Familial hypercholesterolemia

From genetic and clinical diversity towards tailored therapy

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FAMILIAL HYPERCHOLESTEROLEMIA

FROM GENETIC AND CLINICAL DIVERSITY
TOWARDS TAILORED THERAPY

MEREL LOUISA HARTGERS

FAMILIAL HYPERCHOLESTEROLEMIA:

from genetic and clinical diversity towards tailored therapy

Merel Louisa Hartgers

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FAMILIAL HYPERCHOLESTEROLEMIA:

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INTRODUCTION AND GENERAL OUTLINE

INTRODUCTION

'Cardiovascular diseases (CVDs) are the number 1 cause of death globally: more people die annually from CVDs than from any other cause. An estimated 17.9 million people died from CVDs in 2016, representing 31% of all global deaths.' – WHO fact sheet on CVD, updated May 2017.

Atherosclerosis is the most prominent underlying disease ultimately leading to CVD events. Diabetes, hypertension, smoking and hyperlipidemia are well-established risk factors for atherosclerosis and current clinical guidelines are strongly focused on addressing these risk factors. The detrimental role of dyslipidemia, and most notably high plasma levels of low-density lipoprotein cholesterol (LDL-c), has been well established as an independent risk factor, with overwhelming data derived from epidemiological, preclinical and intervention studies, in addition to genetic studies. The notion that patients with familial hypercholesterolemia (FH), a common monogenic disorder with a prevalence of approximately in 1:200-250 in the general population, are at extreme risk for CVD has been a major determinant of our understanding of the role of elevated LDL-c levels in CVD.[1] FH is an autosomal dominant disease caused by pathogenic variants in one of the three known FH genes: *LDLR*, *APOB* and *PCSK9*. The lifelong exposure to these extreme plasma levels of LDL-c leads to accumulation of cholesterol in the arterial wall resulting in premature atherosclerosis. If left untreated, a large proportion of patients with FH will suffer from the clinical consequences thereof, and will experience their first CVD event before the age of 60 years.[2]

CVD RISK IN FH AND THE IMPORTANCE OF EARLY DETECTION AND TREATMENT

Multiple studies have been conducted in FH patients, and the risk for CVD in FH patients has been shown to vary to a large extent in these studies. One study found that FH patients that were not treated with lipid lowering therapy were at a 13-fold higher risk for coronary artery disease (CAD) compared to individuals without FH.[3] Another study showed that heterozygous carriers of a *LDLR* mutation were at 4.2-fold higher risk for an acute myocardial infarction (AMI), which was even 13-fold in those carrying an *LDLR* negative mutation.[4] A recent study shows that patients carrying a pathogenic variant with an LDL-c of 4.9 mmol/L or higher, were at a 22-fold increased CAD risk in comparison to non-carriers, and this extreme risk is directly related to the lifelong exposure to high LDL-c levels.[5] It is well accepted that CVD risk is driven by LDL-c levels and that patients with more severe pathogenic variants resulting in higher LDL-c levels are at higher CVD risk. However, currently there is no consensus on how to classify the CVD risk in patients with FH. A generally applicable accurate tool for CVD risk stratification may be of help in counseling

of FH patients on their CVD risk caused by the variant found in their family. In addition, knowledge of severity of variants can in particularly convince patients with the most severe variants to get their relatives tested early in life and to start aggressive measures to prevent CVD events to occur.

Since a couple of decades, safe and efficacious LDL-c lowering therapies are available, and there is overwhelming evidence that the most widely used class of lipid lowering therapies, statins (HMG-CoA reductase inhibitors), reduce the risk for CVD. Moderate- to high intensity statin therapy lowers the risk for CAD and mortality by approximately 44% in patients with FH.[6] This implies that there is still a residual risk in statin treated FH patients, which could be caused by either insufficient LDL-c lowering or late initiation of lipid lowering therapy, in a phase when atherosclerosis is already in an advanced state. The importance of early treatment is highlighted by the results of a recently published study that showed that the carotid intima-media thickness, a validated measure of the extent of atherosclerosis, was not different in FH patients who started on a statin in childhood and who were treated for 20 years compared to their non-FH siblings. Moreover, it showed that at the age of 39, the cumulative cardiovascular disease-free survival was 99% for FH patients that had been treated with statins from childhood, thus much more favorable in comparison to the 74% cumulative cardiovascular disease-free survival in their affected parents.[7] These findings underline the importance of early detection of FH, as it allows for early initiation of lipid lowering therapy in order to prevent mortality and morbidity due to CVD.

There are different approaches to conduct screening in order to identify patients with FH. In universal screening, all individuals in a predefined age or risk category are screened. This approach was taken in Slovenia, where standardized nationwide measuring cholesterol levels in children at the age of 5 led to the molecular diagnosis of FH in more than half of the children with high LDL-c levels.[8] Universal screening in specific areas allows to identify index patients that lead to subsequent targeted cascade screening, a method that has been used in 'child-parent screening' models.[9] Cascade screening has been shown to be a (cost) effective way of screening.[10] This approach has been implemented in several European countries and a leading example is the nationwide genetic cascade screening program for FH in the Netherlands from the early 1990s to 2013, which has led to the identification of almost 27,000 FH patients.

ADVANCES IN GENETICS

In the Netherlands, FH is typically diagnosed based on the Dutch Lipid Clinic Network (DLCN) criteria. The DLCN algorithm is a validated clinical tool that comprises clinical FH characteristics such as LDL-c levels, the presence of corneal arcus, tendon xanthoma, as

well as family and medical history of cardiovascular events. In patients with a high score (score > 5), genetic analysis is commonly performed to confirm the clinical diagnosis. Ideally, one would sequence all genes involved in cholesterol metabolism at once, in order to identify the pathogenic variant carried by the patients. However, due to unavailability, labor intensity and high cost, sequencing is not performed in most of the clinics around the world.

The three established genes in FH are those encoding for the low-density lipoprotein receptor (*LDLR*), apolipoprotein B (*APOB*) and proprotein convertase subtilisin-kexin type 9 (*PCSK9*). However, a pathogenic mutation in the coding regions of these genes is not identified in a substantial part of patients with a clinical diagnosis of FH. Depending on the clinical cohorts studied, in up to 60% of clinical FH patients no pathogenic variant is found[11-15] In these patients, yet to be discovered genes might be the cause of the hypercholesterolemia phenotype.

Traditionally, the identification of causal new variants is done using linkage analysis studies in large pedigrees. In fact, this method was used to identify the gain of function (GOF) variant in *PCSK9* as an FH causing defect.[16] Recently, advances in genetic diagnostics, however, such as next generation sequencing has led to other ways to identify novel genes or new regions in known FH genes previously not associated with FH. Multiple other candidate genes have been proposed as FH genes: *ABCG5*, *ABCG8*,[17] *LIPA,*[18] *APOE,*[19] *CCDC22*[20] and *STAP1*.[21] All these genes, except for *STAP1*, have been shown to play a role in cholesterol metabolism. STAP1 is primarily expressed in immune tissue such a spleen lymph nodes and bone marrow and not in hepatic cells, and it was therefore unclear how *STAP1* variants are linked to lipid homeostasis.

Recent advances in genetic analytical methods allow us to fast and reliably sequence the exome or even the whole genome, which facilitates studies to identify novel genomic variants causing FH. While the traditional Sanger sequencing was used mainly for coding regions of known regions, next generation sequencing (NGS) enables to explore the impact of variants in genomic regions that hitherto have not been widely studied. For instance, it has been shown that variants in non-coding regions of well-established FH genes such as the intronic regions of *LDLR* might cause FH.[22,23] Thus, intronic variants in *LDLR* might explain a part of the pathogenesis of FH patients who were considered to be “mutation negative” based on the analysis of the exonic regions.

NEW THERAPIES

The identification of GOF variants in *PCSK9* as a cause for FH in 2003 revolutionized not only our understanding of lipid metabolism, but also resulted in a new class of medication.[24] The two registered monoclonal antibodies directed against PCSK9, evolocumab and alirocumab, decreased LDL-c levels by approximately 50-60% irrespective of the use of other LLT. Statins and ezetimibe lower LDL-c levels by approximately 50% and 20% respectively.[25] This is generally not sufficient to reach LDL-c target levels, in particular for FH patients with high LDL-c levels. In fact, only 21% of 1,249 patients in five large outpatient lipid clinics in the Netherlands reached LDL-c targets, even though they could have been treated with potent combination therapy with statins and ezetimibe by then.[26] The effect of PCSK9 inhibition has been extensively studied in patients with FH, and the randomized clinical trials showed that the once every twee week injections resulted in LDL-c reductions of up to 60% on top of the effects of high intensity statins.[27-29] However, since trial results might not be representative for the real world, the question arises whether this beneficial effect on LDL-c reduction is also observed in routine care where 'the general FH patient', that is not heavily selected in order to meet trial inclusion criteria, is treated.

In addition, the efficacy of PCSK9 inhibitors in patients who carry biallelic variants, has been matter of clinical attention, as these patients present with exceedingly high LDL-c levels (typically above 13 mmol/L), and extreme risk for CVD. Unfortunately, these patients respond less to the conventional oral lipid lowering agents compared to patients with a residual LDLR function.

AIM AND OUTLINE OF THIS THESIS

The overall aim of the studies described in this thesis is to enlarge our understanding of the phenotypic differences among patients with FH. Moreover, I set out to investigate the role of putative and novel genomic variants in FH. Lastly, I addressed whether PCSK9 inhibition, a relatively novel LDL-c lowering therapy, is safe and efficacious in a specific patient category (i.e. biallelic FH), what the potential impact of wide use of PCSK9 inhibition would be, and what the effect of PCSK9 inhibition is in a real-world clinical setting.

Chapter 2 provides an overview on FH: the definition, diagnosis, screening and treatment strategies.

Part I focuses on the pheno- and genotypic characteristics of familial hypercholesterolemia. In **chapter 3**, we describe the wide variation of LDL-c levels and CAD risk among FH

patients, and we generate a model where the impact of the *LDLR* variant on LDL-c levels is translated into CAD risk. In **chapter 4, 5 and 6,** we elaborate on novel variants as a cause for familial hypercholesterolemia. The study described in **chapter 4** focuses on the consequences of novel *PCSK9* variants that were found in Cape Town, South Africa and the subsequent segregation and *in vitro* studies to determine the impact of these variants on LDL-c metabolism. The discovery of a novel deep intronic variant of the *LDLR* in a large family with patients with severe FH where no mutations could be identified with standard care DNA analysis in the coding regions of the *LDLR*, *APOB* or *PCSK9* is described in **chapter 5.** Cosegregation analysis is done as well as cDNA sequencing in order to establish pathogenicity of this novel deep variant. In recent years, the role of STAP1 in FH has remained controversial. In **chapter 6** we therefore describe a large number of studies conducted to unravel whether *STAP1* is a candidate gene.

