure 2a* and *2b*, illustrating the excess of lipoproteins in the VLDL-IDL zone and absent LDL, typical for a dysbetalipoproteinemia lipid phenotype. Polyacrylamide gradient gel electrophoresis (PGGE) was performed by the Laboratory of Chemical Pathology at the University of Cape Town, South Africa.

Retinyl palmitate analyses
Examination of retinyl palmitate levels in plasma was performed with high-performance liquid chromatography (HPLC). Plasma samples (100µL) were prepared by a protein precipitation with ethanol and then liquid-liquid extraction followed with hexane. The hexane extract was evaporated under nitrogen and reconstituted with injection solvent containing butylated hydroxytoluene as a preservative. Fifty µL of reconstituted sample was injected onto the HPLC. Quantification was done by preparing calibration standards with pooled plasma that was spiked with known concentrations of certified reference standards of retinyl palmitate. Quality control (QC) samples were prepared in a similar manner to the calibrators – both the calibrators and QCs were extracted as described above.

The extracted standards, QCs and patient samples were analysed on an Agilent 1260 Infinity High Performance Liquid Chromatography system, with a Diode Array Detector (DAD). Reverse phase chromatography was used, and separation was achieved on an Agilent Poroshell C18 column held at 40°C. A 12-minute gradient elution was used with mobile phases A and B set up as water and Methanol: Acetonitrile (80:20, v/v) adjusted to pH 5 with acetic acid, respectively. The DAD was set to 325nm for analyte detection. Data acquisition and quantitation was done using MassHunter software. Linear calibration curves with weighted regression were used to quantify patient samples in µgram/L. Retinyl palmitate analyses were performed by the Laboratory of Chemical Pathology at the University of Cape Town, South Africa.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Each lipoprotein fraction (CM, VLDL, IDL and LDL) was analyzed for (apolipo)protein composition by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 4-20% gradient gels (mini-PROTEAN TGX Precast Gels, product 4568096, Biorad). Proteins were stained overnight with Pageblue Protein Staining Solution (product 24620, Thermo Scientific) after washing with demineralized water. Gels were scanned with an Amersham Imager 600+ (Cytiva). Analysis was performed with ImageQuant V8.2. All separate proteins were calculated as percentage of total protein in the fraction, and expressed in µg/mL. SDS-PAGE was performed by the Laboratory of Vascular Medicine in Erasmus Medical Center, Rotterdam, the Netherlands.

Statistical analyses

Baseline characteristics were presented as means with standard deviations (SD) or medians with interquartile range (IQR) when appropriate. Categorical variables were shown as number with percentage. The post fat load increase was expressed as incremental AUC (iAUC), which is based on the AUC that was calculated with the trapezoidal rule. The iAUC was calculated after adjustment for fasting lipid levels by subtracting eight (hours)*(value at time point 0) from the AUC. Since the data on the difference between placebo and evolocumab was very skewed, median absolute (for fasting levels and iAUC) and percentage difference (for fasting levels) between two treatment arms were calculated. Bootstrapping (1000 samples with replacement) was used to obtain robust confidence intervals (CIs) with corresponding *p*-values. The composition (cholesterol vs TG) per lipoprotein fraction after placebo and after evolocumab was compared. Also, the lipoprotein content (cholesterol and TG) was compared with the lipoprotein particle number (apoB) and expressed as (cholesterol+TG)/apoB ratio for every particle. There were no missing biochemical variables, but in three patients the retinyl palmitate data were removed because they were unrealistic (one patient had extremely high retinyl palmitate concentrations in the fasting state, and two patients had lipemic samples. Carryover and period effects were assessed with an independent samples t-test and no carry-over (p=0.65) or period effect (p=0.13) were observed. All *p*-values were two-tailed and $p<0.05$ was considered statistically significant. All analyses were performed with R statistical software (version 3.5.1; R foundation for Statistical Computing, Vienna, Austria).

Results

Baseline characteristics

In total, 31 patients were randomized and 28 patients completed the study. The patient disposition and reasons for screening failure and dropout are described in *Supplementary Table 1*. There were no clinically relevant baseline differences between the patients who dropped out and those who completed the study. The baseline characteristics of the 28 patients whose data were used for the analyses are shown in *Table 1*. The mean age was 62 ± 9 years and 57% were male. Most patients had an ε2ε2 genotype (93%), two patients had a dominant variant in the *APOE* gene and one patient had an ε2ε2 genotype and a dominant variant in *APOE*. Twenty-five percent of the patients had a history of CVD and 32% had T2DM. Most patients (93%) were on lipid-lowering medication, mostly a combination of a statin and ezetimibe (29%) or a statin and a fibrate (29%). One in four patients used high-intensity statins at baseline. Mean cholesterol level was 4.9 ± 1.9 mmol/L, mean non-HDL-C level was 3.6 ± 1.7 mmol/L and median triglyceride level was 2.8 (IQR 1.8–3.5) mmol/L.

Table 1. Baseline characteristics

	Patients n=28
Age (years)	62 ± 9
Female sex	12 (43)
APOE genotype	
• *ε2ε2*	26 (93)
• Dominant *APOE* variant	3 (11)
Cardiovascular disease	7 (25)
• Coronary heart disease	2 (7)
• Peripheral vascular disease	1 (4)
• Cerebrovascular disease	3 (11)
• Abdominal aortic aneurysm	1 (4)
Diabetes mellitus type 2	9 (32)
Hypertension	22 (79)
Metabolic Syndrome	21 (75)
Family history of premature CVD	7 (25)
Lipid-lowering treatment	26 (93)
• Statin only	6 (21)
• Ezetimibe only	2 (7)
• Fibrate only	1 (4)
• Statin + ezetimibe	8 (29)
• Statin + fibrate	8 (29)
• Statin + ezetimibe + fibrate	1 (4)
High intensity statin	7 (25)
Body mass index (kg/m^2)	29.5 ± 3.6
Waist circumference (cm)	107 ± 11
Laboratory measurements	
• Total cholesterol (mmol/L)	4.9 ± 1.9
• Triglycerides[a] (mmol/L)	2.8 (1.8 - 3.5)
• Non-HDL-cholesterol (mmol/L)	3.6 ± 1.7
• HDL-cholesterol (mmol/L)	1.3 ± 0.4
• Apolipoprotein B (g/L)	0.8 ± 0.2
• Lipoprotein (a)[a] (mg/dL)	8.2 (3.3 - 31.2)
• Glucose (mmol/L)	6.1 ± 1.5

Data are shown as n (%), mean ± standard deviation, or when not-normally distributed as median (interquartile range), indicated by [a]

Number and distribution of lipoproteins

The particle number (expressed as apoB concentration) was significantly reduced for all lipoproteins. Median reductions were 29% (95%CI 10–61%), 33% (95%CI 16–50%), 49% (95%CI 41–59%) and 58% (95%CI 50–73) for fasting CM-apoB, VLDL-apoB, IDL-apoB and LDL-apoB, respectively (*Figure 1* and *Supplementary Table 2*). For all lipoproteins there was no significant change in 8-hours post fat load apoB iAUC (*Supplementary Table 3*).

Results from the PGGE gels are shown in *Figure 2*.The relative contribution of neutral lipid (i.e. cholesterol and TG) to the total amount of neutral lipid, was higher in the larger lipoproteins (i.e. CM, VLDL1 and VLDL2) after treatment with evolocumab compared to placebo. For example, 34% of all neutral lipid was present in VLDL2 after placebo and was significantly different compared to 38% after evolocumab (p=0.007). In contrast, the relative contribution of neutral lipid was lower in the smaller lipoproteins (IDL and LDL) after treatment with evolocumab compared to placebo. IDL neutral lipid significantly decreased from 20% of all neutral lipid after placebo to 15% after evolocumab (p=0.005). For LDL this was 13% and 10% after placebo and evolocumab, respectively.

Composition of lipoproteins

In absolute terms, compared to placebo, cholesterol levels in all lipoproteins (CM, VLDL, IDL and LDL) were significantly reduced after 12 weeks treatment with evolocumab. The median reductions in fasting CM-C, VLDL-C, IDL-C and LDL-C were 58% (95%CI 36–71%); 48% (95%CI 29–63%); 53% (95%CI 36–64%) and 52% (36–65%), respectively (*Figure 3* and *Supplementary Table 4*). After treatment with evolocumab, fasting levels of CM-TG were significantly reduced by 26% (95%CI 20–40%) compared to placebo and fasting VLDL-TG were reduced by 20% (95%CI -6.3–41%). Finally, although absolute TG levels were very low, IDL-TG and LDL-TG were significantly reduced by 33% (95%CI 19–42) and 50% (95%CI 31–60%), respectively (*Figure 4* and *Supplementary Table 5*). The 8-hour post fat load response of cholesterol and TG (iAUC) in all lipoprotein fractions was not reduced after evolocumab (*Supplementary Table 3*).

Cholesterol levels were reduced more than TG levels in all lipoproteins, expressed by a decrease in the cholesterol to TG ratio after treatment with evolocumab, compared with placebo. For example, VLDL consisted of 49% cholesterol and 51% TG after treatment with placebo, and of 39% cholesterol and 61% TG after evolocumab (*Figure 5*).

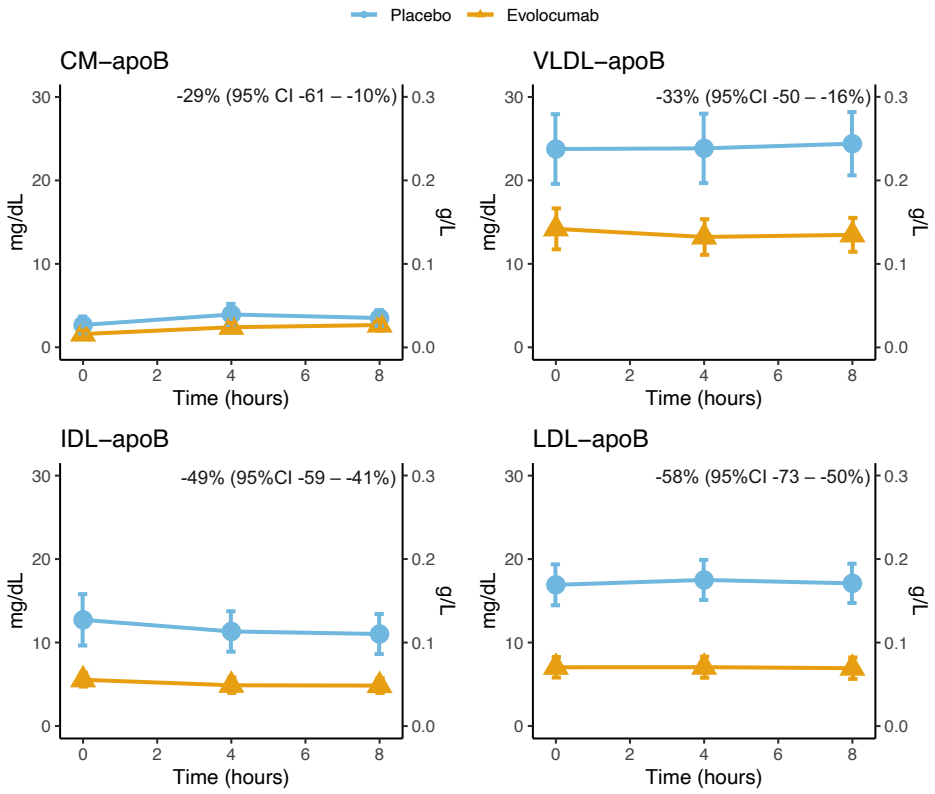

Figure 1. Effect of evolocumab on fasting and 8-hour post fat load apoB levels in lipoproteins

Fasting and 8-hour post fat load apoB levels after an oral fat load, after treatment with evolocumab (orange) or placebo (blue). In top right the median percentage difference in fasting value after treatment with evolocumab compared with placebo. Abbreviations: CM-apoB = chylomicron-apolipoprotein B, VLDL-apoB very-low-density lipoprotein- apolipoprotein B, IDL-apoB = intermediate-density lipoprotein- apolipoprotein B, LDL-apoB = low-density lipoprotein- apolipoprotein B

In addition, for VLDL, IDL and LDL, the number of particles were reduced more than the content (cholesterol and TG) of the particle. Although not significant, the ((TG plus cholesterol)/apoB) ratio increased for VLDL (3.5% (95% CI -11.2-16.3)) and IDL (9.8% (95%CI -4.6-19.4)), indicating that the size of these lipoproteins increased after treatment with evolocumab. The LDL ratio increased significantly with 12% (95% CI 6.9 – 32.9) (*Supplementary Table 6*).

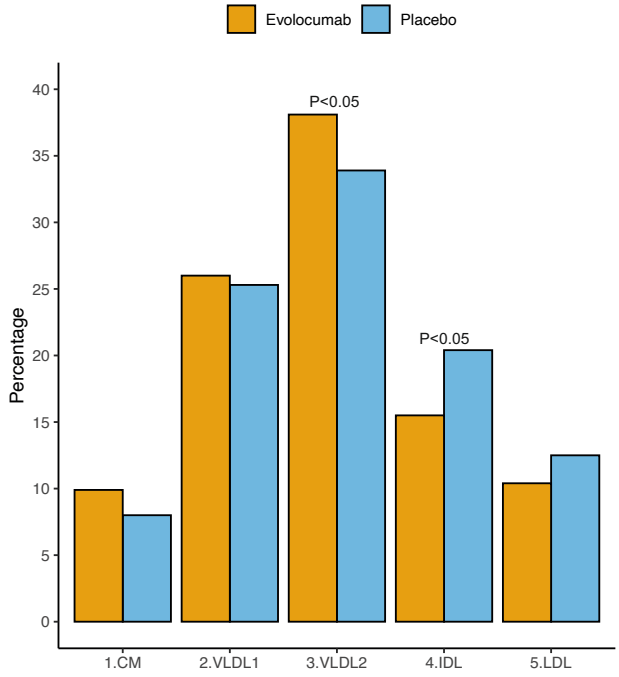

Figure 2. Relative distribution of lipids in lipoproteins after evolocumab, compared with placebo in the fasting state

Distribution of neutral lipid (cholesterol and triglycerides) in lipoprotein fractions after placebo (blue) and after evolocumab (orange).