Part II deals with the treatment of FH patients: from conventional therapies to novel treatment strategies and in particular PCSK9 inhibitors. In **chapter 7,** therapies to lower LDL-c in FH are described. **Chapter 8** provides the study in which the efficacy and safety of alirocumab, a fully human monoclonal antibody directed against PCSK9, is evaluated in patients with double heterozygous, compound heterozygous, or homozygous familial hypercholesterolemia. In **chapter 9** a theoretical model is applied to calculate the proportion of FH patients, identified by the national cascade screening program in the Netherlands, that would reach their LDL-c treatment target using conventional therapy (high intensity statins and ezetimibe) and additional lipid lowering agents such as CETP- and PCSK9 inhibitors. Lastly, in **chapter 10**, we provide the data to show the efficacy and tolerability of PCSK9 inhibitors in routine outpatient care in a large academic hospital in the Netherlands.

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2

NEW APPROACHES IN DETECTION AND TREATMENT OF FAMILIAL HYPERCHOLESTEROLEMIA

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ABSTRACT

Familial hypercholesterolemia (FH) is an autosomal dominant genetic disorder that clinically leads to increased low-density lipoprotein-cholesterol (LDL-c) levels. As a consequence, FH patients are at high risk for cardiovascular disease (CVD). Mutations are found in genes coding for the LDLR, apoB, and PCSK9, although FH cannot be ruled out in the absence of a mutation in one of these genes. It is pivotal to diagnose FH at an early age, since lipid lowering results in a decreased risk of cardiovascular complications especially if initiated early but unfortunately, FH is largely underdiagnosed. While a number of clinical criteria are available, identification of a pathogenic mutation in any of the three aforementioned genes is seen by many as a way to establish a definitive diagnosis of FH. It should be remembered that clinical treatment is based on LDL-c levels and not solely on presence or absence of genetic mutations as LDL-c is what drives risk. Traditionally, mutation detection has been done by means of dideoxy sequencing. However, novel molecular testing methods are gradually being introduced. These next generation sequencing-based methods are likely to be applied on broader scale once their efficacy and effect on cost are being established. Statins are the first-line therapy of choice for FH patients as they have been proven to reduce CVD risk across a range of conditions including hypercholesterolemia (though not specifically tested in FH). However, in a significant proportion of FH patients, LDL-c goals are not met, despite the use of maximal statin doses and additional lipid-lowering therapies. This underlines the need for additional therapies, and inhibition of PCSK9 and CETP is among the most promising new therapeutic options. In this review, we aim to provide an overview of the latest information about the definition, diagnosis, screening, and current and novel therapies for FH.

INTRODUCTION

Familial hypercholesterolemia (FH), a common autosomal dominant inherited disorder, is characterized by high plasma levels of low density lipoprotein-cholesterol (LDL-c) and, as a consequence, high risk for the premature development of atherosclerosis and cardiovascular disease (CVD).[1] The pathological substrate of FH is related to the dysfunctional uptake of LDL particles via its receptor and this can either be caused by mutations in the genes encoding for the LDL receptor (LDLR), apolipoprotein B (apoB), or pro-protein convertase subtilisin/kexin 9 (PCSK9). It is important to diagnose FH at an early age in order to prevent vascular events.

The diagnosis is based on clinical parameters such as lipid levels, presence of xanthomas, family history, and vascular disease, and a definite diagnosis is based either on the identification of a pathogenic mutation in any of the three well established FH causing genes or a probably score derived from clinical characteristics.[2] It has also been postulated that a polygenic form of FH is present in patients meeting the clinical criteria for FH (i.e. according to the Dutch Lipid Criteria Score, Simon Broome Criteria) who do not carry a mutation in one of these genes.[3] There is a wide range in the lipid levels amongst patients with FH, and this is largely related to the severity of the mutation and the specific gene; patients carrying a mutation in the *LDLR* gene for example, tend to suffer from a more severe phenotype than *APOB* mutation carriers.[4] The CVD outcome differs amongst heterozygous carriers of FH mutations, who, in general, typically suffer from CVD events in their fourth decade of life, while patients suffering from homozygous FH, the much rarer form of FH, might already have experienced serious cardiovascular complications in the second decade of life or even in childhood.[5]

HMG-coenzyme reductase inhibitors ('statins') are the therapy of first choice in FH patients.[6] It is of note however, that both the magnitude of CVD risk in untreated FH patients, as well as the CVD risk reduction of statins, is not well-established as randomized controlled trials have not been conducted in this regard. We aim to provide a comprehensive overview of the pathophysiology, epidemiology, screening programs as well as current and future therapies of FH.

PATHOPHYSIOLOGY AND GENETICS

LDLR

FH is caused by a mutation in the gene encoding the LDLR in more than 90% of the molecular diagnosed cases, and this mutation leads to absent or dysfunctional LDLR at the surface of the hepatocytes.[7] As a consequence, hepatic uptake of LDL-c is decreased

which results in elevated plasma levels of LDL-c.[1] The *LDLR* gene is located on the short arm of chromosome, 19 and to date, over 1,700 mutations in the *LDLR* gene have been described (http://www.ucl.ac.uk/ldlr/Current/). Five different classes of *LDLR* mutations have been identified, dependent on the effect on the phenotype. Class 1 mutations are null mutations that result in no detectable LDLR protein. In class 2 mutations, the transport of the LDLR from the endoplasmic reticulum to the Golgi apparatus is blocked completely (class 2a) or partially (class 2b). A class 3 mutation leads to expression of a non-functional LDLR. Class 4 mutations result in LDL binding but the LDLR-LDL complexes cannot be internalized, and in class 5 mutations, recycling of the LDLR is not efficient and therefore do not reach the cell surface.[8]

APOB

In 5% of the molecular-diagnosed FH cases, a pathogenic mutation is found in the gene encoding for the apoB protein and this disease is also referred to as familial defective apoB.[9] The impaired binding of LDL particles to the LDLR therefore results in higher circulating LDL-c concentrations.

PCSK9

In 2003, gain-of-function mutations in a third gene, encoding for PCSK9, were identified as a cause of FH.[10] PCSK9, when forming a complex with the LDLR, is internalized by modification of the LDLR confirmation and interferes with LDLR recycling. This leads to LDLR degradation and therefore reduction of the number of receptors available at the hepatocyte surface to bind circulating LDL particles. [10]

HOMOZYGOUS FH

Patients suffering from homozygous FH (HoFH) are characterized by severely elevated LDL-c levels (typically above 13 mmol/L) and due to this extreme dyslipidemia, patients have been reported to suffer from cardiovascular events in the first decade of life.[11] The molecular defect can either be caused by homozygosity, or more frequently, compound heterozygosity for mutations predominantly in the *LDLR* gene. Moreover, combined mutations in *APOB* and *PCSK9* have also been described (double heterozygotes).[11]

EPIDEMIOLOGY

Unfortunately, the number of individuals diagnosed with FH in most countries is < 1%, except for countries where active screening does take place such as in the Netherlands and Norway, where 71 and 43% of the patients have been described to be diagnosed.[7] These

numbers however, were based on an estimated prevalence of heterozygous FH (HeFH) of 1 in every 500.[12] However, a recent study showed that HeFH was present twice as often in a large Danish population (1 per 200-250). In this study Benn and co-workers applied the Dutch Lipid Network Criteria to quantify the prevalence of FH in the Copenhagen Heart Study, a prospective study comprising over 69,000 Caucasians of Danish descent. In this cohort, 7.76 % were found to meet the "probable or definite FH" criteria using the Dutch Lipid Network criteria.[13] These numbers are very much in line with the numbers that were found in the study of Sjouke et al. who used a genetic approach focusing on a molecular diagnosis.[14] The database of the nationwide molecular diagnostic center was used to identify 49 HoFH patients. The Hardy-Weinberg equilibrium was used to calculate the prevalence, resulting in a prevalence of 1 in 4,180,597 for HoFH and 1 in 319 for HeFH. In another study where large scale exome sequencing was performed, approximately 1 in 217 of the patients who were free of CVD were found to carry a mutation in *LDLR*, whereas the prevalence of mutations was 1 in 50 in those patients who suffered from a premature CVD event.[15] This clearly shows that the true prevalence of FH is probably in the order of 1 in every 200 inhabitants, which would translate in a total of approximately 4.5 million patients with FH in Europe and presumably 35 million people globally. It is of note that regional differences in the prevalence of FH has been described, with a higher prevalence in certain populations due to founder effect, for example in South-Africa and Quebec.[16]

CONSEQUENCES AND CLINICAL HALLMARKS OF FAMILIAL HYPERCHOLESTEROLEMIA

Due to impaired clearance, LDL-particles accumulate in the arterial wall leading to an inflammatory response. The endothelial tissue becomes damaged and atherosclerotic plaques are formed.[17] Endothelial damage begins at a young age, which was shown by studies by de Jongh and co-workers, who measured endothelial function by means of flow mediated dilation (FMD) in children who were diagnosed with HeFH and their non-affected brothers and sisters. It was shown that endothelial function was already impaired in these asymptomatic HeFH patients at the age of 9 to 18 years.[18] The extent of atherosclerosis is further enhanced by other risk factors.[19] It is of note that some risk factors, such as elevated levels of the proatherogenic lipoprotein (a) (Lp(a)), are commonly observed in FH patients.[20] Moreover, triglyceride-rich lipoprotein remnants might contribute to increased CVD risk and premature atherosclerosis in FH.[21] Atherosclerotic plaques are predominantly found in the coronaries, peripheral arteries and aortic valve.[2] Furthermore, cholesterol can accumulate in the skin leading to xanthomas, which are primarily observed in the tendons at the elbows, hands and Achilles. Cholesterol depositions are also found around the eyes in the form

of xanthelasmata or in the cornea, where it can be observed as an arcus lipoides. The presence of xanthoma is pathognomonic for FH and one of the diagnostic criteria for FH.[7] In FH patients, presence of xanthomas is associated with a threefold increased risk of CVD compared to FH patients without xanthomas.[22] Xanthomas are more frequently seen in HoFH, even at birth or during early childhood,[11] but can also be seen in HeFH later in life.

HOW TO DIAGNOSE FAMILIAL HYPERCHOLESTEROLEMIA

CLINICAL VERSUS MOLECULAR DIAGNOSIS

There are several diagnostic criteria to diagnose FH based on different phenotypical and molecular scoring algorithms, and the prediction based on these criteria sets do not differ to a great deal.[23] The Dutch Lipid Network Criteria are widely accepted and commonly used.[24] These can be used to calculate a score predicting the likelihood of FH, whereas a score higher than five makes the diagnosis probable (Table 1). Other criteria that are used and internationally validated are the Simon Broom system criteria,[25] the MEDPED criteria (Make Early Diagnosis to Prevent Early Death)[26] and the Japanese criteria.[27] Secondary causes of hypercholesterolemia such as proteinuria, hypothyroidism or medication must be excluded.[24]

Finding a pathogenic mutation in the *LDLR, APOB or PCSK9* gene is considered to be the gold standard for diagnosing monogenic causes of FH. However, FH cannot be ruled out in the absence of a known mutation being identified. In fact, in a substantial number of cases no monogenic defect can be identified.[28] Reported mutation detection rates range from 20 to 95%.[29-31] It is possible that in these patients, mutations in hitherto unidentified FH genes are present. In line with this assumption is the recent finding of mutations in *STAP1* in patients with FH.[32] Carriers of mutations in this gene were characterized by an FH-like phenotype. Little is known however about the role of STAP1 in lipid metabolism. The gene does not seem to be expressed in tissue with an established role in LDL-metabolism, and clearly, the unravelling of the effect of *STAP1* mutations warrants further studies. Identification of such novel "FH genes" might have a huge impact on our understanding and treatment of dyslipidemia.[33] Alternatively, the FH phenotype might be due to a number of relatively benign variations in a number of genes, a so-called polygenic form of FH. This hypothesis was tested by Talmud, Humphries and co-workers,[3] who indeed showed that the number of LDL-c increasing variations was higher in FH patients in whom no monogenic form was identified compared to controls. It should be emphasized, however, that the LDL-c levels in these subjects were in general lower compared to patients in whom a monogenic defect was identified (LDL-c of 5.87 and

7.03 mmol/L, respectively, p = 0.002).[3] It is important to note that mode of inheritance of these SNP's is not dominant, since the SNPs are located over the whole genome. [20] Furthermore, it is unclear whether this is indeed familial, as the former has implications for genetic screening of relatives whereas non-Mendelian inheritance would not.

Table 1. Dutch Lipid Clinic Network criteria for diagnosis of heterozygous familial hypercholesterolemia

Group 1: family history	**Points**
• First-degree relative with known premature coronary heart disease (CHD) (< 55 years for men; < 60 years for women)	1
• First-degree relative with known LDL cholesterol > 95th percentile by age and gender for country	1
• First-degree relative with tendon xanthoma and/or corneal arcus OR	2
• Child(ren) < 18 years with LDL cholesterol > 95th percentile by age and gender for country	2
Group 2: clinical history	
• Subject has premature CHD (< 55 years for men; < 60 years for women)	2
• Subject has premature cerebral or peripheral vascular disease (< 55 years for men; < 60 years for women)	1
Group 3: physical examination	
• Tendon xanthoma	6
• Corneal arcus in a person < 45 years	4
Group 4: biochemical results (LDL cholesterol)	
• > 8.5 mmol/L (> 325 mg/dL)	8
• 6.5–8.4 mmol/L (251–325 mg/dL)	5
• 5.0–6.4 mmol/L (191–250 mg/dL)	3
• 4.0–4.9 mmol/L (155–190 mg/dL)	1
Group 5: molecular genetic testing (DNA analysis)	
• Causative mutation shown in the *LDLR*, *APOB*, or *PCSK9* genes	8

With the algorithm a numerical score can be calculated which predicts the change that a subject has FH. It is only possible to score once per group. The highest applicable can be chosen. 'Definite FH' > 8 points, 'Probable FH'6-8 points, 'Possible FH' 3-5 points.

SCREENING FOR FAMILIAL HYPERCHOLESTEROLEMIA

Because of the high prevalence of FH, a systemic approach in screening is a legitimate strategy for public health. There are several screening methods for FH. In the Netherlands, cascade screening has been exploited for over two decades. Upon identification of the index case, family directed cascade screening takes place. Index cases can, for example, be identified by opportunistic screening among patients with a (family) history of a CVD event at a young age. Measuring LDL-c levels in such patients should be performed on a routine basis and, while applying any of the clinical criteria metrics, this can be followed by DNA analysis.[35] When a pathogenic mutation is found, cascade screening can take place identifying the same mutation in first-degree relatives. In the Netherlands, over 27,000 individuals have been diagnosed with FH by this method. Multiple studies have

shown that cascade screening is the most cost-effective screenings strategy for FH.[36,37] Although genetic testing has been widely accepted as the gold standard for the diagnosis, measuring LDL-c levels is obligatory since these levels tailor the extent of therapy being prescribed.[38] It is of note that some patients with pathogenic mutations do not show the expected phenotype of elevated LDL-c levels[39] and given the fact that the LDL-c rather than the mutation is the driver of the CVD risk, any preventive therapy measure should be focused on the clinical phenotype, rather than on the presence of a molecular defect.[40]

Another type of screening is universal screening, which involves screening of all individuals in a certain category, for example children of a certain age. So far, this only has been introduced in Slovenia for children at the age of 5.[41] Universal screening in the US at 9 and 11 years has been proposed, for example when vaccination takes place. Recently, it has been proposed to consider universal screening in patients under 20 years of age and preferable before puberty.[35] Ideally this screening strategy would be integrated with cascade screening afterwards, to maximize the detection rate.[42]

MOLECULAR DIAGNOSIS: NEW DETECTION METHODS

Several molecular testing methods are being used to detect mutations in any of the established FH genes, including dideoxy sequencing, array-based sequencing (in case of a relatively limited number of mutations in the population), or denaturing high performance liquid chromatography (DHPLC) and melting analysis.[43] For detection of large insertions of deletions, multiplex probe amplification (MLPA) is used.[43] The large disadvantage of the Sanger based sequence methods is that it is relatively time and labor intensive and this problem is largely overcome by next generation sequencing (NGS) where multiple genes can be analyzed at once. NGS can produce billions of nucleotide reads from a sample of one patient and is relatively inexpensive.[44] NGS can either be used to perform whole genome sequencing (WGS), whole exome sequencing or targeted subgenome analysis,[45] and with these techniques, causative mutations have been identified in a number of patients with monogenetic disease.[44] For FH, NGS has been used in a number of laboratories with differences in success rates.[46]

CVD RISK IN FAMILIAL HYPERCHOLESTEROLEMIA

In the pre-statin era, patients were considered to be at a 100-fold increased risk for coronary heart disease mortality when aged 20-39.[25] Statins were introduced in the 1990s and numerous studies have shown that the lowering LDL-c levels by statin therapy results in a reduction in cardiovascular mortality and morbidity.[47] The effect of statins on CVD events

in FH patients, however, has not been well addressed. It is widely considered that the aforementioned 100-fold increased risk might be an overestimate. Several studies have been published about the CVD risk in FH patients and the risk ratio (RR) associated with FH range from 3 to 16.[13,15,48] Benn et al. (2010) used The Danish General Population to estimate the risk of coronary artery disease (CAD) in probable or definite FH using the Dutch Lipid Network criteria and found that FH patients, who were not treated with lipid-lowering therapy, were at 13-fold risk for CAD compared to non-FH individuals.[13] When using lipid-lowering therapy, compared to non-FH subject, patients with FH were at a 10-fold risk for CAD. In another study by Huijgen et al., patients with a pathogenic *LDLR* mutation had a shorter event-free survival than their relatives who did not carry that mutation (HR 3.64, 95% CI = 3.24-4.08, P < 0.001).[48] In a recent study by Do and co-workers, exome sequencing was performed in nearly 10,000 genomes of patients with myocardial infarction (MI) at a young age, as well as controls. It was found that carriers of non-synonymous mutations in the gene coding for the *LDLR*, were at 4.2-fold higher increased risk for MI. This risk was even higher in carriers of an *LDLR* null mutation (13-fold difference)[15] (see Table 2 for an overview).

Table 2. Cardiovascular disease in FH

	CVD risk
Simon Broome Register Group (1991)[25]	
• Deaths from coronary disease FH vs. non FH	SMR: 386 (95% CI 210 to 639)
Benn et al (2012)[13]	
• CAD in FH vs. non FH (no lipid lowering therapy)	OR: 13.2 (95% CI 10.0-17.4)
• CAD in FH vs. non FH (with lipid lowering therapy)	OR: 10.3 (95% CI 7.8-13.8)
Huijgen et al (2012)[48]	
• Event free survival in carriers of pathogenic *LDLR* mutation vs. non affected relatives	HR: 3.64 (95% CI = 3.24-4.08, p < 0.001)
Do et al (2015)[15]	
• MI in carriers of non-synonymous mutations *LDLR* gene vs. no non-synonynous mutations *LDLR* gene	OR 4.2 ($p = 3 \times 10^{-11}$)
• MI in *LDLR* null mutation vs. no null mutation	OR 13.0 ($p = 9 \times 10^{-5}$)

FH: familial hypercholesterolemia, CVD: cardiovascular disease, SMR: standardized mortality ratio, CI: confidence interval, CAD: coronary artery disease, OR: odds ratio, HR: hazard ratio, p = p-value, MI: myocardial infarction, LDLR: LDL receptor

CURRENT THERAPY

In a number of international guidelines, different LDL-c targets for therapy in FH have been published over the last five years. The target for LDL-c varies in these guidelines and range from a minimal 50% reduction of plasma LDL-c to a target LDL-c level below 2.5 or 1.8

mmol/L in FH patients without or with sharply increased risk for CVD, respectively. The recently published ESC/EAS guideline on FH recommends an LDL-c target level below 2.5 mmol/L for adults, below 1.8 mmol/L for adults with CHD or diabetes, and below 3.5 mmol/L for children. Targets are the same for HeFH and HoFH, regardless of age.[7] The Canadian Guidelines by the Canadian Society also recommend target LDL-c based on different risk categories. For patients with high risk (i.e. CAD, peripheral vascular disease, atherosclerosis or diabetes) and moderate risk, LDL-c target is < 2 mmol/L, in the low risk category (Framingham risk score < 10%) an LDL-c reduction of > 50% is recommended.[49] However, the Framingham risk score is not reliable in FH. The recently updated ACC/AHA guidelines (US) recommend a reduction of > 50% plasma LDL-c levels, acknowledging the fact that no evidence is available supporting a pre-defined target LDL-c level.[6]

It is of note that statins, albeit their efficacy in lowering LDL-c, are widely underused in FH patients.[13] Moreover, the LDL-c levels recommended by the EAS/ESC are not met in over half of the FH patients using statins, even in patients who are treated with maximal doses.[50]

STATINS

Statin therapy is the cornerstone in the treatment of patients with FH. There is a large and robust amount of evidence showing reduction in cardiovascular events by use of statins.[24,47] The effectiveness of statins is based up on upregulation of LDLR by inhibiting HMG-CoA reductase, resulting in lower plasma LDL-c.[21] In their study, Versmissen and colleagues specifically addressed the effect of statins in patients with HeFH.[51] In this non-randomized, retrospective study, it was shown that when treated with statins before onset of CHD, there was a risk reduction of 76%. Moreover, the risk of myocardial infarction in this statin treated group was similar to that in the general population. In the EAS consensus paper of the EAS of Nordestgaard et al, it is advised to start with a maximal potent statin dose in FH patients, if tolerated.[7] In case LDL-c levels are not achieved, adding ezetimibe, a cholesterol absorption inhibitor, is advised.[7]

In some HoFH patients, statins may be effective, although the effects on plasma LDL-c levels are known to be relatively modest,[52] which is due to the severe deficiency in LDLR function. Therapy of choice for HoFH patients is weekly LDL-c apheresis.[53] If apheresis is not available, lomitapide (oral microsomal triglyceride transfer protein (MTP) inhibitor) or mipomersen (antisense apoB) can be given in addition to statins to further lower LDL-c.[35]

EZETIMIBE

Addition of ezetimibe may be necessary to achieve LDL-c targets. Due to reduced absorption of cholesterol in the bowel, there is a compensatory increase in LDLR on hepatocytes

and consequently a 20% reduction in LDL-c.[5] Ezetimibe could also be prescribed as monotherapy for individuals who are not able to tolerate statins, but is preferably given in combination.[54] In the ENHANCE trial, treatment with a combination of ezetimibe and simvastatin did not result in a significant difference in intima-media thickness (cIMT) in comparison to monotherapy with simvastatin.[55] This unexpected result is likely related to the fact that the cIMT values at the time of enrollment were not increased, and as a consequence, a difference over time was less likely to be reached. However, in the recently published IMPROVE-IT trial, cardiovascular outcomes were evaluated in more than 18,000 patients who were hospitalized for an acute coronary syndrome. The results showed that a combination of ezetimibe and statins, resulted in additional LDL-c lowering and improved cardiovascular outcomes.[56]

NOVEL THERAPIES

As mentioned before, many individuals with FH are not able to achieve sufficient reduction in LDL-c levels.[50] This unmet need has driven the development of novel therapies for further LDL-c lowering.