Fasting and post fat load chylomicron response

Ultracentrifugation showed a significant reduction in fasting CM-apoB, CM-C and CM-TG after evolocumab compared to placebo and an unchanged post fat load iAUC of CM-apoB, CM-C and CM-TG. The 8-hour post fat load retinyl palmitate iAUC was not significantly different after evolocumab (7%, 95%CI -15–22%) compared to placebo (*Figure 6* and *Supplementary Table 7*). The results of SDS-PAGE showed that the lipoproteins in the chylomicron fraction as isolated by ultracentrifugation mainly consisted of buoyant VLDL particles, because apoB in the CM fraction consisted of mainly apoB100 proteins and hardly any apoB48 proteins (*Supplementary Figure 3*).

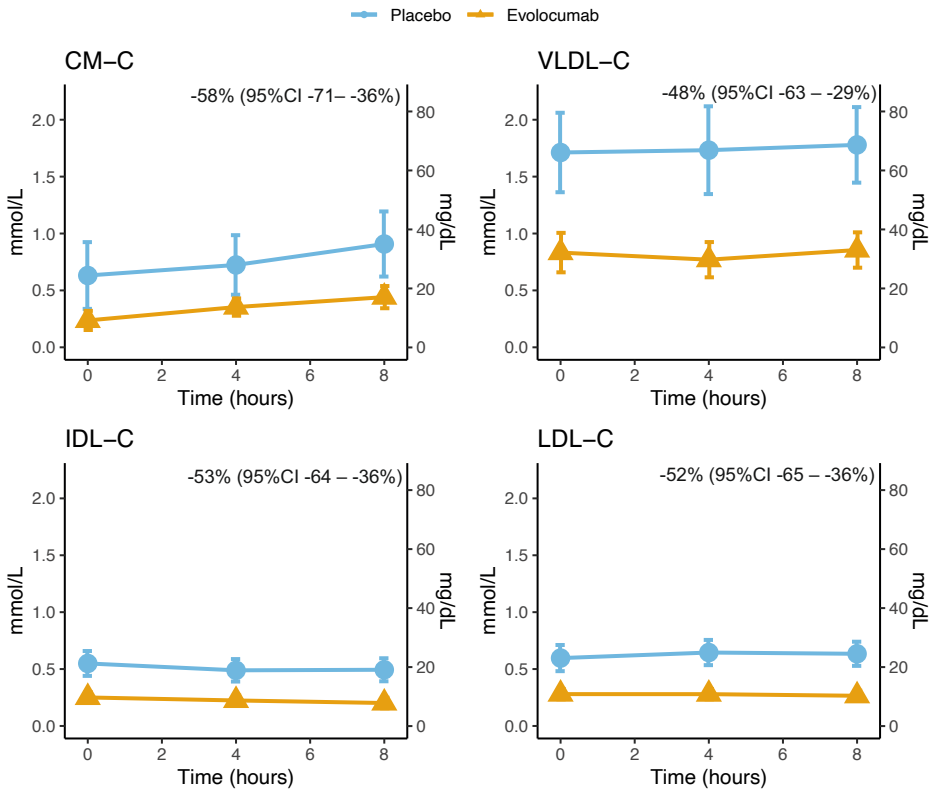

Figure 3. Effect of evolocumab on fasting and 8-hour post fat load cholesterol levels in lipoproteins

Fasting and 8-hour post fat load cholesterol levels after an oral fat load, after treatment with evolocumab (orange) or placebo (blue). In top right the median percentage difference in fasting value after treatment with evolocumab compared with placebo.

Abbreviations: CM-C = chylomicron-cholesterol, VLDL-C very-low-density lipoprotein-cholesterol, IDL-C = intermediate-density lipoprotein-cholesterol, LDL-C = low-density lipoprotein-cholesterol.

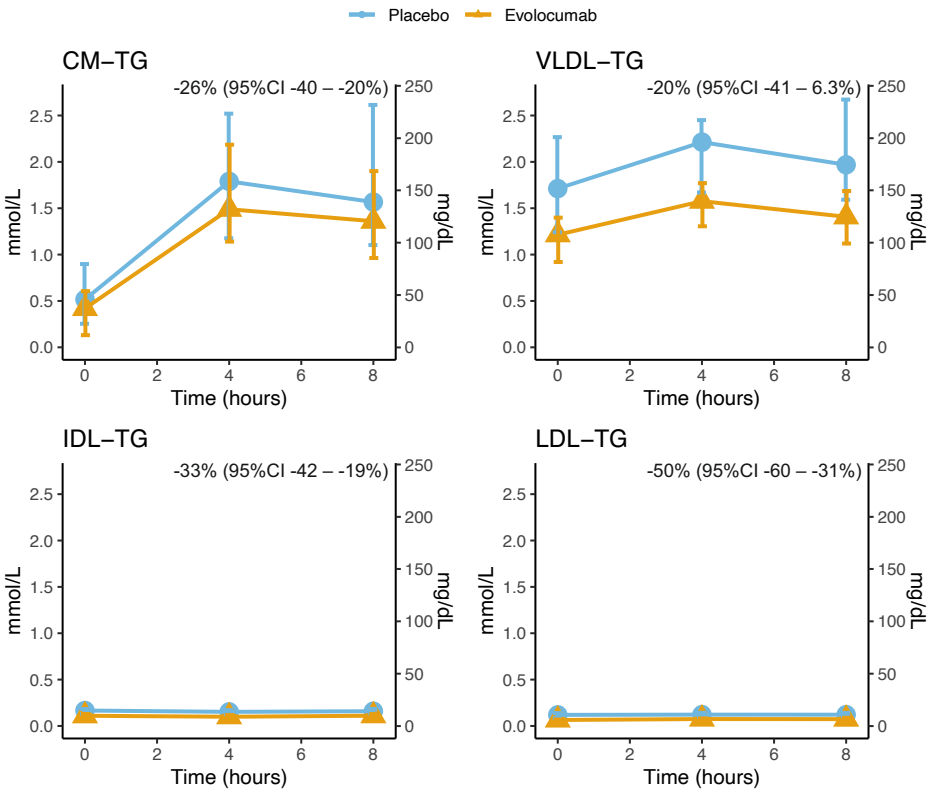

Figure 4. Effect of evolocumab on fasting and 8-hour post fat load triglyceride levels in lipoproteins

Fasting and 8-hour post fat load triglyceride levels after an oral fat load, after treatment with evolocumab (orange) or placebo (blue). Triglyceride concentrations in medians, with bootstrapped 95% CI. In top right the median percentage difference in fasting value after treatment with evolocumab compared with placebo.

Abbreviations: CI = confidence interval, CM-TG = chylomicron-triglycerides, VLDL-TG very-low-density lipoprotein-triglycerides, IDL-TG = intermediate-density lipoprotein-triglycerides, LDL-TG = low-density lipoprotein-triglycerides

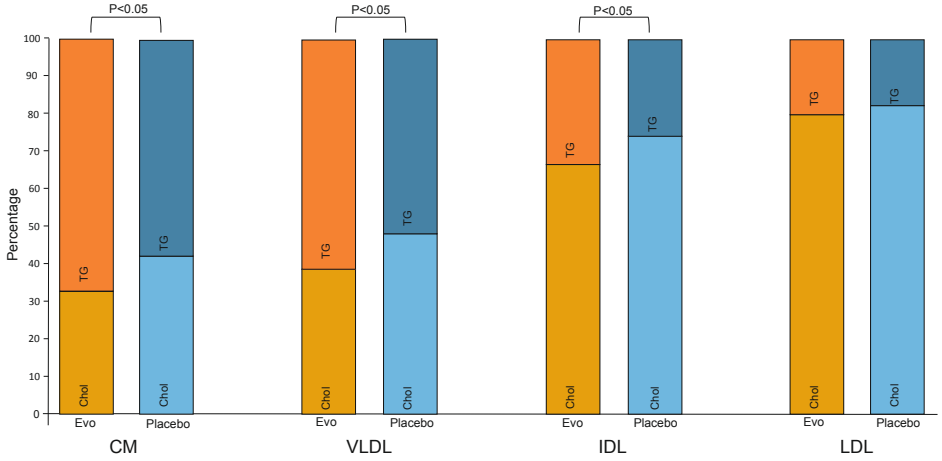

Figure 5. Cholesterol and TG distribution per lipoprotein particle after evolocumab, compared with placebo in the fasting state

Distribution of cholesterol (bottom) and triglycerides (top) per lipoprotein particle after placebo (blue) and after evolocumab (orange).
P-value for difference in cholesterol in each fraction between evolocumab and placebo.

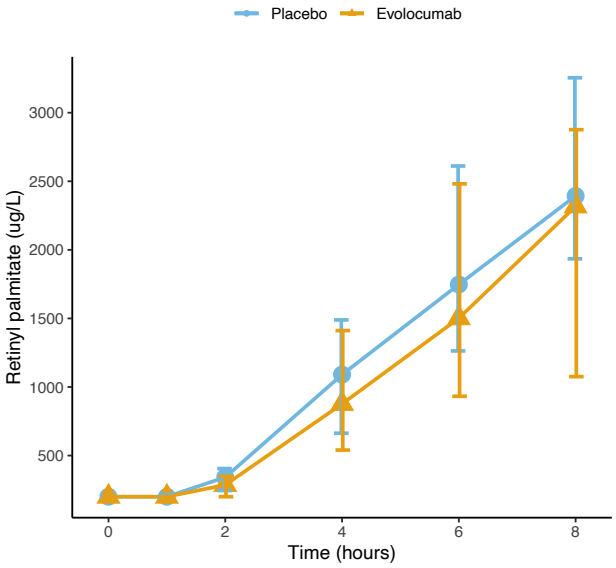

Figure 6. Effect of evolocumab on fasting and 8-hour post fat load retinyl palmitate levels

Retinyl palmitate levels as a marker of apoB48-containing lipoprotein clearance. Retinyl palmitate analyses were performed in 25 patients.

Discussion

In this study in 28 patients with genetically confirmed FD, several effects of the PCSK9 mAbs evolocumab on lipoprotein distribution and composition were observed. First, in absolute terms, VLDL, IDL and LDL particle numbers were significantly reduced after treatment with evolocumab. This reduction was larger for the smaller lipoprotein particles (IDL and LDL) compared to the larger VLDL particles. Second, absolute levels of cholesterol and TG in VLDL, IDL and LDL were reduced, with a larger reduction in cholesterol than TG. Third, evolocumab did not affect the number or composition of chylomicrons.

In the main report of the EVOLVE-FD trial, it was demonstrated that fasting and post fat load levels of non-HDL-C, TG, TC, apoB, VLDL-C and IDL-C were significantly decreased after treatment of 12 weeks with evolocumab, compared with placebo.[15] Post fat load non-HDL-C levels (AUC) were significantly decreased with 49% (95%CI 42–55) and post fat load TG levels were significantly decreased with 20% (95%CI 10–29).The effects of PCSK9 mAbs in FD have been investigated in one other study. That non-randomized study evaluated the effect of PCSK9 mAbs on fasting lipids in three patients with FD and vascular disease who were intolerant or resistant to treatment with statins and fibrates. The study showed that cholesterol and apoB levels were decreased in all apoB100-containing lipoprotein fractions, although confidence intervals could not be provided due the limited sample size.[22]

The present study showed that PCSK9 mAbs reduced the number of small cholesterol-rich particles (49% and 58% for IDL and LDL, respectively) more than the number of larger TG-rich particles (33% for VLDL), compared to placebo. This is in line with a stable isotope study in 18 healthy individuals that showed that IDL-apoB was reduced by 30% and LDL-apoB was reduced by 56% after 10-week treatment with alirocumab 150 mg.[14] In contrast to the present findings, no effect on VLDL-apoB was found in that study, and the effect on IDL was smaller (30% versus 49% in the present study). These differences could be due to the fact that the study used healthy subjects with TG levels in the normal range, compared to FD patients that have hypertriglyceridemia. However, another study in 80 healthy men found that evolocumab 420 mg every 2 weeks significantly reduced fasting and post fat load VLDL-apoB levels.[10,11] In patients with T2DM, who have a lipoprotein phenotype that is more similar to FD, a stable isotope study showed that evolocumab had little effect on the VLDL1-apoB100 concentration, but did lead to a significant reduction in VLDL2-apoB100 concentration.[9] In conclusion, PCSK9 mAbs seem to reduce particle number of LDL, IDL and probably VLDL in different populations including FD.

The present study showed that PCSK9 mAbs reduce cholesterol and TG in VLDL, IDL and LDL. The reduction in cholesterol was more pronounced than the reduction in TG. The increased reduction in cholesterol could be due to increased clearance of cholesterol-rich particles. Although we found a small increase in lipoprotein size after treatment with evolocumab (calculated as the sum of TG and cholesterol to apoB in VLDL, IDL and LDL particles), it is unknown whether this is a clinically relevant difference. The increase in VLDL particles size is in line with an observational longitudinal study that showed that after treatment with PCSK9 mAbs, VLDL particle size was increased (based on TG to apoB ratio in VLDL), indicating accelerated degradation of smaller VLDL particles.[23] In addition, we found a significant increase in LDL size, suggesting an increased clearance of smaller LDL. However, this is not in line with previous studies that found that the LDL size (reflected by the cholesterol to apoB ratio in LDL), decreased after treatment with evolocumab.[11,23] The decrease in LDL size could be explained by the effect that smaller LDL particles have a reduced affinity for the LDL-R compared to larger LDL particles,[24,25] but this was not observed in our study. This difference might be explained by different methods to estimate the lipoprotein size, in which the previous studies assumed that VLDL and LDL consisted of respectively TG and cholesterol only. Changes in composition and distribution of particles could be clinically relevant since smaller, cholesterol-rich particles (IDL and LDL), or cholesterol-enriched larger particles (such as beta-VLDL) are more atherogenic compared to other apoB lipoproteins, potentially leading to a reduced CVD risk. Detailed insight in the distribution and composition also provides insight in the mechanism of action of PCSK9 mAbs in FD.

Although the results of the ultracentrifugation showed a significant reduction in CM particle number and particle content (CM-C and CM-TG), this is most likely in reality large VLDL. SDS-PAGE results namely showed that the CM fraction mainly consisted of CM-sized VLDL (apoB100 carrying) particles. In FD, often more buoyant VLDL particles are seen,[26] this likely due to the reduced lipolysis in FD. Therefore, the decrease in apoB, cholesterol and TG in chylomicrons is likely due to a decrease of apoB, cholesterol and TG in VLDL particles. This might have led to an underestimation of the effect of evolocumab on VLDL in FD patients. In addition, the post fat load CM-TG response (iAUC) after treatment with evolocumab and placebo was identical and there was no change in retinyl palmitate concentrations after evolocumab, indicating no effect on chylomicron metabolism. Therefore, it seems most likely that PCSK9 mAbs have no effect on chylomicron production and clearance in patients with FD. This in line with most previous studies demonstrating that PCSK9 mAbs do not substantially affect chylomicron metabolism, including chylomicron formation in the intestine, chylomicron transport in the thoracic duct, and chylomicron (or chylomicron-remnant) clearance in the liver.[8-10,14]

In healthy subjects, clearance of TRLs can take place directly through binding of apoE to the LDL-R, or indirectly by conversion of IDL to LDL. Given that all patients with FD have dysfunctional apoE, that has a greatly reduced affinity (>98% reduction)[3] for the LDL-R, it is intriguing that LDL-R upregulation (the primary mode of action of PCSK9 mAbs) leads to significant reductions in (small) TRLs in this FD population. For this we pose two hypotheses.

The first hypothesis is that PCSK9 mAbs stimulate the conversion of IDL to LDL. In healthy people, IDL is converted to LDL through lipolysis by lipoprotein lipase (LPL) and hepatic lipase (HL). In FD, this process is inhibited because apoE2 displaces apoC2,[3] thus limiting the co-factor for proper LPL mediated lipolysis.[27] In addition, the action of HL on IDL is impaired by apoE2, although the exact mechanisms are unknown.[28,29] Previous studies showed that PCSK9 mAbs did not affect LPL, HL or apoC3 activity.[8,9] In addition, previous kinetic studies with stable isotopes in healthy subjects and patients with T2DM showed that there was a decreased conversion from IDL to the LDL fraction after treatment with PCSK9 mAbs.[9,10,14] Taken together, the existing evidence does not support increased conversion from IDL to LDL as an explanation for the effects of PCSK9 mAbs in patients with FD.

The second hypothesis is that PCSK9 mAbs increase hepatic clearance of IDL particles. IDL can be cleared by binding of apoE to three hepatic clearance receptors, namely the LDL-R, heparan sulphate proteoglycans (HSPG) and LDL-R related protein 1 (LRP1).[30,31] The LDL-R can clear remnant lipoproteins directly, using apoE as a ligand, as well as LDL with apoB100 as a ligand. HSPG can clear remnant lipoproteins alone, and the LRP1 depends on HSPG for clearance of remnants. In FD, the affinity of the apoE protein is low for the LDL-R, but binding to the other receptors is not impaired.

The first argument for the increased IDL clearance hypothesis is that the upregulation of LDL-R by PCSK9 mAbs can lead to rapid clearance of LDL-apoB and therefore to less competition with apoE to bind to the LDL-R. A stable isotope study in healthy subjects and in patients with T2DM found an increased direct clearance of IDL by the liver after PCSK9 mAb treatment. Although it is unknown by which receptor this clearance was mediated, the authors suggest that very low levels of LDL achieved with PCSK9 mAbs allow other lipoproteins (with less affinity for the LDL-R) to enter the extremely upregulated LDL-R pathway.[9]

A second argument to support increased IDL clearance is that the severely reduced apoE affinity in FD is likely enough for proper functioning of PCKS9 mAb. In a study in ApoE3*Leiden.CETP mice (a translational model for FD, in which mice express both

mouse apoE and the human mutant apoE3*Leiden), it was found that PCSK9 antibodies resulted in a significant reduction of cholesterol (-45%) and TG levels (-36%).[32] In the same study the FD mice were compared to mice without any functional ApoE. In this group PCSK9 antibodies did not have any effect on cholesterol or TG. This shows that at least some apoE binding to LDL-R is necessary for PCSK9 antibodies to function, and that the limited apoE affinity in FD is likely enough to establish these effects.

A third argument for increased IDL clearance is that statins have also been shown to be effective in FD patients. Although statins also reduce cholesterol production by decreasing the rate of intracellular cholesterol production, the main mechanism of action of statins is upregulation of the LDL-R. Statins have been shown to reduce particle number, and cholesterol and TG content in all apoB100 particles, including IDL in FD.[33-35] The mode of action of statins in FD is not fully understood, but these observations do support the notion that LDL-R upregulation can lead to a decrease in atherogenic lipid fractions, including TRLs, even in FD.

A fourth argument for increased IDL clearance might be that PCSK9 mAbs affect non-LDL-R clearance pathways, since the LDL-R is not the major receptor for IDL clearance. However, it has been shown that the number of LRP1 receptors is not affected by PSCK9 mAbs,[36] and the effects of PCSK9 mAbs on HSPGs are unknown. In theory, LDL reduction by PCSK9 mAb might create more space for (small) IDL in the space of Disse, leading to easier uptake of IDL by the HSPG and LRP1 systems. Recently, it was shown that another receptor, the VLDL-receptor (VLDL-R) also plays a role in IDL clearance, and that its expression is regulated by PCSK9.[37,38] However, this receptor is located in peripheral tissues and not in the liver. In conclusion, the hypothesis that IDL reduction of PCSK9 mAbs in FD is caused by increased clearance seems likely, but the exact mechanisms remain unknown.

The main strength of this study is that it is part of the largest RCT conducted in patients with FD, with extensive determination of lipoprotein fractions, both in the fasting and post fat load state.

Some limitations need to be considered. First, since FD is a typical IDL (remnant) disease with extremely elevated IDL particles and relatively low LDL-C levels, it is noteworthy that LDL-C levels measured with ultracentrifugation were higher than IDL-C levels (LDL-C 0.6 ± 0.3 mmol/L *vs* IDL-C 0.5 ± 0.3 mmol/L). This might be due to the ultracentrifugation itself, because in FD IDL may contaminate LDL fractions.[39] Therefore, it is difficult to distinguish the IDL and LDL fractions in FD since they overlap. The same is true for CM and VLDL. Although during insulin resistance, CMs may be present in the fasting state,[40]

the CM fraction is expected to be zero. As was discussed before, VLDL-apoB100 was found in the CM fraction. This could have led to an overestimation of apolipoproteins and lipids in CM and LDL and an underestimation of them in VLDL and IDL. Furthermore, we were unable to differentiate which part of the IDL fraction was cleared by hepatic receptors directly and which part was converted to LDL. Studies with stable isotopes in patients with FD are needed to assess this.

In the present study it is shown that evolocumab significantly reduced all apoB100-containing lipoproteins (VLDL, IDL and LDL) in FD patients, including very atherogenic remnant lipoproteins that are causally related to atherosclerosis and CVD.[41] Therefore, evolocumab is expected to reduce CVD risk in this high-risk population. The potential of PCSK9 mAbs, not only with respect to LDL-C lowering, but also with respect to IDL-cholesterol lowering, in patients with and without functional apoE, should be highlighted.

In conclusion, evolocumab added to standard lipid-lowering therapy in FD patients significantly reduced lipoprotein particle number, in particular the smaller and more cholesterol-rich lipoproteins (i.e. IDL and LDL). In addition, cholesterol levels were reduced more than TG levels. Evolocumab did not affect chylomicron metabolism. It seems likely that the reduction in TRLs is achieved by increased hepatic clearance, but the specific contribution of the LDL-receptor and non-LDL-receptor pathways remains to be elucidated.

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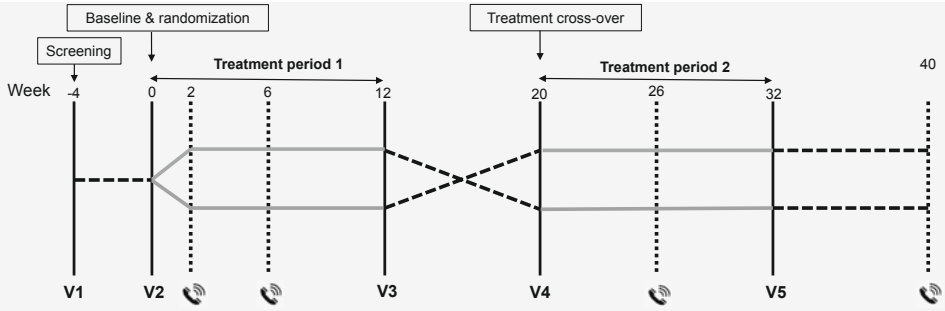
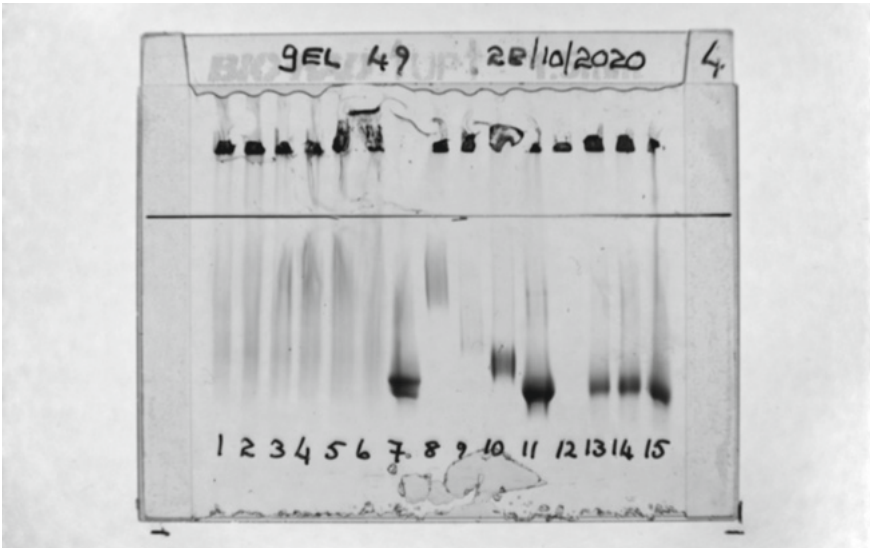
Supplementary Material



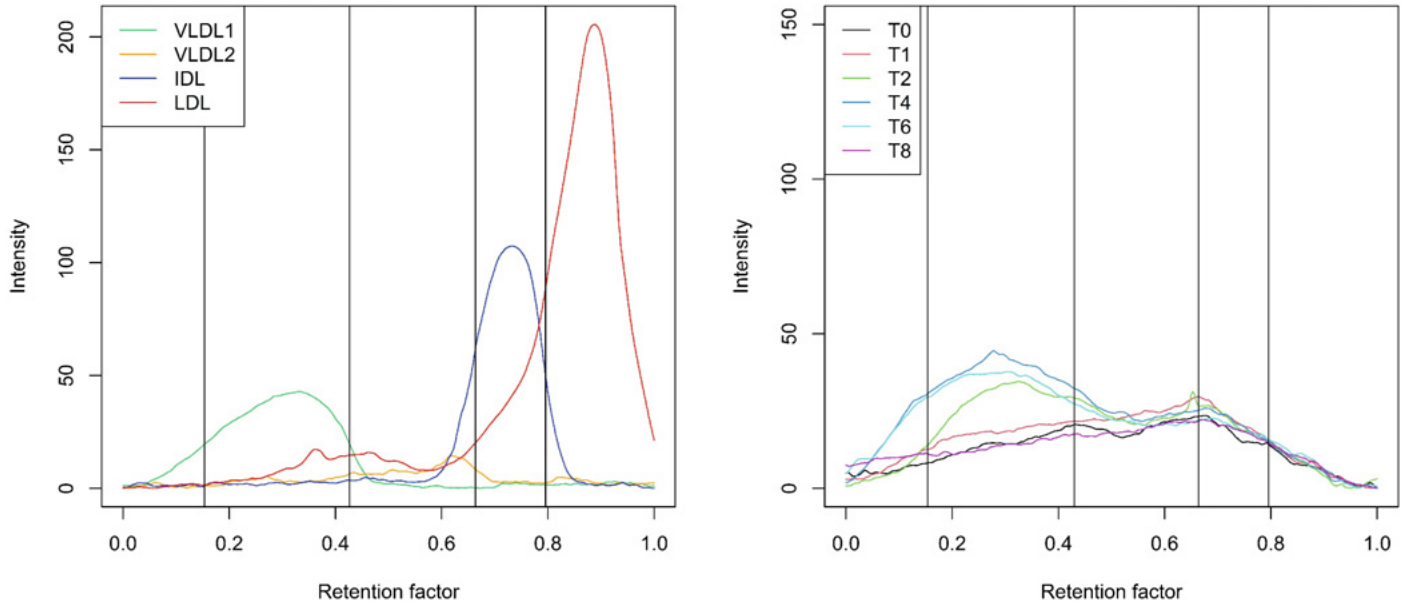
Supplementary Figure 1. Study design EVOLVE-FD

At four identical visits (two at the beginning of the treatment period and two at the end of the treatment period) an oral fat load was administered and blood samples were collected up to eight hours after the oral fat load. Patients were randomized to treatment order, meaning that all patients used evolocumab (orange) and placebo (blue) during the study. During weeks 2, 6, 26 and 40, phone calls were made to evaluate medication use and possible adverse events.