PCSK9 INHIBITION

PCSK9 is a serine protease secreted by hepatocytes and is involved in the degradation of the LDLR.[10] Monoclonal antibodies against PCSK 9 have been developed, and result in increased expression of the LDLR and therefore lowering of LDL-c levels. Following phase 2 studies, PCSK9 inhibitors alirocumab[57] and evolocumab[58] were compared to placebo in subjects with heterozygous FH. Use of alirocumab every two weeks resulted in LDL-c reduction of 67% and subcutaneous injections of evolocumab every four weeks resulted in a 70% lowering of LDL-c. Recently, the results of the OSLER trial showed that evolocumab was efficacious and safe.[59]

CETP INHIBITION

Cholesterylester transfer protein (CETP) is a protein that facilitates exchange of cholesteryl esters for triglycerides between and among HDL particles and apoB-containing lipoproteins (VLDL, intermediate-density protein (IDL) and LDL particles).[60] Blocking this transport by inhibiting CETP, results in an increase in HDL-c and apolipoprotein A1 and a decrease in atherogenic lipoproteins such as LDL-c and Lp(a).[61] The recently published results of the REALIZE trial, showed that treatment with the CETP inhibitor anacetrapib for one year resulted in substantial reductions in LDL-c concentration.[62] The mechanism by which CETP reduction gives a reduction in LDL-c is unknown. Rader and co-workers recently showed

that CETP inhibition by anacetrapib, reduces LDL-apoB-100 levels by increasing the rate of apoB-100 fractional clearance and increasing affinity for the LDLR.[63] Whether CETP inhibtion leads to a reduction in cardiovascular risks still has to be established in an outcome study.

NOVEL THERAPIES FOR THE TREATMENT OF HOMOZYGOUS FH

Mipomersen is an antisense oligonucleotide that binds to apoB messenger RNA which results in a decreased apoB synthesis.[64] It is approved for treatment in patients with HoFH in the USA but not in Europe. It has shown to lower plasma LDL-c in 21% in patients with HoFH[65] and 28% in patients with HeFH.[66]

Lomitapide inhibits MTP at the hepatocytes and blocks the transfer of triglycerides into VLDL in the liver and chylomicrons in the bowel.[65] Lomitapide is approved for treatment of HoFH. LDL-c reductions of 50, 44 and 38% respectively at 26, 56 and 78 weeks have been described.[67]

CONCLUSION

FH is a common inherited disease which leads to premature CVD and atherosclerosis, and early treatment is needed. FH is widely underdiagnosed and LDL-c targets are often not achieved so new therapies are being developed to overcome this problem. This review gives an up to date overview of clinically relevant information on FH.

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PART I

GENO- AND PHENOTYPICAL DIVERSITY IN FAMILIAL HYPERCHOLESTEROLEMIA

3

LDLR GENOTYPE SPECIFIC CORONARY ARTERY DISEASE RISK ESTIMATION BASED ON LDL-C LEVELS IN FAMILIAL HYPERCHOLESTEROLEMIA

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ABSTRACT

INTRODUCTION

Both low-density lipoprotein cholesterol levels and risk for coronary artery disease (CAD) vary among patients diagnosed with heterozygous familial hypercholesterolemia (heFH). This variation is partly explained by the severity of the underlying genetic variants. The low-density lipoprotein cholesterol receptor (*LDLR*) variants are classified as 'deficient' and 'defective' based on prediction tools. This dichotomous approach results in a crude CAD risk estimation.

AIMS

To assess whether and to what extent an *LDLR* genotype specific CAD risk estimation based on LDL-c levels is useful in patients with heFH, and to assess its performance compared to the dichotomous classification recommended by international heFH guidelines.

METHODS

All individuals who were screened for the presence of pathogenic *LDLR* variants as part of the Dutch nationwide FH cascade screening in the period between 1994 and 2010 were enrolled. First, we classified variants by calculating pre-treatment LDL-c levels in each individual and generated age and gender adjusted LDL-c percentiles. For each variant the mean percentile LDL-c was assessed and variants were subsequently stratified into six strata using the 75th, 88th, 92nd, 96.5th and 98th percentile as cut-offs. Alternatively, classification of *LDLR* variants was done according to class 1 – i.e. receptor deficient – versus non-class 1 (most often receptor defective). Second, a Cox proportional hazard model was used to calculate the risk of major CAD (myocardial infarction, percutaneous coronary intervention, coronary artery bypass graft) for carriers of different variants versus their unaffected relatives.

RESULTS

A total of 35,257 individuals were tested for 456 different *LDLR* variants. LDL-c percentiles could be determined for 35,067 (99.5%). In 12,485 (36%) individuals a pathogenic *LDLR* variant was identified. CAD risk (HR with 95% confidence interval) compared to their unaffected family members increased gradually from 2.2 (0.97-5.0), 2.5 (1.3-5.0), 3.8 (2.6-5.7), 4.1 (2.9-5.9), 8.8 (6.2-13) up to a 12 (5.5-24.8) fold higher risk for those with variants that were associated with an LDL-c below the 75th percentile (LDL-c 3.3 ± 1.1 mmol/L), between the 75-88th (LDL-c 4.3 ± 1.5 mmol/L), 88-92th (LDL-c 5.0 ± 1.6 mmol/L), 92-96.5th (5.6 ± 1.9 mmol/L), 96.5-98th (LDL-c 6.2 ± 2.1 mmol/L) and higher than the 98th percentile (LDL-c 6.5 ± 1.9 mmol/L) respectively. In comparison, carriers of a non-class-1 variant had a 3.9 (3.0-4.9)

fold higher risk than their family controls. Carriers of class 1 variant had even a 7.3 (5.4-9.3) higher CAD risk than their unaffected relatives.

CONCLUSIONS

Our approach provides an objective and readily applicable tool allowing a more personalized and accurate CAD risk estimate based on the type of *LDLR* variant and the associated LDL-c percentile.

INTRODUCTION

Heterozygous familial hypercholesterolemia (heFH) is a common genetic disorder characterized by elevated plasma low-density lipoprotein cholesterol (LDL-c) levels and an increased risk of coronary artery disease (CAD).[1-7] HeFH is caused by variants in the low-density lipoprotein receptor gene (*LDLR*), apolipoprotein B (*APOB*) and proprotein convertase subtilisin-kexin type 9 (*PCSK9*).[8] Of the three heFH causing genes *LDLR* variants are most common and to date over 1,700 pathogenic variants have been described, although the effect of different variants on residual LDLR activity varies widely.[9] As a consequence, LDL-c levels and CAD risk vary from one heFH patient to another, and this clinical variability is partly explained by the severity of the underlying variant.[10,11]

In most international guidelines, *LDLR* variants are classified as either receptor 'deficient' (also known as 'negative' or 'null', with residual LDLR activity < 2%) or 'defective' (residual LDLR activity 2-25%) with the former having a more detrimental effect on LDLR function with concomitant higher plasma LDL-c levels.[12,13] However, this classification is likely an oversimplification, as the wide range of impact on LDLR function of the different variants is not well captured in a dichotomous variable. In addition, this traditional classification is derived from studies on LDLR functionality in fibroblasts, which, in general, can only be reliably interpreted in homozygous carriers of such variants.[14] For variants where no *in vitro* studies have been performed, the classification is based on *in silico* prediction tools. However, these tools cannot be used for all type of variants and the effect of a considerable number of variants remains unclassified.[11] Moreover, experts often disagree on how to label an *LDLR* variant based on *in silico* prediction tools.[15] Thus, to determine functionality or pathogenicity of variants can be a complex and challenging process, in which LDL-c phenotype or CAD risk is not taken into account.[16] In the current study, we try to overcome these issues by using the LDL-c levels as a readout for (loss of) functionality of the LDLR. To set out for this, we assessed the severity of pathogenic variants based on LDL-c levels in a large cohort of heFH patients and related this to CAD risk in order to generate a more individualized risk estimate. This may help physicians to not only better understand, but also to communicate about the CAD risk in the individual patient.

METHODS

STUDY POPULATION

Individuals were eligible if they underwent family screening for *LDLR* variants between 1994 and 2010 as part of the Dutch nationwide cascade screening program, as described

before.[17] Index patients were excluded from the current analysis to avoid clinical sampling bias. Homozygous FH patients as well as subjects tested for variants in *APOB* and/or *PCSK9* variants were excluded.[18] This program was approved by the Medical Ethical Committee of the Academic Medical Centre, University of Amsterdam, the Netherlands and this study was approved by the scientific board of LEEFH, a non-profit organization responsible for data collection and storage. All participants gave written informed consent.

LIPID MEASUREMENT AND DNA ANALYSIS

Lipid levels were not measured in all participants before August 2003, however, thereafter lipids were measured in all participants using the LDX-analyzer. LDL-c levels were calculated based on the Friedewald formula unless triglycerides were above 4.5 mmol/L (400 mg/dL).[19] Ten milliliters of blood were collected in EDTA containing tubes by venipuncture. DNA testing procedures (Sanger sequencing) have been described previously.[20]

CLASSIFICATION OF VARIANTS

Age- and sex-specific levels for LDL-c were calculated based on levels measured in untreated non-carriers according to a previously described method.[10] These reference values were used to calculate age and sex specific LDL-c percentiles for each individual. All individuals were assigned to a group with the same age and same sex. Subsequently, the *z score* of the LDL-c of each individual was determined, as a standardized deviation from the mean of the group from the same sex and age category (LDL-c group). The *z score* was calculated as the difference between the individual LDL-c level and the LDL-c group divided by the SD of the group. This *z score* was used to calculate the age- and sex-specific percentile LDL-c for each individual.[10] In case only on-treatment lipid levels were available, pre-treatment levels were calculated as described and validated before.[21] For each specific *LDLR* variant, a mean percentile LDL-c was calculated based on all carriers of that variant. Subsequently, this variant specific percentile LDL-c was used as a measure of lifetime exposure to the extent of hypercholesterolemia. Hot deck imputation was performed in carriers of a specific variant in whom no LDL-c level was measured (as was often the case in individuals who were screened before 2003); these individuals were assigned to the LDL-c percentile derived from patients with the same variant with known LDL-c levels.[22] Subsequently, the total cohort of variant carriers were categorized in six strata according to the effect on LDL-c levels; below the 75th, 75th-88th, 88th-92nd, 92nd-96.5th and above the 98th percentile. These cutoffs were used to obtain by and large similar sized groups.