Supplementary Figure 2a. Example of PGGE

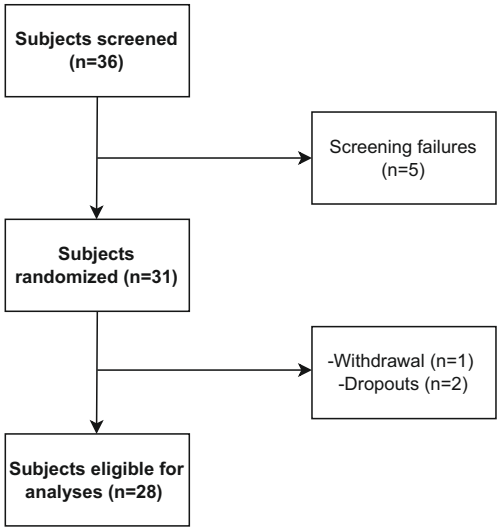
Example of polyacrylamide gradient gel electrophoresis of one patient with FD. Lanes 1 to 6 are the fasting and 1, 2, 4, 6 and 8 hours after the oral fat load, respectively. In lanes 8 to 11 contain ultracentrifugally prepared lipoprotein fractions which serve as markers of that particular fraction. Lane 8 is the VLDL1 marker, lane 9 is the VLDL2 marker, lane 10 is the IDL marker and lane 11 is the LDL marker. Lanes 12 to 15 contain different concentrations of LDL-C. Lane 12 is 0 mmol/L LDL-C, lane 13 is 0.3 mmol/L LDL-C, lane 14 is 0.7 mmol/L and lane 16 is 2.6 mmol/L LDL-C.



Supplementary Figure 2b. Corresponding density plot of marker lanes (left) and the post fat load lanes (right)

On the left is an example of the density plot for the markers of the gel from Supplementary Figure 2a. The cut-offs of the markers were automatically defined for each gel separately, and were set when the relative intensity of the next marker was higher than the previous marker.

On the right, the density plot for the post fat load lanes of the patient is shown. On the x-axis, the retention factor is given with large lipoproteins (CM) starting at retention factor 0.0 to the smallest lipoproteins (small LDL) at a retention factor of 1.0. The black vertical lines are the cut-offs between the lipoprotein fractions. The AUC of every lipoprotein fraction (CM, VLDL1, VLDL2, IDL and LDL) was calculated. In this figure it is clear that this FD patient has very little LDL compared to the larger lipoproteins and that at 2, 4 and 6 hours (green and blue lines) the number of large lipoproteins (between retention factor 0.1 and 0.4) was increased.



Supplementary Table 1. Patient disposition

Reasons for screening failures (n=5); severe dyslipidemia requiring initiation of lipid-lowering treatment first (n=2) and not having an A*POE* genotype that was associated with FD (n=3).
Reason for withdrawal (n=1); withdrawal of consent because of pain due to pre-existent peripheral artery disease. Reason for dropout (n=2); no completion of all measurements to assess the primary endpoint; one patient was not able to ingest fresh cream due to a recent cholecystectomy, therefore only fasting samples were drawn at that visit. One patient was not feeling well after ingestion of the oral fat load, therefore two post fat load samples were missing at one visit.

Supplementary Table 2. Effect of evolocumab compared to placebo on fasting apoB levels in lipoproteins

	After placebo mean ± SD	**After evolocumab** mean ± SD	**Difference** median (IQR)	**% Difference** median (95%CI)	***P*-value**
CM-apoB					
mg/dL	2.7 ± 2.7	1.6 ± 1.1	-0.6 (-1.3 – -0.1)	-29 (-61 – -10)	<0.001
VLDL-apoB					
mg/dL	24 ± 11	14 ± 7.0	-6.2 (-14 – -1.5)	-33 (-50 – -16)	<0.001
IDL-apoB					
mg/dL	13 ± 8	5.5 ± 2.0	-5.3 (-9.0 – -2.9)	-49 (-59 – -41)	<0.001
LDL-apoB					
mg/dL	17 ± 7	7.0 ± 3.0	-8.8 (-15 – -5.3)	-58 (-73 – -50)	<0.001

To calculate apoB levels in g/L divide by 100.
Abbreviations: ApoB = apolipoprotein B, CI = confidence interval, CM= chylomicron, IDL = intermediate-density lipoprotein, IQR = interquartile range, LDL = low-density lipoprotein, VLDL = very-low-density lipoprotein

Supplementary Table 3. Effect of evolocumab compared to placebo on 8-hour post fat load (iAUC) of apoB, cholesterol and TG levels in lipoproteins

	After placebo	**After evolocumab**	**Difference**	***P*-value**
	mean ± SD	mean ± SD	median (95%CI)	
ApoB				
CM-apoB				
mg/dL.8h	6.7 ± 20	5.4 ± 4.8	-0.9 (-4.2 - 2.4)	0.85
VLDL-apoB				
mg/dL.8h	1.6 ± 28	-5.3 ± 16	-7.7 (-20 - 1.6)	0.34
IDL-apoB				
mg/dL.8h	-9.0 ± 14	-4.0 ± 3.9	2.2 (-0.9 - 6.8)	0.35
LDL-apoB				
mg/dL.8h	2.7 ± 7.0	-0.2 ± 5.9	-3.4 (-5.6 - -0.2)	0.052
Cholesterol				
CM-C				
mmol/L.8h	0.9 ± 1.1	0.9 ± 0.9	-0.2 (-0.4 - 0.2)	0.34
VLDL-C				
mmol/L.8h	0.2 ± 1.9	-0.2 ± 1.0	0.1 (-1.4 - 0.6)	1.0
IDL-C				
mmol/L.8h	-0.4 ± 0.6	-0.2 ± 0.4	0.1 (-0.2 - 0.6)	1.0
LDL-C				
mmol/L.8h	0.3 ± 0.8	0.0 ± 0.3	-0.1 (-0.3 - 0.1)	0.57
Triglycerides				
CM-TG				
mmol/L.8h	7.4 (4.1 - 10.5)	5.8 (4.4 - 9.9)	-0.3 (-1.7 - -0.01)	0.09
VLDL-TG				
mmol/L.8h	2.2 (0.9 - 4.5)	2.1 (0.5 - 2.9)	-0.6 (-2.3 - 0.5)	0.34
IDL-TG				
mmol/L.8h	-0.04 (-0.1 - 0.02)	-0.02 (-0.08 - 0.03)	0.01 (-0.04 - 0.09)	0.35
LDL-TG				
mmol/L.8h	0.02 (0.0 - 0.08)	0.05 (0.01 - 0.08)	0.0 (-0.04 - 0.05)	1.0

Supplementary Table 4. Effect of evolocumab compared to placebo on fasting cholesterol levels in lipoproteins

	After placebo	**After evolocumab**	**Difference**	**% Difference**	***P*-value**
	mean ± SD	mean ± SD	median (IQR)	median (95%CI)	
CM-C					
mmol/L	0.6 ± 0.8	0.2 ± 0.2	-0.2 (-0.4 – -0.03)	-58 (-71 – -36)	0.004
VLDL-C					
mmol/L	1.7 ± 0.9	0.8 ± 0.5	-0.8 (-1.3 – -0.2)	-48 (-63 – -29)	<0.001
IDL-C					
mmol/L	0.5 ± 0.3	0.3 ± 0.1	-0.2 (-0.5 – -0.1)	-53 (-64 – -36)	<0.001
LDL-C					
mmol/L	0.6 ± 0.3	0.3 ± 0.1	-0.3 (-0.5 – -0.06)	-52 (-65 – -36)	<0.001

Abbreviations: C = cholesterol, CI = confidence interval, CM = chylomicron, IDL = intermediate-density lipoprotein, IQR = interquartile range, LDL = low-density lipoprotein, VLDL = very-low-density lipoprotein

Supplementary Table 5. Effect of evolocumab compared to placebo on fasting triglyceride levels in lipoproteins

	After placebo	**After evolocumab**	**Difference**	**% Difference**	***P*-value**
	median (IQR)	median (IQR)	median (IQR)	median (95%CI)	
CM-TG					
mmol/L	0.52 (0.24 – 0.97)	0.42 (0.13 – 0.7)	-0.14 (-0.3 – -0.03)	-26 (-40 – -20)	<0.001
VLDL-TG					
mmol/L	1.71 (0.98 – 2.51)	1.21 (0.77 – 1.56)	-0.28 (-1.15 – 0.19)	-20 (-41 – 6.3)	0.18
IDL-TG					
mmol/L	0.17 (0.12 – 0.19)	0.11 (0.1 – 0.13)	-0.06 (-0.08 – -0.02)	-33 (-42 – -19)	<0.001
LDL-TG					
mmol/L	0.12 (0.07 – 0.15)	0.06 (0.04 – 0.09)	-0.05 (-0.07 – -0.03)	-50 (-60 – -31)	<0.001

Abbreviations: CM = chylomicron, CI = confidence interval, IDL = intermediate-density lipoprotein, IQR = interquartile range, LDL = low-density lipoprotein, TG = triglycerides, VLDL = very-low-density lipoprotein

Supplementary Table 6. Effect of evolocumab compared to placebo on lipoprotein size, expressed by fasting lipid to apoB ratios

	After placebo	**After evolocumab**	**Difference**	**% Difference**	***P*-value**
	mean ± SD	mean ± SD	median (IQR)	median (95%CI)	
VLDL-C+VLDL-TG/VLDL-apoB	14.5 ± 3.1	14.7 ± 2.9	0.5 (-2.1 – 2.8)	3.5 (-11.2 – 16.3)	0.57
IDL-C+IDL-TG/IDL-apoB	6.1 ± 1.6	6.5 ± 3.1	0.5 (-0.4 – 1.4)	9.8 (-4.6 – 19.4)	0.34
LDL-C+LDL-TG/LDL-apoB	4.3 ± 1.0	5.6 ± 2.5	0.5 (-0.2 – 2.0)	12.1 (6.9 – 32.9)	0.04

Abbreviations: apoB = apolipoprotein B, C = cholesterol, CM = chylomicron, CI = confidence interval, LDL = low-density lipoprotein, TG = triglycerides, VLDL = very-low-density lipoprotein

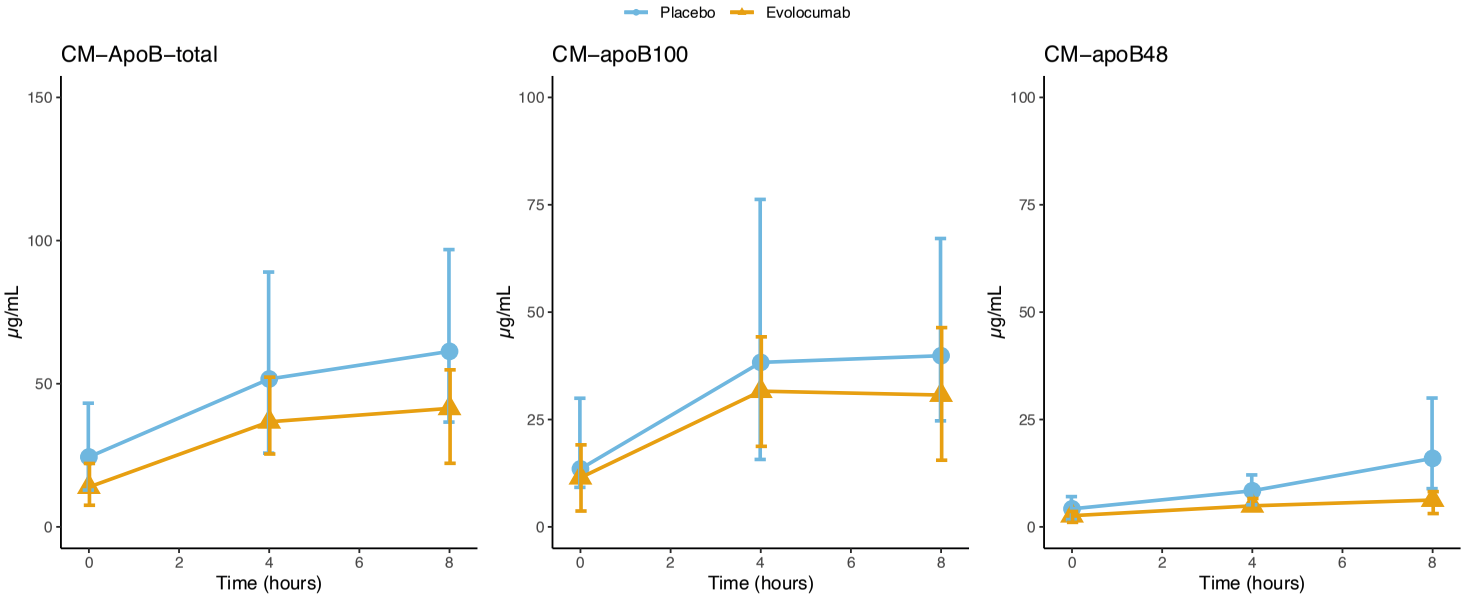
Supplementary Table 7. Effect of evolocumab on fasting and 8-hour post fat load chylomicrons

	After placebo	**After evolocumab**	**Difference**	**%Difference**	***P*-value**
	median (IQR)	median (IQR)	median (95% CI)	median (95% CI)	
Fasting					
Retinyl palmitate					
ug/L.8h	200 (200 - 201)	200 (200 - 200)	0 (0 - 0)	0 (0 - 0)	1.0
iAUC					
Retinyl palmitate					
ug/L.8h	7452 (3740 - 10938)	6144 (2746 - 10024)	-292 (-1627 - 1101)	-7 (-22 - 15)	0.23

The minimum retinyl palmitate concentration that could be measured was 200 ug/L.

Retinyl palmitate analyses were performed in 25 patients.

Abbreviations: CI = confidence interval, iAUC = incremental area under the curve, IQR = interquartile range.



Supplementary Figure 3. ApoB100 and apoB48 in the chylomicron fraction

Fasting and 8-hour post fat load of CM-ApoB-total (sum of CM-apoB100 and CM-apoB48) and CM-apoB100 and CM-apoB48 separately, measured with SDS-PAGE.

Chapter 11

General discussion

General discussion

In this thesis, the general objectives were to investigate the relationship between genes, lipoproteins and cardiovascular disease (CVD) risk. We focused on the genetic lipid disorder Familial Dysbetalipoproteinemia (FD) and sought to evaluate etiologic pathways, diagnostic criteria and new therapeutic options.

What is the role of triglyceride-rich lipoproteins (TRLs) in the development of CVD?
Until recently, the focus regarding lipids and related CVD risk was on low-density lipoproteins (LDL) which in clinical practice is reported as LDL-cholesterol (LDL-C). Numerous clinical trials and Mendelian randomization studies have shown that lowering LDL-C lowers CVD risk.[1] LDL particles are small and cholesterol-rich, allowing them to invade the endothelial wall, leading to cholesterol-laden foam cells and consequently atherosclerosis.[2] However, since with effective medications the reduction (or in some cases even the elimination[3]) of LDL-C does not eliminate CVD risk, attention has shifted to other lipoproteins and their potential to cause atherosclerosis and CVD. Another class of lipoproteins that is gaining attention are the triglyceride-rich lipoproteins (TRLs). TRLs consist of varying density, size and composition and include very-low-density lipoproteins (VLDL) and chylomicrons (CM) (in the non-fasting state) and their lipolytic remnants. Since part of the TG in large TRLs, such as VLDLs and CMs, are lipolyzed almost immediately when they enter the circulation, almost all TRLs reflect some form of remnant lipoproteins.[4] However, in healthy subjects TRLs are removed or converted to LDL very quickly, with an estimated plasma residence time of less than 4 hours. Therefore, in the general population, TRLs are generally low or even absent, in contrast to LDL which has a plasma residence time of 2.5–3.5 days on average.[5] Therefore, LDL is the dominant lipoprotein in the fasting state in almost all healthy subjects, whereas TRLs are particularly increased in an insulin resistant or obese state.[6]

Although many observational studies have found a strong relation between TRLs and CVD, there has been disagreement about the causal role of TRLs in the development of CVD.[6] One of the reasons may be the unclear role of triglycerides (TGs), which are the main component of TRLs, in the process of atherosclerosis, but for sure give rise to an increased risk of pancreatitis (**chapter 2.1**).[4] Furthermore, intervention studies aiming to reduce TGs showed conflicting results regarding the incidence of CVD events.[7,8] Nowadays there is consensus that it is not TG in TRLs, but cholesterol in TRLs that is associated with atherosclerosis. This is called remnant cholesterol (remnant-C) and includes all cholesterol that is not in LDL or high-density lipoproteins (HDL). The distinction between TG and remnant-C is thus more conceptual; high TGs should be seen as a marker of high cholesterol levels in TRLs, because LDL and HDL usually do

not contain TGs. Clinically, therefore, it seems more meaningful to investigate how to lower cholesterol levels in TRLs instead of TGs. It was demonstrated that not only LDLs are small enough to enter the endothelial wall, but that all lipoproteins <70 nm (which includes small TRLs) can enter the arterial wall.[6] Moreover, it is suggested that TRLs are associated with low-grade inflammation (which also plays a role in atherosclerosis) whereas LDLs are not.[9,10] Also, when TRLs accumulate in plasma, they become enriched in cholesterol (through the effect of cholesteryl ester transfer protein (CETP)) and in this situation TRLs can contain up to four times as many cholesterol molecules per particle as LDL.[11] In **chapter 3**, we evaluated the relationship between cholesterol in TRLs (remnant-C) and recurrent CVD in patients with established CVD. We found that elevated remnant-C (in the fasting state and therefore called VLDL-C) was associated with major adverse limb events (MALE), but there was no relation with major adverse cardiovascular events (MACE) and all-cause mortality. This association was independent of LDL-C levels and use of lipid-lowering medication. The observation that remnant-C is more strongly associated with peripheral vascular events than cardiac and cerebral vascular events is consistent with other studies. Another study in the Copenhagen General Population Study, in which more than 100.000 subjects from the general population were included, found that elevated remnant-C was associated with a five-fold increased risk of peripheral artery disease (PAD), which was higher than the four- and two-fold increased risk for myocardial infarction (MI) and ischemic stroke, respectively.[12] The Women's Health Study showed that remnant-C was strongly associated with both peripheral and coronary events, whereas small dense LDL-C was only associated with MI.[13] These results suggest that cholesterol content in different lipoproteins may affect vascular beds differently and that remnant-C could be a specific risk factor for the development of peripheral artery events. Mechanisms why remnant-C is more strongly associated with peripheral events than coronary or cerebral events remain to be elucidated, but it is hypothesized that this association underscores the fact that remnant-C itself causes atherosclerosis. PAD is typically an atherosclerotic disease, and often a symptom of extensive systemic atherosclerosis, with concomitant coronary and carotid atherosclerosis. Stroke, on the other hand, has multiple causes, likely leading to a less strong association with remnant-C than PAD.[12]

What is the role of genetic susceptibility for certain risk factors in the development of CVD?

Single rare pathogenic variants in genes that affect the function of proteins involved in lipoprotein metabolism can lead to a markedly increased risk of CVD or other relevant clinical outcomes. Interestingly, even in a monogenic lipid disorder, such as monogenic chylomicronemia, there can be considerable heterogeneity in clinical presentation. In **chapter 2.1** we described three patients with hypertriglyceridemia, all caused by

monogenic chylomicronemia. All three patients had different affected genes, different numbers of affected alleles, and different symptoms, outcomes and prognosis, varying from no symptoms (besides mildly increased TGs) to life-threatening pancreatitis with TGs up to 66 mmol/L, indicating that in patients with hypertriglyceridemia, the absence of pancreatitis or the presence of mild hypertriglyceridemia does not exclude monogenic chylomicronemia.

In **chapter 2.2**, we described a woman with HDL-C levels up to 3.5 mmol/L, a rare variant in the *LIPC* gene, and recurrent transient ischemic attacks (TIAs). The variant in the *LIPC* gene, which codes for hepatic lipase, likely caused subtle changes in the remodeling and lipolysis of HDL particles. Additional analyses revealed that she had no cholesterol in HDL, indicating that the direct homogenous HDL-C assay may have led to falsely increased HDL-C levels. However, these observations should be replicated. Furthermore, the link of this variant to the occurrence of her recurrent TIAs could be not be established nor excluded. For this, evaluation of other patients with (the same) variant in the *LIPC* gene is needed.

At the population level the minor contribution of several variants in genes can also play an important role in the development of CVD. A previous study in 438.952 subjects from the general population examined the combined effect of variants in genes lowering LDL-C and systolic blood pressure (SBP).[14] Compared to the reference group (i.e. patients with less than the median number of genetic variants that lower LDL-C and SBP; a genetically unfavorable profile) patients with a genetically favorable profile (i.e. more than the median number of genetic variants that lower LDL-C and SBP) had 0.4 mmol/L lower LDL-C and their SBP was on average 3.1 mmHg lower. Compared with the reference group, this led to an impressive decrease in lifetime risk of developing major coronary events with an odds ratio (OR) of 0.61 (95%CI 0.59–0.64).[14] This study demonstrated that relatively small absolute differences in LDL-C and SBP can significantly affect the lifetime risk for CVD. **Chapter 4** evaluated the effect of genetic variants associated with LDL-C and SBP and the risk of recurrent CVD in patients with manifest CVD. Although LDL-C levels in patients with an unfavorable genetic profile were 0.18 mmol/L higher (95%CI 0.15–0.21) and SBP was 3.2 mmHg higher (95%CI 2.60–3.78) compared to the patients with a favorable genetic profile, neither the separate polygenetic risk scores (PRS) nor their combination resulted in a higher risk for recurrent CVD (hazard ratio (HR) 1.09, 95%CI 0.93–1.28). This shows the limited effect of genetic susceptibility on recurrent CVD events in patients who had previously experienced CVD. These findings suggest that genetically determined LDL-C and SBP do not explain differences in residual cardiovascular risk in patients with established vascular disease. The difference in findings between a healthy population

and a population with CVD might be explained by the sample size of our cohort, index-event bias, and the use of lipid-lowering and/or antihypertensive medication in patients with an unfavorable genetic profile. The latter two may lead to attenuation of the estimates found compared to healthy subjects. Although we evaluated the etiologic relation and not the predictive value of using such PRS in clinical practice, the effect of including such PRS in existing risk prediction scores for patients with established CVD is probably limited. Currently, genetic risk scores are considered of limited utility for the prediction of CVD events.[15] Moreover, in the scenario that PRSs will play a role in clinical practice in the future, it is likely that its greatest benefit lies in the first decades of life, before clinical events and even before definable development of atherosclerosis. How and when to use genetic risk scores in clinical practice deserves further investigation.

What causes a dysbetalipoproteinemia phenotype in healthy ε2ε2 subjects?

Approximately 1% of the general population is homozygous for the ε2 allele in the *APOE* gene.[16] Compared to wild-type (homozygosity for the ε3 allele in the *APOE* gene), these ε2ε2 subjects have lower LDL-C levels and a lower risk of CVD.[16] Of these hypolipidemic subjects, however, an estimated 10-15% develops the atherogenic dysbetalipoproteinemia phenotype, characterized by the presence of cholesterol-enriched remnant lipoproteins.[17,18] The combination of a specific genotype (*ε2ε2* genotype or other specific dominant variants in the *APOE* gene) with a specific phenotype (dysbetalipoproteinemia phenotype) leads to the diagnosis of Familial Dysbetalipoproteinemia (FD). Why some *ε2ε2* subjects develop this specific lipoprotein phenotype and others do not, is not fully understood.

In general, any situation leading to either an overproduction of atherogenic lipoproteins (insulin resistance, obesity), reduced lipolysis (insulin resistance) or reduced clearance of remnant lipoproteins (hypothyroidism, menopause, medication) can disrupt the delicate balance of production and clearance of remnant lipoproteins in *ε2ε2* subjects and consequently lead to FD.[19] Previous studies aimed at finding determinants for the development of FD in subjects with an ε2ε2 genotype suggested a primary role for insulin resistance and obesity.[18,20-23] Since the inclusion of healthy ε2ε2 subjects is difficult, due to their relative rarity in the general population, all previous studies were however limited to a cross-sectional design, which limits the evaluation of the direction of an association. **Chapter 6** describes the first longitudinal analysis in healthy *ε2ε2* subjects from the general population. The initial sample consisted of 18.987 subjects from two large prospective Dutch population-based cohorts (PREVEND and Rotterdam Study) of which 118 subjects (0.6%) had an ε2ε2 genotype, indicating the rarity of this *APOE* genotype. Of these, 69 *ε2ε2* subjects were available for the prospective analyses, as they had no FD at baseline. Although we could not distinguish between an ordinary

hypertriglyceridemia or a dysbetalipoproteinemia phenotype, we assumed that all ε2ε2 subjects who became hyperlipidemic or used lipid-lowering medication during follow-up had developed FD. A total of 11 of these 69 ε2ε2 subjects developed FD during follow-up (median follow-up PREVEND 4.2 (IQR 4.0–4.3) years and Rotterdam Study 10.4 (IQR 5.6–10.7) years). Age, sex and cohort-adjusted risk factors for the development of FD were BMI (OR 1.19 (95%CI 1.04–1.39), waist circumference (OR 1.26 95%CI 1.01–1.61) and presence of non-TG metabolic syndrome (OR 4.39 95%CI 1.04–18.4) at baseline. Interestingly, change in adiposity during follow-up was not associated with development of FD. Although the exact underlying mechanism to develop FD is unknown, the fact that obesity at baseline, prior to development of a certain lipid phenotype, predisposes patients to the development of FD in the future, suggest that the development of FD is a slow and gradual process.