Variants were also categorized the variants according to the widely used and previously described methodology by Hobbs, Brown and Goldstein.[14] In essence, class 1 variants are those variants that are not able to produce any functional LDLR protein (receptor deficient

or null variants). *LDLR* deficient variants were defined as LDLR activity < 2%. *LDLR* defective variants were defined as LDLR activity 2-25% in fibroblasts. Class 1 variants are generally the most severe variants since no LDLR protein is being synthesized at all and therefore there is no residual LDLR activity. In non-class-1 variants, there is often some residual receptor activity. These are mostly receptor defective and are in general considered less severe. However, for the non-class 1 variants, some will still have a detrimental effect on LDLR function so these will not by definition be *LDLR* defective with some residual activity, and classifying these can be challenging and arbitrary.

CLINICAL OUTCOME: CORONARY ARTERY DISEASE IN PRE-STATIN ERA

All study subjects were alive at the time of genetic testing and no structural follow up after the moment of genetic test was available. CAD was therefore defined as a history of one of the following non-fatal cardiac outcomes at the time of genetic testing: myocardial infarction, percutaneous coronary intervention and coronary artery bypass graft. CAD event-free survival was defined as the period from birth until the date of the first CAD event or censoring at the 1st of January 1990, whichever came first. This date was chosen since statins became available in the Netherlands in 1990. Data on CAD events collected after 1990 would be confounded by statin use, and was censored for that reason.

STATISTICAL ANALYSIS

Differences in normally distributed variables such as LDL-c were compared by means of unpaired Student's *t*-tests. Dichotomous variables were compared with chi-square tests. Cumulative event rate between carriers or subgroup of carriers and unaffected relatives were compared using Kaplan Meier survival analysis, with a log rank test. The Cox proportional hazard model was used to compare the risk of CAD for the different *LDLR* variant carriers, where unaffected relatives were the reference group. We adjusted for gender, age, presence of hypertension, presence of diabetes, smoking habit, and body mass index. A two-sided p-value < 0.05 was considered statistically significant. Data were analyzed with SPSS for Windows 23 (Chicago IL).

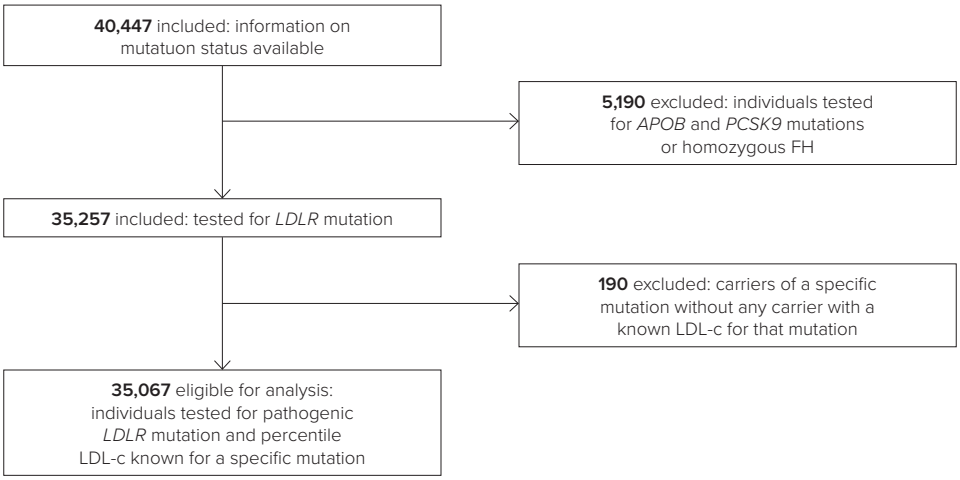
RESULTS

STUDY POPULATION

Between 1994 and 2010, a total of 40,447 individuals underwent molecular cascade screening for familial hypercholesterolemia. We excluded 5,190 patients analyzed for *APOB* or *PCSK9* variants. In 190 persons, LDL-c levels were not available for any of the carriers and therefore no mean percentile LDL-c could be calculated for those specific variants

(0.5%). The remaining study population consisted of 35,067 persons, who were screened for the presence of the specific *LDLR* variant identified in the index patient of their family (Figure 1). A total of 456 different *LDLR* variants were analyzed, all of which were considered to be pathogenic at the time of initiating genetic cascade screening in these families (Supplemental Table 1). A total of 12,485 (36%) individuals were heterozygous carriers of the FH causing *LDLR* variant. For the calculation of CAD event free survival in the pre-statin era, only data were used from 31,217 persons who were born before 1990.

Figure 1. Flow diagram of study population



Demographic and clinical characteristics of the total cohort are shown in Table 1. FH patients were younger (mean age in years ± SD: 38.0 ± 20.0 vs 43.7 ± 19.0) had a lower Body Mass Index (BMI) (in kg/m^2 ± SD: 24.0 ± 4.9 vs 25.0 ± 4.6) and the prevalence of classical cardiovascular risk factors such as smoking, diabetes and hypertension was lower in carriers compared to family controls (28% vs 34%, 2.2% vs 3.2% and 8.5% vs 12%, respectively). LDL-c levels were significantly higher in patients with FH (mean LDL-c in mmol/L ± SD: 4.2 ± 1.4 vs 2.9 ± 0.95 in non-carriers). CAD was more prevalent among FH variant carriers compared to their unaffected family members (6.9% vs 3.6%).

Table 1. Characteristics of heterozygous carriers of a variant in *LDLR* and non-carriers

	FH patients	Non carriers	P-value
Number of patients	12,485	22,582	
Age years	38.0 ± 20.0	43.7 ± 19.0	< 0.001
Male sex n (%)	5,941 (47.7%)	10,637 (47.1%)	0.39
BMI kg/m^2	24.0 ± 4.9	25.0 ± 4.6	< 0.001
Hypertension n (%)	1,055 (8.5%)	2,720 (12.0%)	< 0.001
Smoking ever n (%)	3,507 (28.2%)	7,586 (33.6%)	< 0.001
Diabetes n (%)	275 (2.2%)	717 (3.2%)	< 0.001
Lipid lowering medication n (%)	4,982 (39.9%)	2,193 (9.7%)	< 0.001
Total cholesterol mmol/L	6.0 ± 1.5	4.9 ± 1.1	< 0.001
LDL-cholesterol mmol/L	4.2 ± 1.4	2.9 ± 0.95	< 0.001
LDL-cholesterol, off - treatment† mmol/L	5.3 ± 2.0	3.1 ± 1.0	< 0.001
HDL-cholesterol mmol/L	1.2 ± 0.38	1.3 ± 0.38	< 0.001
Triglycerides mmol/L	1.3 ± 0.88	1.5 ± 0.96	< 0.001
CAD n (%)	859 (6.9%)	819 (3.6%)	< 0.001

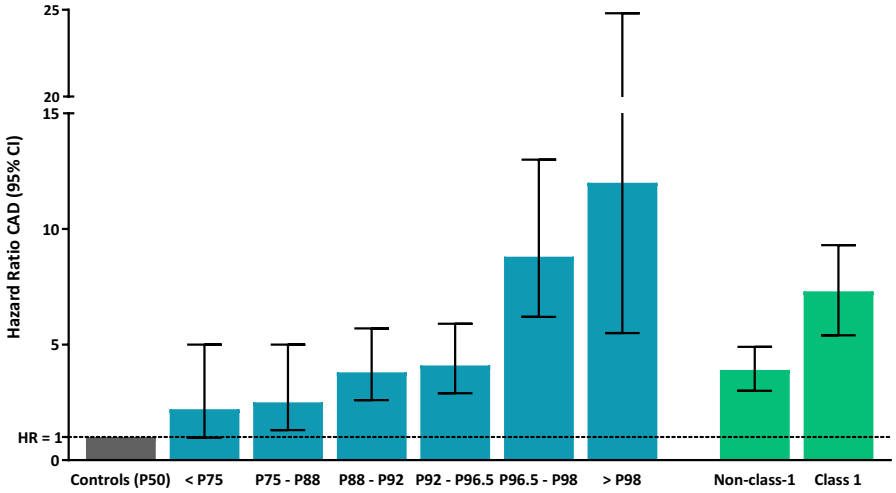
Continuous variables with a normal distribution are reported as mean ± standard deviation. N: number; BMI: body mass index; LDL: low-density lipoprotein; HDL: high-density lipoprotein. CAD: coronary artery disease, defined as myocardial infarction, coronary bypass surgery and percutaneous transluminal coronary angioplasty in medical history. †Off treatment LDL cholesterol levels are calculated based on type and dose of lipid lowering treatment.

The clinical characteristics of the groups stratified are listed in Table 2. After dividing the FH population into six strata, it was shown that patients in the lowest percentile LDL-c stratum were slightly older than those in the highest percentile LDL-c stratum (mean age in years ± SD: 40.4 ± 20.2 vs 37.7 ± 19.4 years). Patients from the lowest percentile LDL-c stratum were less often treated with lipid lowering therapy at genetic diagnosis (16% vs 48%). Class 1 variant carriers were present in every stratum: in the lowest percentile LDL-c stratum, 7.9% of the patients carried a class 1 variant, while 53% of the patients in the highest percentile LDL-c stratum carried a class 1 variant (Table 2).

CLINICAL OUTCOME: CAD CUMULATIVE EVENT RATE IN PRE-STATIN ERA

Overall, CAD risk was 5-fold higher in carriers compared to family controls (HR 5.0, 95% CI: 4.1 to 6.0). The risk for CAD was increased across the different strata of LDL-c percentiles; 2.2 (HR, 95% CI: 0.97-5.0), 2.5 (HR, 95% CI: 1.3-5.0), 3.8 (HR, 95% CI: 2.6-5.7), 4.1 (HR, 95% CI: 2.9-5.9), 8.8 (HR, 95% CI: 6.2-13), versus 12 (HR, 95% CI: 5.5-24.8) for carriers of *LDLR* variants associated with a percentile LDL-c of < P75, P75-P88, P88-P92, P92-P96.5, P96.5-P98 and > P98, respectively (Figure 2). In comparison, carriers of a non-class-1 *LDLR* variant had a 3.9-fold (HR, 95% CI: 3.0-4.9) higher CAD risk than their unaffected relatives. Carriers of a class 1 *LDLR* variant had a 7.3-fold (HR, 95% CI: 5.4-9.3) higher CAD risk compared with their unaffected relatives.

Figure 2: Association between variant category based on mean LDL-c percentile and CAD risk in the pre-statin era



LDL-c: low-density lipoprotein cholesterol; CAD: coronary artery disease; CI: confidence interval; P: percentile. A Cox proportional hazard model was used to compare CAD risk between carriers and non-carriers with adjustments for age, sex, hypertension, diabetes, body mass index and exposure ever to smoking. The non-carriers served as a reference group (controls (p50)). CAD was defined as myocardial infarction, coronary bypass surgery and percutaneous transluminal coronary angioplasty in medical history. Pre-statin era was defined as the period from the data of birth until the date of the first CAD event or censoring at the 1 January 1990, whichever came first. Classification of *LDLR* variants was done according to class 1 – i.e. receptor deficient – versus non-class 1 (most often receptor defective).