It is hypothesized that in ε2ε2 subjects, the limited clearance of TRLs by the LDL-receptor (LDL-R) (due to dysfunctional apolipoprotein E2 (apoE2): <2% binding affinity to LDL-R compared to wild-type ε3ε3) is of little importance. Because the most important part of remnant clearance happens through the heparan sulphate proteoglycans (HSPGs) system. Since remnant clearance by HSPGs is not affected in healthy ε2ε2 subjects, these subjects can still adequately clear remnant lipoproteins. It is not completely known why ε2ε2 subjects accumulate remnant lipoproteins when they become insulin resistant. Based on studies in mice, it is hypothesized that in an insulin resistant state, the *SULF2* gene is dysregulated, leading to an overproduction of the sulfatase-2 protein, and consequently to a decreased binding of TRLs to HSPGs.[24,25] In humans, one specific variant (rs2281279) in the *SULF2* gene has been studied in several populations.[26-29] In **chapter 5** the effects of this variant on metabolic parameters and the development of (recurrent) CVD was evaluated in a cohort consisting of 4386 patients at high CVD risk. There was no effect of this particular variant on metabolic parameters (including non-HDL-C, TG, insulin and quantitative insulin sensitivity check index), and consequently no effect on CVD events. We aimed to investigate mechanisms related to the development of FD in healthy ε2ε2 subjects, in relation to these TRL clearance pathways. Since some studies reported that the presence of the minor allele G of this variant was associated with a favorable metabolic profile,[26,29] we used this variant as a model for HSPG function. For this, we used the following assumptions: an ε2ε2 genotype in combination with an AA genotype in rs2281279 might mimic an FD model, because in this case both the LDL-R and HSPG TRL clearing pathways do not function properly. Patients with an ε2ε2 genotype in combination with a GG genotype in rs2281279 G might in that case mimic 'healthy' ε2ε2 subjects. However, due to the rarity of homozygosity for the ε2 allele, this resulted in only 29 patients with the 'FD model' (HSPG-/LDL-R-) and only 4 patients with the 'healthy ε2ε2 model' (HSPG+/LDL-R-), limiting our ability to draw conclusions.

Although speculative, it seemed that the 'FD' (HSPG-/LDL-R-) group compared to the 'healthy ε2ε2 group' (HSPG+/LDL-R-) had a less favorable lipid profile, reminiscent of an FD metabolic profile, possibly due to an interaction of the *SULF2* genotype with the ε2 allele.

In **chapter 6** it was demonstrated that in ε2ε2 subjects obesity at baseline was a determinant for the development of FD in the future. Therefore, it could be hypothesized that the HSPG clearance system functions normally for a long time, even when part of the HSPGs are damaged by sulfatase-2 upregulation due to adiposity or insulin resistance. In that case, the 'switch' to FD will only take place when a certain threshold of damage to the number of HSPGs occurs. **Chapter 5** examined only one variant in the *SULF2* gene, and not the (dys)function of the sulfatase-2 protein itself. Therefore, more detailed studies of the role of sulfatase-2 in the development of FD in humans are needed. Ideally, studies in large cohorts with humans should be performed. Humans should be stratified for rs2281279 genotype and sulfatase-2 expression (in liver biopsies) and postprandial responses should be evaluated. Also, sulfatase-2 expression in healthy ε2ε2 subjects should be compared with sulfatase-2 expression in patients with FD. In addition, the effect of sulfatase-2 inhibitors on remnant-C levels should be evaluated in patients with FD, because they might be a promising treatment modality for this patient group. In mice with type 2 diabetes mellitus (T2DM), sulfatase-2 antisense oligonucleotides targeting (and thereby inhibiting) sulfatase-2 were able to completely resolve postprandial plasma TG excursions.[25]

How to measure LDL-C in FD – and should it?

The dysbetalipoproteinemia phenotype is characterized by accumulation of remnant lipoproteins and low LDL-C levels.[30] The most commonly used method to estimate LDL-C levels in clinical practice is the Friedewald formula. To accurately estimate LDL-C levels based on the Friedewald formula, a fixed ratio of VLDL-C to VLDL-TG is assumed.[31] Since the introduction of the Friedewald formula in 1972, FD patients are known to be an exception for the use of the Friedewald formula, because they have cholesterol-enriched VLDL and consequently a different ratio of VLDL-C to VLDL-TG, leading to falsely increased estimated LDL-C levels. However, the ESC guidelines and most laboratories do not make an explicit exception for FD for the use of the Friedewald formula.[32] Currently there are other options for determining LDL-C levels, including the Martin-Hopkins formula (which uses a modifiable factor based on TG and non-HDL-C levels). This formula is particularly appropriate when LDL-C levels are low or when TG levels are slightly elevated. Furthermore, a direct or homogenous assay to measure LDL-C or derivation by polyacrylamide gradient gels (PGGE) could be used, but it is unknown how well these methods estimate LDL-C levels in patients with FD. In

chapter 8 we compared the different methods with ultracentrifugation in 28 patients with FD. We found that Friedewald, Martin-Hopkins and the direct assay significantly overestimated LDL-C levels compared to ultracentrifugation, and that PGGE significantly underestimated LDL-C levels. However, the presence of many remnant lipoproteins complicates the determination of LDL-C levels, since there is a gradual transition from small remnants to LDL. This could also make the reference method inaccurate. Even beta-quantification, the generally accepted reference standard for LDL, includes some remnant lipoproteins as well as Lp(a) in the LDL fraction, and may therefore also lead to falsely increased LDL-C levels.[33] Therefore, it cannot be excluded that actual LDL-C levels may be lower than determined by ultracentrifugation and that PGGE turns out to be the most appropriate alternative for the measurement of LDL-C in FD. Therefore, we conclude that there is currently no method to accurately measure LDL-C in FD. In addition to limited accuracy, plasma LDL-C levels do not adequately reflect the very high CVD risk in FD, which is caused by remnant accumulation, rather than increased LDL-C levels. Therefore, the use of LDL-C for patients with FD is not recommended in clinical practice, and LDL-C cannot be used as treatment goal in FD patients. The ESC guidelines recommend using non-HDL-C as treatment goal in patients with high TG levels, T2DM, obesity or very low LDL-C levels,[32] which is somewhat consistent with a dysbetalipoprotenemia phenotype, but do not recommend non-HDL-C as a standard treatment goal in all patients with FD. In **chapter 8** we demonstrated that non-HDL-C levels (measured as total cholesterol minus HDL-C) are in good agreement with non-HDL-C levels measured by ultracentrifugation, emphasizing that non-HDL-C should be the treatment goal of choice in FD.

How to evaluate the relationship of variants in the *APOE* gene and FD?

Next Generation Sequencing (NGS) has been increasingly used in recent years, and involves the complete sequencing of genes. For lipid disorders, NGS is often done with standard panels that include genes involved in lipoprotein metabolism.[34] Consequently, healthcare providers can currently face a clinical dilemma, because many new variants of unknown significance are found. With regard to variants in the *APOE* gene and their relationship with FD, there are two important questions. Firstly, is this variant pathogenic? In other words, does this particular variant play a clinically significant role or is this variant just an innocent bystander? The second question is whether the variant is associated with specific biochemical properties and a specific lipoprotein phenotype.

To answer the first question, the American Clinical Medical Genetics and Genomics (ACMG) guidelines can be followed. However, options to investigate pathogenicity with a high level of evidence are expensive and labor-intensive and cannot be performed

for every variant currently found. To answer the second question, the lipoprotein phenotype should be analyzed and *in vitro* functional tests should be performed. However, this is challenging because demonstrating the specific dysbetalipoproteinemia phenotype is not straightforward and cannot be done with standard laboratory measurements. For example, a patient with FD may be falsely diagnosed as having Familial Hypercholesterolemia (FH), if LDL-C levels are falsely increased by using the Friedewald formula. Also, functional tests are limited to a research setting.

In **chapter 7** we proposed two approaches to establish the relation between *APOE* variants and FD. First, we propose a comprehensive method. This method consists of evaluation of the FD lipoprotein phenotype with one the reference standards (ultracentrifugation or PGGE) in several, unrelated patients. To assess the causal relationship of the variant with FD, *in vitro* functional assays should be performed to evaluate the binding of apoE to the LDL-R and/or HSPG and, optionally, *in vivo* postprandial clearance studies should be performed, as impaired binding of the apoE protein and an impaired postprandial remnant clearance are characteristic of FD. To assess pathogenicity, the ACMG guidelines can be followed. The proposed *in vitro* and *in vivo* tests can be part of the pathogenicity assessment according to the ACMG guidelines. When a variant is classified as (likely) pathogenic and the causal relationship with FD is established, it can be concluded the *APOE* variant is FD-causing.

However, this approach is often not feasible in clinical practice. Therefore we also proposed a more practicable method. Regarding pathogenicity, we still suggest following the ACMG guidelines, and it remains important to confirm the dysbetalipoproteinemia phenotype in each patient. In clinical practice, the reference standards are usually not available, but the non-HDL-C/apolipoprotein B (apoB) >4.91 mmol/g algorithm is considered highly specific for the dysbetalipoproteinemia phenotype (compared with ultracentrifugation).[35] When a patient has a (likely) pathogenic *APOE* variant and the dysbetalipoproteinemia phenotype, according to the apoB algorithm, is present, this can be classified as presumptive FD. However, a definitive diagnosis can only be made following the comprehensive approach. If the patient has an *APOE* variant of unknown significance, but meets the non-HDLC/apoB criterion for the presence of a dysbetalipoproteinemia phenotype, it can be classified as possible FD. Both a presumptive and possible FD can be treated as FD. With this approach, patients are treated adequately, even when the exact role of their variant is not (yet) known. If the patient has a (likely) pathogenic *APOE* variant but does not have a dysbetalipoproteinemia phenotype, the diagnosis of FD cannot be made for the present. It is possible, however, that the dyslipoproteinemia phenotype may be expressed later in life. The variant may later also be associated with another type of dyslipidemia. If the variant is classified as variant of unknown significance and the patient

does not have a dysbetalipoproteinemia phenotype, FD can be excluded (at this time), but changes in the pathogenicity label of the variant and lipid phenotype of the patients should be monitored. We recognize that this approach is based on expert opinion, but since no other alternatives are available, it should create conformity in the diagnostic challenges that healthcare providers currently face. Further studies to substantiate these approaches are warranted. Implementation in clinical practice should be based on an intensive collaboration between genetic laboratories and expertise centers with physicians involved in lipidology. This is important because genetic laboratories generally do not have access to (detailed) lipoprotein phenotypes while physicians may struggle to interpret genetic results.

What is the optimal treatment strategy in patients with FD?

The treatment goal for FD patients is non-HDL-C, which includes cholesterol in all atherogenic apoB containing lipoproteins (TRLs and LDL).[17,32] FD is often caused by a second metabolic hit, such as insulin resistance or T2DM, but may also be precipitated by other risk factors that place stress on the metabolic system, such as hypothyroidism or alcohol consumption. Therefore, the underlying cause must be appropriately treated or minimized. Because most metabolic hits are not reversible (such as insulin resistance and ageing), the dysbetalipoproteinemia phenotype with its associated CVD risk and therefore the need for appropriate treatment persists in these high-risk patients. However, the exact risk for CVD in FD is unknown, since longitudinal cohort studies in FD patients are lacking.

Dietary interventions are the first step in treatment of hyperlipidemia in FD patients. Patients with FD generally respond well to changes in diet. A previous study demonstrated that a low glycemic diet was very effective in weight reduction and lowering total cholesterol and TG in FD patients.[36] According to the ESC guidelines, the first step in lipid-lowering medication in FD are statins.[32] Statins lower cholesterol levels by LDL-R upregulation and because they decrease de novo cholesterol production by the liver. Although the exact underlying lipid-lowering mechanism of statins in FD is unknown, they are very effective in reducing atherogenic lipids.[37-42] The ESC guidelines advise to start a fibrate in FD when the lipid profile is dominated by hypertriglyceridemia, despite the use of a statin.[32] A recent randomized controlled trial in 15 patients with FD showed that bezafibrate significantly reduced fasting and 6-hour post fat load area under the curve (AUC) of non-HDL-C and TG, in addition to a reduction in the post fat load incremental AUC (iAUC) of TG and apoB.[43] Fibrates act as PPAR-alpha agonists, improving lipolysis and therefore lowering TGs. Since fibrates and statins have different molecular mechanisms, and based on the results of that study, combination therapy with statins and fibrates could be considered as standard lipid-lowering therapy in patients with FD.[43]

However, it was found that in clinical practice, only 10% of the FD patients used this combination therapy, and even when the combination was used, 60% did not achieve their non-HDL-C treatment goal, indicating the need for more intensive lipid-lowering medication.[44]

Proprotein convertase subtilisin/kexin type 9 (PCSK9) monoclonal antibodies (mAbs) are a relatively new class of lipid-lowering medication. PCSK9 mAbs have been approved in the European Union since 2015. There is increasing evidence that they are highly effective in lowering LDL-C, and other atherogenic lipoproteins, and the resulting CVD risk in several high-risk populations.[45,46] PCSK9 mAbs neutralize the PCSK9 protein, and thus prevent degradation of the LDL-R, leading to LDL-R upregulation and increased hepatic lipoprotein clearance by the LDL-R.[47] Since the hallmark of FD is postprandial remnant accumulation, the post fat load effects of evolocumab are of specific interest. In patients with T2DM, PCSK9 mAbs were shown to effectively reduce postprandial TRLs by 30-40%.[48-51] However, the (fasting and post fat load) effects of PCSK9 mAbs in FD are unknown. We wondered whether the effect of PCSK9 mAbs would be similar as those in T2DM, as FD patients generally have low LDL-C levels and dysfunctional apoE that binds the LDL-R with severely decreased affinity. Therefore, we conducted a randomized, multicenter, placebo-controlled, double-blind, cross-over trial to evaluate the effects of evolocumab 140 mg every 2 weeks, added to standard lipid-lowering therapy, on fasting and post fat load lipid and lipoprotein levels in 28 patients with FD (**chapter 9**). Although we assumed a relatively limited effect of evolocumab on atherogenic lipoproteins, we found a striking reduction in the 8-hour post fat load non-HDL-C AUC of 49% (95%CI 42–55) and TG AUC of 20% (95%CI 10–29). Moreover, other lipids and lipoproteins, including apoB, VLDL-C and remnant cholesterol were significantly reduced by evolocumab. Evolocumab had no effect on the post fat load increase in TG. This study showed that after treatment with a PCSK9 mAb patients with FD have significantly lower levels of atherogenic lipids and lipoproteins. The ultimate goal of lipid-lowering is, of course, to reduce CVD risk, but currently no longitudinal studies are available that evaluate the effect of lipid-lowering medication on CVD risk in FD patients. However, it can be expected that the clinically significant decrease in atherogenic lipids will translate into a reduction in the risk of CVD in these high-risk patients. Therefore, PCSK9 mAbs could be added to the list of lipid-lowering options in patients with FD.

What is the mechanism of action of PCSK9 monoclonal antibodies in FD?

Given the underlying pathophysiology of FD with a greatly reduced affinity of TRLs for the LDL-R, it seems somewhat surprising that PCSK9 mAbs could reduce apoB100-containing lipoproteins to the extent described in **chapter 9**. In **chapter 10** we described

the detailed effects of evolocumab on lipoprotein distribution and composition in 28 patients with FD. Since the main mechanism of action of PCSK9 mAbs is upregulation of LDL-R,[47] it is intriguing that administration of evolocumab for 12 weeks led to a 53% (95%CI 36-64) reduction in fasting remnant-cholesterol in FD. In **chapter 10**, it was found that particle numbers were significantly reduced for all lipoproteins after evolocumab. but this reduction was larger for remnant and LDL particles. Also, absolute levels of cholesterol and TG in all apoB-containing lipoproteins were reduced, but the cholesterol levels were reduced more than TG levels. Evolocumab did not affect the number or composition of chylomicrons.

Based on the observation that evolocumab leads to significant reductions in (small) TRLs, we proposed two hypotheses about the mechanism of action of PCSK9 mAbs in FD. The first hypothesis is that PCSK9 mAbs increase the conversion, or lipolysis of remnants to LDL. However, a previous study showed that PCSK9 mAbs did not influence lipoprotein lipase (LPL), HL or apolipoprotein C3 (apoC3) activity[49,50], making this hypothesis less likely.

Another hypothesis is that PCSK9 mAbs increase the direct clearance of remnant particles by the liver. There are several arguments for this hypothesis. The first argument is that in a previous study with stable isotopes in patients with T2DM, treatment of PCSK9 mAbs increased the direct uptake of remnants by the liver.[50] Upregulation of the LDL-R by PCSK9 mAbs leads to a rapid clearance of LDL-apoB (since LDLs are cleared by apoB100 with normal affinity for the LDL-R). It is suggested that other lipoproteins (which depend on apoE with less affinity for the LDL-R) are more easily cleared by the highly upregulated LDL-R pathway, facing less competition of LDL. However, T2DM patients have a normal binding affinity of apoE for the LDL-R, whereas in FD the affinity of apoE for the LDL-R is extremely reduced. Since we found an effect on remnant-cholesterol almost similar to the effect of PCSK9 mAbs in patients with T2DM, the reduced affinity of apoE in FD seems sufficient for increased uptake of remnants by apoE mediated pathways through the LDL-R. To investigate how much affinity of apoE for the LDL-R is required for a clinically relevant effect of PCSK9 mAbs, it would be interesting to study patients with an apoE deficiency (leading to no production of the apoE protein at all). Because this is a very rare condition, the effects of PCSK9 mAbs in patients with an apoE deficiency are not known. However, several studies have been performed in apoE deficient (apoE-/-) mice. It was found that in these apoE-/- mice PCSK9 overexpression[52], PCSK9 gene deletion, or the administration of anti-PCSK9 antibodies[53] have no effect on lipoprotein profiles. In contrast, in ApoE3*Leiden. CETP mice (a translational model for FD, in which mice express both mouse apoE and the human mutant apoE3*Leiden), it was found that PCSK9 antibodies resulted

in a reduction in cholesterol levels (45%) and TG levels (36%).[53] Although the human apoE3*Leiden protein has a higher affinity for the LDL-R (11-25%)[54] compared with <2% in ApoE2, this indicates that at least some LDL-R binding affinity of the apoE protein must be present for the lipid-lowering effect of PCSK9 mAbs. Based on the results of our study, it appears that the limited affinity in FD is likely enough to establish an effect on remnant clearance through LDL-R upregulation in FD.

It would be very interesting to conduct a trial in patients with apoE deficiency and in patients with different heterozygous apoE variants (thereby including patients with apoE proteins with different affinities for the LDL-R) to evaluate the effects of PCSK9 mAbs on their lipoprotein profiles. Such studies could elucidate the role of the LDL-R binding affinity in the mechanism of action of PCSK9 mAbs in FD.

The evidence described above suggests that the response to neutralization of PCSK9 is attributable exclusively to LDL-R upregulation. TRLs, including remnants however, are cleared primarily by HSPG and LDL-R-related protein 1 (LRP1),[55,56] and not by the LDL-R. It has previously been shown that the number of LRP1 receptors is not affected by PSCK9 mAbs,[57] and the effects of PCSK9 mAbs on HSPGs are unknown. In theory, LDL reduction by PCSK9 mAb might create more space for (small) TRLs in the space of Disse, leading to easier uptake of TRLs by the HSPG and LRP1 systems. This could increase the uptake of TRLs, even when the HSPG and LRP1 systems themselves are not upregulated by PCSK9 mAbs. To further elucidate the role of PCSK9 mAbs in patients with FD, stable isotope studies with labeled TRLs could be undertaken to prove the mechanism of action of PCSK9 mAbs, by differentiation of the production, clearance and conversion rates of the different lipoprotein fractions after treatment with PCSK9 mAbs. In addition, studies in apoE deficient, ApoE3*Leiden.CETP mice with HSPG-/-, LRP1-/- and LDL-R-/- on and off PCSK9 mAbs are needed to elucidate by which clearance systems PCSK9 mAbs lower apoB100-containing lipoproteins in FD. To conclude, it seems likely that the reduction in TRLs by evolocumab is achieved by increased hepatic clearance, but the specific contribution of the LDL-R and non-LDL-R pathways remains to be elucidated.

Concluding remarks

For healthcare professionals, it is important to consider a genetic cause when other common, secondary causes of dyslipidemia have been ruled out. FD is a relatively common monogenic dyslipidemia, characterized by accumulation of TRLs. The causal role of TRLs in atherosclerosis has already been established, but future work should consider the differential effects of TRLs on different vascular beds. FD appears to be more prevalent than previously thought, in part because it is difficult to recognize FD

in clinical practice. The diagnostic pathway is limited by difficulties in determining the specific dysbetalipoproteinemia phenotype, and sometimes also the genetic basis of FD is unclear. Although apoB can be easily measured by standard laboratories and improves differentiation between FD and other causes of mixed dyslipidemias, the actual reference standards for demonstrating the dysbetalipoproteinemia phenotype are not readily accessible in clinical practice. In addition, LDL-C in FD may not be calculated or measured accurately. Moreover, a specific genotype causally related to FD is required to confirm the diagnosis. In the future, more attention should be paid to the interpretation of the increasing number of newly found *APOE* variants. A systematic method should be developed to investigate the association of all newly found variants in the *APOE* gene with lipid phenotypes.

Although the specific underlying mechanisms that apply when a healthy subject with an ε2ε2 genotype transitions into to the highly atherogenic dysbetalipoproteinemia phenotype are unclear, it is important to avoid obesity in these healthy ε2ε2 subjects to prevent the development of a dysbetalipoproteinemia phenotype. In addition, the role of sulfatase-2 in the degradation of HSPG in the pathophysiology of FD remains to be further elucidated. When patients are diagnosed with FD there is an increased risk of CVD. To lower that risk, several lipid-lowering options are available. However, only a minority of medical practitioners prescribe the recommended combination therapy of a statin and a fibrate, and when this combination is used, the majority of patients still do not achieve non-HDL-C treatment goals. This thesis demonstrated that in patients with FD, addition of evolocumab to standard lipid-lowering therapy significantly improved atherogenic fasting and postprandial lipids and lipoproteins compared with standard lipid-lowering therapy alone. Although the underlying mechanisms that permit PCSK9 mAbs to reduce TRLs in patients without functional apoE were not researched, it has been shown that small, cholesterol-rich lipoproteins such as remnants and LDL are lowered most and the effect is therefore most likely mediated by increased clearance. Future work should investigate the mechanism of action of PCSK9 inhibition in patients with FD, the LDL-R-independent effects of PCSK9 mAbs and their role in TRL clearance.

Highlights of this thesis

- The clinical characteristics, disease severity and prognosis of patients with monogenic chylomicronemia can vary widely.
- The direct homogenous HDL-C assays used in clinical practice might not be accurate in some situations where HDL composition is affected by subtle changes in remodeling and lipolysis (for example due to partial HL deficiency by heterozygous variants in the *LIPC* gene).

- VLDL-C, a measure of the cholesterol levels in TRLs, is associated with MALE, but not with MACE or all-cause mortality, independent of LDL-C and lipid-lowering medication, in patients with stable CVD.
- Genetic variants associated with LDL-C and SBP are not associated with the risk of recurrent cardiovascular events in patients with established vascular disease.
- A specific variant in the *SULF2* gene (rs2281279), is not associated with metabolic parameters, including TG metabolism, and does not increase the risk of vascular events or T2DM in patients at high risk for cardiovascular disease.
- Adiposity increases the risk of developing an FD-like lipid phenotype in homozygous *APOE* ε2 subjects from the general population.
- To establish the causal relationship between FD and unknown variants in the *APOE* gene, the specific lipoprotein phenotype in several unrelated patients should be determined, and *in vitro* functional tests and, optionally, *in vivo* postprandial clearance studies should be performed.
- There is currently no convenient method to accurately measure LDL-C in FD in clinical practice. The Friedewald formula, the Martin-Hopkins formula and the direct homogeneous assay severely overestimate LDL-C levels compared with the reference method, and polyacrylamide gels severely underestimate LDL-C levels.
- Evolocumab, a PCSK9 monoclonal antibody, added to standard lipid-lowering therapy significantly reduces fasting and post fat load levels of all atherogenic lipids and lipoproteins in patients with FD. This decrease is mainly caused by a decrease in cholesterol in remnant and LDL particles.

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Appendix

Summary
Samenvatting (voor niet-ingewijden)
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Summary

Cardiovascular diseases (CVD) remain the most important cause of mortality worldwide. One of the most important risk factors for CVD is dyslipidemia. The main focus has been on low-density lipoprotein cholesterol (LDL-C), but dyslipidemia involves the metabolism of many more lipids and lipoproteins, and any imbalance in this metabolism can potentially cause an atherogenic lipid profile and CVD. Dyslipidemia is caused by lifestyle factors, polygenic susceptibility and/or rare monogenic variants of large effect in genes involved in lipoprotein metabolism. In this thesis, the general objectives were to investigate the relationship between genes, lipoproteins and cardiovascular disease (CVD) risk. We focused on the genetic lipid disorder Familial Dysbetalipoproteinemia (FD) and sought to evaluate etiologic pathways, diagnostic criteria and new therapeutic options.

In **chapter 2.1** we described three patients with monogenic chylomicronemia. Monogenic chylomicronemia results in hypertriglyceridemia due to loss-of-function variants in genes involved in the lipolysis of triglycerides (TG). One patient had mild hypertriglyceridemia without other symptoms whereas the other two patients experienced severe hypertriglyceridemia and pancreatitis. This case report illustrated the heterogeneity in the clinical presentation of monogenic chylomicronemia, depending on the gene involved and the number of alleles affected. In **chapter 2.2** we described a female in her 50s with high-density lipoprotein cholesterol (HDL-C) levels up to 3.5 mmol/L and recurrent transient ischemic attacks (TIAs), without important secondary risk factors. Genetic analysis revealed a heterozygous variant in the *LIPC* gene, encoding for hepatic lipase (HL). HL deficiency leads to a reduced lipolysis of TG in HDL and remnant lipoproteins. Heterozygous variants in *LIPC* can result in a partial HL deficiency and lead to heterogeneous lipid and lipoprotein profiles. Interestingly, in this case, additional laboratory analyses revealed that her HDL species was rich in TG, but did not contain cholesterol. This suggests that direct homogenous HDL-C assays used in clinical practice might not be accurate in some situations where HDL composition is affected by subtle changes in remodeling and lipolysis. In **chapter 3** we examined the relation between very-low density lipoprotein cholesterol (VLDL-C) and risk of cardiovascular events in patients with manifest cardiovascular disease. We found that VLDL-C was associated with an increased risk of major adverse limb events (MALE) with a hazard ratio of 1.49 (95%CI 1.16–1.93), but not for recurrent cardiovascular events or all-cause mortality, after adjustment for well-established risk factors such as LDL-C and lipid-lowering medication. In **chapter 4** we evaluated the relationship between genetic variants associated with LDL-C and systolic blood pressure (SBP) and the risk of recurrent cardiovascular disease in patients with established vascular

disease. We found that a weighted polygenic risk score (PRS) with LDL-C-related single nucleotide polymorphisms (SNPs) and a PRS with SBP-related SNPs were significantly associated with LDL-C and SBP values, respectively. However, the LDL-C PRS and SBP PRS, neither individually, nor in combination, were significantly associated with recurrent cardiovascular events.