Table 2. Characteristics within the different LDL-c percentile strata

	< P75	P75 - P88	P88 - P92	P92 - P96.5	P96.5 - P98	> P98
Number of patients	983	972	3,245	2,793	3,377	1,115
Median year of first visit	2006	2006	2004	2005	2004	2005
Age years	40 ± 20	40 ± 24	38 ± 19	37 ± 19	36 ± 20	36 ± 19
Male sex n (%)	445 (45.3%)	457 (47.0%)	1,560 (48.1%)	1,381 (49.4%)	1,594 (47.2%)	504 (45.2%)
BMI kg/m^2	24 ± 5.0	24 ± 4.8	24 ± 4.5	24 ± 4.8	23 ± 5.0	24 ± 5.2
Hypertension n (%)	114 (11.6%)	119 (12.2%)	231 (7.1%)	247 (8.8%)	259 (7.7%)	85 (7.6%)
Smoking ever n (%)	320 (32.6%)	297 (30.6%)	905 (27.9%)	783 (28.0%)	877 (26.0%)	325 (29.1%)
Diabetes n (%)	43 (4.3%)	43 (4.4%)	45 (1.4%)	58 (2.1%)	60 (1.8%)	26 (2.3%)
Lipid lowering medication n (%)	158 (16.1%)	277 (28.5%)	1,064 (32.8%)	1,317 (47.2%)	1,634 (48.4%)	532 (47.7%)
Total cholesterol mmol/L	5.0 ± 1.2	5.6 ± 1.3	5.9 ± 1.3	6.0 ± 1.5	6.4 ± 1.6	6.5 ± 1.6
HDL-cholesterol mmol/L	1.2 ± 0.36	1.2 ± 0.38	1.2 ± 0.39	1.2 ± 0.36	1.2 ± 0.35	1.2 ± 0.41
Triglycerides mmol/L	1.5 ± 1.0	1.4 ± 0.87	1.3 ± 0.89	1.3 ± 0.82	1.3 ± 0.88	1.3 ± 0.88
LDL-cholesterol mmol/L	3.1 ± 1.0	3.7 ± 1.2	4.0 ± 1.2	4.3 ± 1.4	4.6 ± 1.5	4.7 ± 1.5
LDL-cholesterol, off treatment†	3.3 ± 1.1	4.3 ± 1.5	5.0 ± 1.6	5.6 ± 1.9	6.2 ± 2.1	6.5 ± 1.9
No. of class 1 variant carriers n (%)	78 (7.9%)	58 (6.0%)	70 (2.2%)	1,231(44.1%)	2,152 (63.7%)	592 (53.1%)

Continuous variables with a normal distribution are reported as mean ± standard deviation. P: percentile; n: number; BMI: body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein. †Off treatment LDL cholesterol levels are calculated based on type and dose of lipid lowering treatment.

DISCUSSION

In this observational study, we show that the LDL-c based (re)classification of *LDLR* variants displays a CAD risk ranging from a 2.2- to a 12-fold increase. In contrast, the conventional risk estimate based on non-class-1 vs class 1 variants show an increased risk of 3.9 and 7.3, respectively. Thus, our approach provides a more refined CAD risk estimate.

We based our findings on a large cohort of more than 35,000 individuals screened for the presence of a pathogenic variant in *LDLR*. Overall, patients with molecularly proven FH were found to be at 5.0 (HR, 95% CI: 4.1-6.0) fold increased risk of CAD compared with unaffected relatives in the pre-statin era. When *LDLR* variants were further categorized according to class 1 and non-class-1, it resulted in a crude refinement in CAD risk. We observed that carriers of a class 1 variant have a CAD risk that is roughly twice as high as carriers of a non-class 1 variant (HR 7.3, 95% CI: 5.4-9.7 vs. HR 3.9, 95% CI 3.0-4.9). Alternatively, variant categories based on percentile LDL-c resulted in CAD risk inclining more gradually, introducing a much larger variation in CAD risk ranging from 2- to 12-fold increased risk depending on which variant is considered. In support, CAD risk is 5-fold higher in heterozygous carriers of *LDLR* variants associated with extreme LDL-c levels (defined as LDL-c > 98th percentile for age and sex) compared to carriers of *LDLR* variants that typically present with LDL-c levels between the 50th and 75th percentile. Thus, classification based on variant-based LDL-c percentile allows a more refined CAD risk estimate. It has been shown that the clinical phenotype varies among FH patients with different variants and we do observe this in our study. Mean pre-treatment LDL-c levels ranged from 2.54 mmol/L (SD ± 1.00) for carriers of the most modest – and likely neutral - *LDLR* variant to 9.18 mmol/L (SD ± 1.80) for carriers of the most severe variants in our cohort. This heterogeneous phenotype in terms of LDL-c levels has large ramifications on the risk estimates as the overall risk is largely driven by the total burden of LDL-c exposure over time. Slightly elevated LDL-c for lifetime has large implications that are reflected in the wide range in CAD risk: carriers of the variants associated with an LDL-c below the 75th percentile results in a risk of 2.2-fold, while carriers with variants associated with an LDL-c above the 98th percentile results in a risk of even a 12-fold higher than unaffected family controls. In the light of observed contributions of other risk factors in the general population risk estimators, such as diabetes which has around 2-fold higher risk, this difference is substantial and therefore of large clinical significance. It is crucial to account for variant severity as accurately as possible when counseling patients about the risk for CAD they are exposed to. In our view, stratification based on observed LDL-c phenotype can be used as a widely applicable and accurate tool to assess variant severity of each *LDLR* variant.

Our findings are in line with three recent publications using large cohorts and exome sequencing for rare *LDLR* variants.[4,7,23] These studies consistently showed an approximately 4-fold higher CAD risk in carriers of a variant in *LDLR*, and an even more pronounced approximately 10-fold higher risk for more severe variants. In these studies, a rigid definition was used to classify the FH variants. Khera and colleagues, for example, sequenced *LDLR* in 26,050 individuals and reported a 4-fold increased risk in FH in general and 10-fold for carriers of loss-of-function (i.e. receptor deficient) *LDLR* variants. Variants were considered to be loss-of-function once all five *in silico* prediction algorithms classified the variant as such.[23] For comparison, our criteria based on the LDL-c percentile classified the most severe *LDLR* variants with a very high CAD risk, similar to the strict definition used in the study by Khera and colleagues. The majority of *LDLR* variants in our cohort did not classify as loss of function while using the prediction tools. Moreover, a large number of variants cannot be ascertained by these tools, such as splice site variants or insertions and deletions.[23] Our method, however, overcomes this phenomenon by using the LDL-c levels as a readout for (loss of) functionality of the LDLR in a large cohort of heFH patients. Thus, our severity stratification is particularly of value in classifying the large remaining group of defective *LDLR* variants into risk categories, ranging from non-pathogenic variants associated with no or only slightly increased CAD risk to variants associated with severe CAD risk.

The two main strengths of our study are the unprecedented large number of carriers of variants, which allows for a relatively precise effect estimation, especially since LDL-c levels in non-carriers of the same families were used as the reference. Moreover, while excluding index cases, we minimized the effect of referral bias. Another strength of our approach to classify variant severity based on percentile LDL-c is that it more inclusive than the conventional receptor deficient or receptor defective status.[14] For the variants where no *in vitro* studies have been performed, the functionality is often assessed with *in silico* prediction tools. However, *in silico* prediction tools cannot be applied for some variants due to their nature, e.g. some insertions or intronic variants. For example, *in silico* prediction with Sorting Intolerant From Tolerant (SIFT) or PolyPhen could only be applied to 43 of the 64 most prevalent *LDLR* variants (67%) in the Netherlands.[11] In addition, it was shown that *in silico* prediction tools misclassify variant severity, in particular with a specificity of below 45%. It was demonstrated that a large proportion of the non-pathogenic *LDLR* variants, who proved to have normal LDL-c levels and no increased CAD risk, were assigned as pathogenic by either SIFT or POLYPHEN or both *in silico* prediction tools.[11] Thus, we consider the method used in the current study to be superior to the dichotomous classification that is widely used nowadays.

A number of considerations and limitations have to be taken into account while interpreting

the current study. One important limitation is the fact that the risk estimates are not derived from prospective studies, but rather from a longitudinal study. Moreover, the CAD risk is based on non-fatal CAD events that occurred prior to 1990, in the era before statins were available. We have chosen this approach to avoid confounding. Since statins are recommended in FH patients, irrespective whether they did or did not suffer from a CAD event, statin use would hamper the evaluation of the effect of the genotype on CAD risk. While only focusing on non-fatal events prior to 1990 (fatal CAD events could not be recorded and were not included while estimating CAD) however, we also introduced a potential underestimation of the effect of the genetic variants analyzed in this study. Participants were screened at a median date of 2005; 15 years after 1990. A patient who suffered from a non-fatal CAD event in the pre-statin era was only included in this study if he or she had survived for several years, sometimes 15 years after this event or even more. It is likely that survival bias has affected those without FH and those with molecularly proven FH in a different manner.[24] To address this selective survival bias, we chose to report relative CAD risk for variant carriers and non-carriers from the pre-statin era, instead of absolute CAD risk in FH.[25]

Lastly, our method of variant classification based on LDL-c percentiles may not be applicable in other regions. In most countries, no structural genetic cascade screening is performed and diagnosis of FH is largely dependent on clinical diagnosis in lipid clinics. In that situation, a reference population of unaffected relatives is lacking, and determining LDL-c distribution in the general population can be more challenging. As a consequence, calculation of LDL-c percentiles for variant carriers can be more bothersome.

In conclusion, our approach to stratify variant severity classified based on LDL-c percentiles allows for a genotype specific CAD risk estimation in heFH patients compared to the current dichotomous classification. Our method enables the physician to counsel the individual patient and his/her relatives about the CAD risk based on the specific genetic variant in the family, with a more than 5-fold risk difference between carriers with variants classified as mild (mean percentile LDL-c < P75) versus those with a severe variant (mean percentile LDL-c > P98).