In order to unravel the pathophysiology of FD and the role of sulfatase-2 in this process, in **chapter 5** we investigated the relation between a specific SNP in the *SULF2* gene (encoding for the sulfatase-2 protein), metabolic parameters and vascular disease in patients at high cardiovascular risk. We found that this SNP (rs2281279) was not associated with metabolic parameters (i.e. TG, non-HDL-C, insulin and quantitative insulin sensitivity check index), nor with recurrent vascular events or type 2 diabetes mellitus (T2DM). In **chapter 6** we investigated the longitudinal association between adiposity and the development of dyslipidemia in *APOE* ε2 homozygous subjects. We showed that risk factors for the development of dyslipidemia in ε2ε2 subjects (i.e. likely FD) were BMI (OR 1.19 95%CI 1.04–1.39), waist circumference (OR 1.26 95%CI 1.01–1.61) and non-TG metabolic syndrome (OR 4.39 95%CI 1.04–18.4). Change in adiposity during follow-up was not associated with development of dyslipidemia. These results suggest that adiposity increases the risk of developing dyslipidemia (likely FD) in homozygous *APOE* ε2 subjects.

In clinical practice, Next Generation Sequencing (NGS) reveals many (new) variants in the *APOE* gene in patients with dyslipidemia for which the relationship with FD is unknown or uncertain. In **chapter 7** we propose two approaches to establish the relationship between FD and genetic variants in the *APOE* gene. First, we propose a comprehensive approach that consists of determining the pathogenicity of the variant (according to existing genetic guidelines) and determining the causal relationship with FD by confirming a dysbetalipoproteinemia phenotype and performing *in vitro* functional tests, and, optionally, *in vivo* postprandial clearance studies. When this is not feasible, a second, pragmatic approach for individual patients is suggested.

In **chapter 8** we compared LDL-C values using the Friedewald formula, the Martin-Hopkins formula, a direct assay and polyacrylamide gradient gel electrophoresis (PGGE) to the reference standard density gradient ultracentrifugation in FD patients. We demonstrated that all four methods over- or underestimated LDL-C levels compared with density gradient ultracentrifugation. Due to the lack of a valid way to measure LDL-C, and because LDL-C is neither a reliable marker to assess risk nor an adequate treatment goal in FD the use of LDL-C in FD is not recommended.

Since the majority of patients with FD do not achieve non-HDL-C treatment goals with current therapeutic options, we investigated the effect of the PCSK9 monoclonal antibody evolocumab in FD. In **chapter 9**, we described the results of a multicenter, randomized, placebo-controlled, double-blind, crossover trial in 28 FD patients. Addition of evolocumab to standard lipid-lowering therapy resulted in a significant reduction in the 8-hour post fat load area under the curve (AUC) of non-HDL-C (49% 95%CI 42–55). In addition, fasting and post fat load lipids and lipoproteins (including TG, total cholesterol, apolipoprotein B (apoB), VLDL-C and remnant-cholesterol) were significantly reduced by evolocumab. In **chapter 10**, we used the data from the trial described in chapter 9, and we further investigated the detailed effects of evolocumab on lipoprotein distribution and composition in FD. Evolocumab added to standard lipid-lowering therapy significantly reduced particle number (expressed as apoB), in particular smaller and more cholesterol-rich lipoproteins. Also, lipoprotein composition changed as cholesterol levels were reduced more than TG levels in all lipoproteins. Furthermore, evolocumab seemed not to alter chylomicron metabolism. Given that patients with FD have apolipoprotein E (apoE) on their triglyceride-rich lipoproteins (TRLs) with greatly reduced affinity for the low-density lipoprotein-receptor (LDL-R), it is intriguing that LDL-R upregulation (the primary mode of action of PCSK9 monoclonal antibodies) leads to significant reductions in (small) TRLs. These results suggest that the reduction in TRLs by evolocumab is achieved by increased hepatic clearance, but the specific contribution of the LDL-R and non-LDL-R pathways remains to be elucidated.

Samenvatting (voor niet-ingewijden)

Hart- en vaatziekten zijn wereldwijd nog altijd de belangrijkste doodsoorzaak. Hart- en vaatziekten worden meestal veroorzaakt door aderverkalking. Aderverkalking kan ontstaan door leefstijlfactoren zoals roken, een hoge bloeddruk, overgewicht of een hoog cholesterol gehalte (dyslipidemie), maar ook door een genetische aanleg. Er zijn twee belangrijke soorten vetten in het bloed, namelijk cholesterol en triglyceriden. Deze zijn van belang voor de aanmaak van hormonen en vitamine D, het functioneren van celmembranen en voor het leveren van energie. Omdat deze vetten niet kunnen worden opgelost in het bloed worden ze vervoerd in verschillende deeltjes (lipoproteïnen). De bekendste zijn LDL (low-density lipoproteïne) en HDL (high-density lipoproteïne), die vaak ook het 'slechte' en 'goede' cholesterol worden genoemd. Daarnaast zijn er nog andere deeltjes zoals chylomicronen, VLDL (very-low density lipoproteïne) en IDL (intermediate-density lipoproteïne), ook wel remnants ('restjes') genoemd. De focus binnen en buiten de onderzoekswereld ligt voornamelijk op LDL-cholesterol omdat is aangetoond dat het verlagen van het LDL-cholesterol het risico op hart- en vaatziekte verlaagt. Echter zijn er steeds meer aanwijzingen dat ook andere deeltjes geassocieerd zijn met hart- en vaatziekten. In dit proefschrift hebben wij ons op de relatie tussen genen, lipoproteïnen en het risico op hart- en vaatziekten gericht, waarbij de focus lag op de genetische aandoening Familiaire Dysbetalipoproteïnemie (FD).

In **hoofdstuk 2.1** beschreven we drie patiënten met monogenetische chylomicronemie. Dit is een aandoening waarbij veranderingen in genen (mutaties) leiden tot (zeer) hoge triglyceriden concentraties. We lieten zien dat één patiënt mild verhoogde triglyceriden had zonder andere klachten, terwijl de andere twee patiënten zeer hoge triglyceriden hadden, waardoor ze beiden een alvleesklierontsteking hebben ontwikkeld. Dit hoofdstuk benadrukt vooral de verschillen in de uiting van monogenetische chylomicronemie tussen verschillende patiënten. In **hoofdstuk 2.2** beschreven we een vrouw met een zeer hoog HDL-cholesterol. Uit genetisch onderzoek bleek dat zij een zeldzame mutatie had in het *LIPC* gen, dit gen codeert voor het hepatisch lipase eiwit. Dit eiwit speelt een rol in de samenstelling van HDL. Uit aanvullend onderzoek bleek dat haar HDL veel triglyceriden bevatte en juist geen cholesterol. Dit duidt erop dat de standaardtesten om HDL-cholesterol te meten in sommige situaties (waarbij de HDL samenstelling is veranderd) mogelijk niet accuraat zijn.

In **hoofdstuk 3** hebben we het effect van verhoogde VLDL-cholesterol concentraties op het optreden van (nieuwe) hart- en vaatziekten onderzocht bij mensen die al hart- en vaatziekten hadden. Hieruit bleek dat VLDL-cholesterol leidde tot een verhoogd risico op vaatziekte in de benen, maar niet tot een verhoogd risico op hart- en vaatziekte in

het algemeen of overlijden. In **hoofdstuk 4** hebben we onderzocht of een genetische gevoeligheid voor het hebben van een hoog LDL-cholesterol en/of een hoge bloeddruk gepaard gaat met het ontwikkelen van nieuwe hart- en vaatziekten in patiënten die eerder al hart- en vaatziekte hadden doorgemaakt. Dit bleek echter niet zo te zijn, mogelijk omdat het onderzoek plaatsvond in mensen die al hart- en vaatziekte hadden doorgemaakt.

Naast een genetische gevoeligheid, die wordt bepaald door kleine effecten in meerdere genen, zijn er specifieke genen die een groot effect kunnen hebben op de vetten in het bloed. Een van deze genen is het *APOE* gen, dit gen codeert voor het apolipoproteïne E (apoE) eiwit. Het apoE eiwit zit op bijna alle lipoproteïnen en speelt een rol bij het verwijderen van deze lipoproteïnen uit het lichaam. In de algemene bevolking komen verschillende vormen van het *APOE* gen voor, namelijk ε2, ε3 en ε4. Omdat een gen altijd bestaat uit twee allelen kunnen de volgende combinaties worden gemaakt; ε2ε2, ε2ε3, ε3ε3, ε2ε4, ε3ε4 en ε4ε4, waarbij ε3ε3 het meest voorkomt en ε2ε2 het minst (ongeveer 1% van de bevolking). Deze variaties hebben invloed op de functie van het apoE eiwit. Mensen met het *APOE* ε2ε2 genotype hebben een apoE eiwit dat niet goed bindt aan de LDL-receptor, die belangrijk is voor het verwijderen van lipoproteïnen uit het bloed. Voor de meeste mensen met dit ε2ε2 genotype heeft dit geen gevolgen, maar sommige mensen ontwikkelen in de loop van hun leven de aandoening Familiaire Dysbetalipoproteïnemie, afgekort als FD. FD wordt gekenmerkt doordat lipoproteïnen (voornamelijk remnants) niet goed door de lever worden verwijderd, en dus in het bloed blijven, waardoor er hoge triglyceriden- en cholesterol waarden ontstaan, met juist vaak een laag LDL-cholesterol gehalte. Mensen met FD hebben een sterk verhoogd risico op het krijgen van hart- en vaatziekten (op jonge leeftijd). Waarom de ene persoon met een ε2ε2 genotype wel en de andere persoon met een ε2ε2 genotype geen FD ontwikkelt is niet helemaal duidelijk. In **hoofdstuk 6** volgden we gezonde mensen met een ε2ε2 genotype, waarbij we de mensen die afwijkende lipiden kregen vergeleken met degenen die dit niet kregen. Het bleek dat het hebben van overgewicht en een brede buikomvang risicofactoren zijn voor het ontwikkelen van FD. Vervolgens is de vraag hoe overgewicht dan leidt tot de ontwikkeling van FD. Daarom hebben wij in **hoofdstuk 5** patiënten onderzocht met een mutatie in het *SULF2* gen, dat codeert voor het sulfatase-2 eiwit. Mogelijk zou dit eiwit een rol spelen bij de ontwikkeling van FD. Echter bleek dat het hebben van een mutatie in het *SULF2* gen geen invloed had op de lipidenwaarden of het ontwikkelen van hart- en vaatziekten of suikerziekte. Dit sluit echter de rol van het sulfatase-2 eiwit bij de ontwikkeling van FD niet uit.

De meerderheid van de FD gevallen wordt veroorzaakt door het *APOE* ε2ε2 genotype, maar in ongeveer één op de tien gevallen wordt FD niet veroorzaakt door ε2ε2. In deze gevallen is één allel in het *APOE* gen aangedaan en heeft daarmee een groot effect

op de lipiden waarden. Tegenwoordig kan er bij mensen met dyslipidemie genetisch onderzoek worden gedaan waarbij een groot aantal genen tegelijk op mutaties wordt onderzocht. Soms wordt dan een nieuwe mutatie in het *APOE* gen gevonden waarvan het verband met FD onzeker of onduidelijk is. In **hoofdstuk 7** stellen we twee benaderingen voor om het verband tussen FD en deze mutaties in het *APOE* gen vast te stellen.

Artsen en laboratoria gebruiken vrijwel voor alle patiënten de Friedewald formule om het LDL-cholesterol te berekenen. Een van de uitzonderingen voor het gebruik van de Friedewald formule is het hebben van FD. Er zijn andere methoden om LDL-cholesterol te bepalen, maar het is niet bekend hoe goed zij het LDL-cholesterol kunnen bepalen in mensen met FD. In **hoofdstuk 8** hebben we verschillende methoden vergeleken met ultracentrifugatie, de 'gouden standaard' voor het bepalen van LDL-cholesterol. Het bleek dat alle methoden het LDL-cholesterol over- of onderschatten. Bovendien is het ook de vraag hoe goed de 'gouden standaard' is in het geval van FD. Aangezien het LDL-cholesterol in FD niet goed te bepalen is, en het niet goed het risico op hart- en vaatziekte weergeeft (bij FD wordt dit risico veroorzaakt door de aanwezigheid van remnants en niet door LDL-cholesterol), wordt het bepalen van LDL-cholesterol bij mensen met FD afgeraden.

In **hoofdstuk 9** hebben we onderzocht wat het effect is van de PCSK9-remmer evolocumab op cholesterolwaarden voor en na het eten in patiënten met FD. We vonden dat evolocumab vrijwel alle lipiden en lipoproteïnen (bijna) halveerde, zowel voor als na het eten. In **hoofdstuk 10** onderzochten we verder het effect van evolocumab op de verdeling en samenstelling van de lipoproteïnen in patiënten met FD. We toonden aan dat de verlaging van de lipidenwaarden voornamelijk werd veroorzaakt door een verbeterde opname van lipoproteïnen uit het bloed in de lever. Het precieze onderliggende werkingsmechanisme van evolocumab in mensen met FD is echter nog niet volledig duidelijk en moet verder worden onderzocht.

Samenvattend laat dit proefschrift zien dat lipidenafwijkingen, naast leefstijlfactoren, ook een genetische oorzaak kunnen hebben. FD komt vaker voor dan gedacht en wordt gekenmerkt door ophoping van remnants. FD wordt veroorzaakt door overgewicht, maar de onderliggende mechanismen zijn nog niet geheel duidelijk. Bovendien is het soms moeilijk om de diagnose FD te stellen, omdat het lastig is om het specifieke lipiden profiel te herkennen, en omdat de genetische basis van FD niet altijd duidelijk is. Patiënten met FD hebben een verhoogd risico op hart- en vaatziekte. De toevoeging van evolocumab kan de lipiden en lipoproteïnen verder verlagen, waardoor het risico op hart- en vaatziekte waarschijnlijk kan worden verminderd.

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