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Supplemental Table 1. Mean LDL-c and LDL-c percentile for prevalent *LDLR* variants

cNomen	pNomen	Variant category (range mean pLDL-c)	Mean pLDL-c	Mean LDL-c in mmol/L ± SD	No of carriers
c.682G>T	p.Glu228*	Most severe percentile LDL-c > 98	100.00	8.06 ± 1.45	8
c.(?_-1)_(67+1_68-1)del	p.?	Most severe percentile LDL-c > 98	100.00	9.18 ± 1.80	5
c.648_649del	p.Cys216*	Most severe percentile LDL-c > 98	100.00	6.52 ± 1.20	8
c.(940+1_941-1)_(2311+1_2312-1)del	p.?	Most severe percentile LDL-c > 98	100.00	7.14 ± 2.05	5
c.2132G>T	p.Cys711Phe	Most severe percentile LDL-c > 98	99.99	6.26 ± 1.51	12
c.1892C>A	Ala631Asp	Most severe percentile LDL-c > 98	99.98	6.91 ± 1.19	8
c.320_332del	p.Lys107Argfs*95	Most severe percentile LDL-c > 98	99.95	7.95 ± 2.31	6
c.501C>A	p.Cys167*	Most severe percentile LDL-c > 98	99.88	6.19 ± 0,86	9
c.1688C>A	p.Pro563His	Most severe percentile LDL-c > 98	99.84	7.56 ± 1,77	9
c.1567G>A	p.Val523Met	Most severe percentile LDL-c > 98	99.84	6.35 ± 1,67	11
c.2478ins of c.2478dup?	p.Val827Argfs*9	Most severe percentile LDL-c > 98	99.82	6.42 ± 1.12	8
c.463T>G	p.Cys155Gly	Most severe percentile LDL-c > 98	99.79	6.65 ± 1.57	9
c.1759del	p.Ser587Alafs*78	Most severe percentile LDL-c > 98	99.74	7.63 ± 1.68	6
c.519C>G	p.Cys173Trp	Most severe percentile LDL-c > 98	99.74	6.97 ± 2.40	15
c.2000G>A	p.Cys667Tyr	Most severe percentile LDL-c > 98	99.71	5.62 ± 1.31	5
c.1A>G	p.? Start loss	Most severe percentile LDL-c > 98	99.69	6.30 ± 1.79	18
c.1297G>T	p.Asp433Tyr	Most severe percentile LDL-c > 98	99.64	7.20 ± 2.34	20
c.986G>A	p.Cys329Tyr	Most severe percentile LDL-c > 98	99.62	6.29 ± 2.19	5
c.1A>C	p.? Start loss	Most severe percentile LDL-c > 98	99.61	7.51 ± 2.12	7
c.(694+1_695-1)_(2140+1_2141-1)del	p.?	Most severe percentile LDL-c > 98	99.56	5.75 ± 1.47	13
c.550T>C	p.Cys184Arg	Most severe percentile LDL-c > 98	99.49	6.22 ± 1.55	27
c.2033_2044del	p.Gln678_Cys681del	Most severe percentile LDL-c > 98	99.41	6.38 ± 3.07	7
c.1871_1873del	p.Ile624del	Most severe percentile LDL-c > 98	99.36	6.66 ± 2.25	6
c.-136C>T	p.?	Most severe percentile LDL-c > 98	99.30	5.75 ± 1.57	14
c.(67+1_68-1)_(2140+1_2141-1)del	p.?	Most severe percentile LDL-c > 98	99.27	5.51 ± 1.57	10
c.-193_-187del	p.?	Most severe percentile LDL-c > 98	99.17	5.67 ± 1.06	9

Supplemental Table 1. Continued

cNomen	pNomen	Variant category (range mean pLDL-c)	Mean pLDL-c	Mean LDL-c in mmol/L ± SD	No of carriers
c.(1186+1_1187-1)_(1845+1_1846-1)del	p.?	Most severe percentile LDL-c > 98	99.12	6.76 ± 1.29	5
c.28T>C	p.Trp10Arg	Most severe percentile LDL-c > 98	98.98	5.50 ± 1.18	20
c.2417_2418insG	p.Phe807Leufs*10	Most severe percentile LDL-c > 98	98.91	6.39 ± 2.65	21
c.[932A>G;939>G]	p.Lys311Arg/p.Cys313Trp	Most severe percentile LDL-c > 98	98.88	5.87 ± 1.12	15
c.1481_1586+16del	p.Val494Alafs*19	Most severe percentile LDL-c > 98	98.88	5.02 ± 0.35	6
c.1048C>T	p.Arg350*	Most severe percentile LDL-c > 98	98.84	5.97 ± 1.68	64
c.518del	p.Cys173Serfs*33	Most severe percentile LDL-c > 98	98.78	6.84 ± 1.94	30
c.1358+1G>A	p.?	Most severe percentile LDL-c > 98	98.75	7.09 ± 2.35	41
c.671A>T	p.Asp224Val	Most severe percentile LDL-c > 98	98.75	6.42 ± 1.48	19
c.429C>A	p.Cys143*	Most severe percentile LDL-c > 98	98.72	6.93 ± 2.12	34
c.1176C>A	p.Cys392*	Most severe percentile LDL-c > 98	98.56	6.30 ± 1.62	28
c.(2311+1_2312-1)_(2547+1_2548-1)del	p.?	Most severe percentile LDL-c > 98	98.46	6.92 ± 1.96	39
c.1486_1487del	p.Gly496Hisfs*39	Most severe percentile LDL-c > 98	98.20	6.27 ± 1.28	13
c.1475A>G	p.Asp492Gly	Most severe percentile LDL-c > 98	98.17	5.55 ± 1.15	7
c.2054C>T	p.Pro685Leu	Very Severe percentile LDL-c 96.5-98	97.96	5.67 ± 1.42	54
c.1329G>C	p.Trp443Cys	Very Severe percentile LDL-c 96.5-98	97.95	6.03 ± 1.68	44
c.1843G>A	p.Glu615Lys	Very Severe percentile LDL-c 96.5-98	97.95	5.81 ± 2.17	24
c.877del	p.Asp293Thrfs*77	Very Severe percentile LDL-c 96.5-98	97.88	6.94 ± 2.96	28
c.(940+1_941-1)_(1186+1_1187-1)del	p.?	Very Severe percentile LDL-c 96.5-98	97.86	6.44 ± 2.42	162
c.(2311+1_2312-1)_(*1_?)del	p.?	Very Severe percentile LDL-c 96.5-98	97.81	6.34 ± 1.95	28
c.1478_1479del	p.Ser493Cysfs*42	Very Severe percentile LDL-c 96.5-98	97.81	6.12 ± 1.69	6
c.191-2A>G	p.?	Very Severe percentile LDL-c 96.5-98	97.67	6.36 ± 2.47	195
c.1833G>C	p.Leu611Phe	Very Severe percentile LDL-c 96.5-98	97.55	6.45 ± 1.83	127
c.2390-2A>G	p.?	Very Severe percentile LDL-c 96.5-98	97.38	5.99 ± 1.79	63
c.1004G>T	p.Gly335Val	Very Severe percentile LDL-c 96.5-98	97.33	6.18 ± 1.62	44
c.1066G>T	p.Asp356Tyr	Very Severe percentile LDL-c 96.5-98	97.32	5.54 ± 1.55	17

Supplemental Table 1. Continued

cNomen	pNomen	Variant category (range mean pLDL-c)	Mean pLDL-c	Mean LDL-c in mmol/L ± SD	No of carriers
c.1646G>A	p.Gly549Asp	Very Severe percentile LDL-c 96.5-98	97.22	6.29 ± 1.65	10
c.(1705+1_1706-1)_(*1_?)del	p.?	Very Severe percentile LDL-c 96.5-98	97.02	6.55 ± 1.57	28
c.2050_2063del	p.Ala684Profs*28	Very Severe percentile LDL-c 96.5-98	96.96	6.05 ± 1.67	7
c.2192_2204dup	p.Val736Glnfs*50	Very Severe percentile LDL-c 96.5-98	96.89	5.76 ± 1.28	13
c.681C>A or c.681C>G	p.Asp227Glu	Very Severe percentile LDL-c 96.5-98	96.84	6.10 ± 3.01	18
c.622G>A	p.Glu208Lys	Very Severe percentile LDL-c 96.5-98	96.84	5.47 ± 2.11	22
c.313+1G>A of c.313+1G>C of c.313+2T>C	p.?	Very Severe percentile LDL-c 96.5-98	96.78	6.19 ± 2.04	492
c.131G>A	p.Trp44*	Very Severe percentile LDL-c 96.5-98	96.76	6.28 ± 1.95	264
c.682G>A	p.Glu228Lys	Very Severe percentile LDL-c 96.5-98	96.70	6.30 ± 2.31	276
c.1285G>A	p.Val429Met	Severe percentile LDL-c 92-96.5	96.43	6.40 ± 2.31	81
c.889A>C	p.Asn297His	Severe percentile LDL-c 92-96.5	96.33	5.64 ± 2.01	12
c.1359-1G>A	p.?	Severe percentile LDL-c 92-96.5	96.16	6.21 ± 2.08	481
c.1865_1866AT	p.Ile623fs	Severe percentile LDL-c 92-96.5	95.99	5.93 ± 1.49	7
c.(1186+1_1187-1)_(1845+1_1846-1)dup	p.?	Severe percentile LDL-c 92-96.5	95.91	6.74 ± 2.57	33
c.660del	p.Asp221Thrfs*44	Severe percentile LDL-c 92-96.5	95.47	6.47 ± 3.51	7
c.979C>T	p.His327Tyr	Severe percentile LDL-c 92-96.5	95.28	5.43 ± 2.43	9
c.1322T>C	p.Ile441Thr	Severe percentile LDL-c 92-96.5	95.27	4.99 ± 0.93	5
c.1118G>A	p.Gly373Asp	Severe percentile LDL-c 92-96.5	95.12	5.20 ± 1.75	9
c.2179_2191dup	p.Val731Glyfs*55	Severe percentile LDL-c 92-96.5	94.89	5.96 ± 2.03	11
c.1291G>A	p.Ala431Thr	Severe percentile LDL-c 92-96.5	94.82	5.33 ± 1.57	96
c.1301C>G	p.Thr434Arg	Severe percentile LDL-c 92-96.5	94.77	5.48 ± 1.53	10
c.917C>T	p.Ser306Leu	Severe percentile LDL-c 92-96.5	94.60	5.13 ± 1.43	274
c.1069G>A	p.Glu357Lys	Severe percentile LDL-c 92-96.5	94.56	6.46 ± 2.04	28
c.305A>C	p.Gln102Pro	Severe percentile LDL-c 92-96.5	94.46	4.65 ± 1.36	13
c.14de	p.Gly5Alafs*201	Severe percentile LDL-c 92-96.5	94.44	6.41 ± 3.25	15
c.1988-1G>A	p.?	Severe percentile LDL-c 92-96.5	94.15	5.38 ± 1.79	11

Supplemental Table 1. Continued

cNomen	pNomen	Variant category (range mean pLDL-c)	Mean pLDL-c	Mean LDL-c in mmol/L ± SD	No of carriers
c.662A>G	p.Asp221Gly	Severe percentile LDL-c 92-96.5	93.89	5.66 ± 1.64	40
c.314-1G>A	p.?	Severe percentile LDL-c 92-96.5	93.67	5.86 ± 1.82	42
c.2113G>C	p.Ala705Pro	Severe percentile LDL-c 92-96.5	93.63	5.38 ± 1.80	148
c.661G>A	p.Asp221Asn	Severe percentile LDL-c 92-96.5	92.77	4.85 ± 1.95	9
c.798T>A	p.Asp266Glu	Severe percentile LDL-c 92-96.5	92.74	4.46 ± 1.00	14
c.1586+2T>C	p.?	Severe percentile LDL-c 92-96.5	92.55	5.29 ± 1.69	19
c.742T>G	p.Cys248Gly	Severe percentile LDL-c 92-96.5	92.40	5.20 ± 1.50	37
c.621C>T	p.Gly207=	Severe percentile LDL-c 92-96.5	92.38	5.16 ± 1.67	241
c.1690A>C/2397_2405del	p.Asn564His/Val800_ Leu802del	Moderate severity percentile LDL-c 88-92	91.89	4.99 ± 1.57	1135
c.2407_2424dup	p.Cys803_Leu808dup	Moderate severity percentile LDL-c 88-92	91.83	4.18 ± 1.04	7
c.763T>C	p.Cys255Arg	Moderate severity percentile LDL-c 88-92	91.69	5.24 ± 2.00	68
c.1586+5G>A	p.?	Moderate severity percentile LDL-c 88-92	91.07	4.74 ± 1.76	23
c.1784G>A	p.Arg595Gln	Moderate severity percentile LDL-c 88-92	90.83	5.91 ± 3.59	7
c.-134C>A	p.?	Moderate severity percentile LDL-c 88-92	90.62	4.69 ± 0.42	5
c.706T>C	p.Cys236Arg	Moderate severity percentile LDL-c 88-92	90.51	4.80 ± 1.04	5
c.2096C>T	p.Pro699L	Moderate severity percentile LDL-c 88-92	90.38	4.74 ± 1.35	65
c.301G>T	p.Glu101*	Moderate severity percentile LDL-c 88-92	89.89	5.53 ± 1.62	8
c.858C>A	p.Ser286Arg	Moderate severity percentile LDL-c 88-92	89.75	5.38 ± 1.96	33
c.1775G>A	p.Gly592Glu	Moderate severity percentile LDL-c 88-92	89.73	4.88 ± 1.63	111
c.148del	p.Ala50Leufs*156	Moderate severity percentile LDL-c 88-92	89.68	5.63 ± 2.15	16
c.1820A>G	p.His607Arg	Moderate severity percentile LDL-c 88-92	89.22	5.16 ± 1.94	19
c.1265T>C	p.Leu422Pro	Moderate severity percentile LDL-c 88-92	89.18	4.84 ± 1.63	65
c.268G>A	p.Asp90Asn	Moderate severity percentile LDL-c 88-92	88.99	4.34 ± 1.15	18
c.337G>A	p.Glu113Lys	Modest severity percentile LDL-c 75-88	87.67	4.02 ± 1.01	5
c.241C>T	p.Arg81Cys	Modest severity percentile LDL-c 75-88	86.57	4.48 ± 1.20	110

Supplemental Table 1. Continued

cNomen	pNomen	Variant category (range mean pLDL-c)	Mean pLDL-c	Mean LDL-c in mmol/L ± SD	No of carriers
c.1340C>G	p.Ser447Cys	Modest severity percentile LDL-c 75-88	86.38	4.52 ± 1.27	7
c.-139C>A	p.?	Modest severity percentile LDL-c 75-88	86.26	4.80 ± 1.80	19
c.1247G>A	p.Arg416Gln	Modest severity percentile LDL-c 75-88	85.81	4.07 ± 1.12	60
c.1027G>A	p.Gly343Ser	Modest severity percentile LDL-c 75-88	85.37	4.44 ± 1.54	212
c.1549T>C	p.Ser517Pro	Modest severity percentile LDL-c 75-88	84.83	4.61 ± 1.56	46
c.1217G>C	p.Arg406Pro	Modest severity percentile LDL-c 75-88	84.65	4.99 ± 1.73	6
c.1444G>A	p.Asp482Asn	Modest severity percentile LDL-c 75-88	84.51	4.25 ± 1.31	17
c.(940+1_941-1)_(1060+1_1061-1)dup	p.?	Modest severity percentile LDL-c 75-88	82.52	4.30 ± 1.30	6
c.139G>C	p.Asp47His	Modest severity percentile LDL-c 75-88	81.94	3.90 ± 1.31	7
c.1195G>A	p.Ala399Thr	Modest severity percentile LDL-c 75-88	81.78	4.27 ± 1.37	34
c.1243G>C	p.Asp415His	Modest severity percentile LDL-c 75-88	81.70	4.26 ± 1.35	23
c.1976C>A	p.Thr659Asn	Modest severity percentile LDL-c 75-88	81.69	5.04 ± 2.74	6
c.1898G>A	p.Arg633His	Modest severity percentile LDL-c 75-88	81.53	4.48 ± 1.75	62
c.2282C>T	p.Thr761Met	Modest severity percentile LDL-c 75-88*	80.65	4.90 ± 2.25	11
c.1898G>T	p.Arg633Leu	Modest severity percentile LDL-c 75-88	80.60	4.15 ± 1.22	37
c.801A>T	p.Glu267Asp	Modest severity percentile LDL-c 75-88	79.98	4.45 ± 1.64	24
c.2390_2407dup	p.Leu802_Cys803ins-LeuLeuLeuValPheLeu	Modest severity percentile	79.74	3.23 ± 0.91	11
c.1432G>A	p.Gly478Arg	Modest severity percentile LDL-c 75-88	79,55	4.52 ± 1.61	5
c.1284C>G	p.Asn428Lys	Modest severity percentile LDL-c 75-88	79,08	3.86 ± 1.20	34
c.1307T>C	p.Val436Ala	Modest severity percentile LDL-c 75-88	78.01	4.16 ± 1.47	42
c.451G>A	p.Ala151Thr	Modest severity percentile LDL-c 75-88	76.66	3.67 ± 1.08	10
c.649G>A	p.Asp217Asn	Modest severity percentile LDL-c 75-88	76.23	4.06 ± 1.00	15
c.-79C>G	p.?	Modest severity percentile LDL-c 75-88	75.16	3.63 ± 1.11	6
c.829G>A	p.Glu277Lys	Mild severity percentile LDL-c < 75*	74.63	3.83 ± 1.25	26
c.1414G>T	p.Asp472Tyr	Mild severity percentile LDL-c < 75	74.37	3.87 ± 0.75	7

Supplemental Table 1. Continued

cNomen	pNomen	Variant category (range mean pLDL-c)	Mean pLDL-c	Mean LDL-c in mmol/L ± SD	No of carriers
c.-153C>T	p.?	Mild severity percentile LDL-c < 75	73.94	3.91 ± 1.50	13
c.1783C>T	p.Arg595Trp	Mild severity percentile LDL-c < 75	73.15	3.69 ± 1.08	34
c.1504G>T	p.Asp502Tyr	Mild severity percentile LDL-c < 75	71.59	3.43 ± 1.15	8
c.343C>T	p.Arg115Cys	Mild severity percentile LDL-c < 75	68.65	3.44 ± 1.52	7
c.2140+5G>A	p.?	Mild severity percentile LDL-c < 75*	67.50	3.57 ± 0.96	12
c.542C>G	p.Pro181Arg	Mild severity percentile LDL-c < 75	67.31	3.45 ± 0.88	5
c.1519A>G	p.Lys507Glu	Mild severity percentile LDL-c < 75	66.97	3.14 ± 1.00	7
c.2479G>A	p.Val827Ile	Mild severity percentile LDL-c < 75*	65.59	3.37 ± 1.06	36
c.1085A>C	p.Asp362Ala	Mild severity percentile LDL-c < 75*	64.76	3.46 ± 1.00	55
c.644G>A	p.Arg215His	Mild severity percentile LDL-c < 75	64.57	2.68 ± 0.44	5
c.2231G>A	p.Arg744Gln	Mild severity percentile LDL-c < 75*	64.45	3.45 ± 0.97	52
c.757C>T	p.Arg253Trp	Mild severity percentile LDL-c < 75*	62.16	3.46 ± 1.42	34
c.1816G>T	p.Ala606Ser	Mild severity percentile LDL-c < 75*	61.58	3.36 ± 0.85	12
c.1825T>C	p.Phe609Leu	Mild severity percentile LDL-c < 75	61.06	3.30 ± 1.01	12
c.1057G>A	p.Glu353Lys	Mild severity percentile LDL-c < 75	60.93	3.55 ± 1.45	55
c.1966C>A	p.His656Asn	Mild severity percentile LDL-c < 75	60.73	3.61 ± 1.35	14
c.263G>A	p.Arg88Lys	Mild severity percentile LDL-c < 75	59.57	3.33 ± 1.30	5
c.1876G>A	p.Glu626Lys	Mild severity percentile LDL-c < 75*	58.90	3.26 ± 0.89	94
c.1658A>G	p.Tyr553Cys	Mild severity percentile LDL-c < 75*	58.75	3.13 ± 0.97	13
c.1761C>G	p.Ser587Arg	Mild severity percentile LDL-c < 75*	56.97	3.23 ± 1.25	38
c.108C>A	p.Asp36Glu	Mild severity percentile LDL-c < 75*	55.94	3.03 ± 0.96	30
c.58G>A	p.Gly20Arg	Mild severity percentile LDL-c < 75	55.16	3.12 ± 1.24	28
c.2101G>A	p.Gly701Ser	Mild severity percentile LDL-c < 75*	55.12	3.28 ± 0.87	14
c.2177C>T	p.Thr726Ile	Mild severity percentile LDL-c < 75*	54.02	3.07 ± 1.16	47
c.1837G>A	p.Val613Ile	Mild severity percentile LDL-c < 75	53.43	3.30 ± 1.20	10
?	p.?	Mild severity percentile LDL-c < 75*	51.95	2.91 ± 1.12	31

Supplemental Table 1. Continued

cNomen	pNomen	Variant category (range mean pLDL-c)	Mean pLDL-c	Mean LDL-c in mmol/L ± SD	No of carriers
c.211G>A	p.Gly71Arg	Mild severity percentile LDL-c < 75	50.93	3.05 ± 1.49	9
c.-127G>C	p.?	Mild severity percentile LDL-c < 75*	49.26	3.06 ± 1.08	14
c.488	p.Pro150Ser	Mild severity percentile LDL-c < 75	45.29	2.93 ± 1.57	7
c.941-4G>A	p.?	Mild severity percentile LDL-c < 75*	39.35	2.54 ± 1.00	18
c.1301C>T	p.Thr434Met	Mild severity percentile LDL-c < 75	39.10	2.81 ± 1.01	6
c.434T>C	p.Val145Ala	Mild severity percentile LDL-c < 75*	35.70	2.69 ± 0.67	6

The *LDLR* variants where at least 5 carriers have been identified in the Netherlands have been included in this table. pLDL-c: percentile LDL-c. Variants were described according to the nomenclature as proposed by den Dunnen and Antonarakis.[26] *Variants that are by now established non-pathogenic, but that were at time of initiation of genetic cascade screening assumed pathogenic.

4

NOVEL PROPROTEIN CONVERTASE SUBTILISIN KEXIN TYPE 9 VARIANTS IN FAMILIAL HYPERCHOLESTEROLEMIA PATIENTS FROM CAPE TOWN

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ABSTRACT

BACKGROUND

Familial hypercholesterolemia (FH) is characterized by elevated low density lipoprotein cholesterol (LDL-c) and markedly increased cardiovascular risk. In patients with a genetic diagnosis low-density-lipoprotein receptor (*LDLR*) mutations account for > 90% of cases, apolipoprotein B (*APOB*) mutations for approximately 5% of cases, while proprotein convertase subtilisin kexin type 9 (*PCSK9*) gain of function (GOF) mutations are rare (< 1% of cases).

OBJECTIVES

To evaluate the functional impact of several novel *PCSK9* mutations in a cohort of FH patients by genetic cascade screening and *in vitro* functionality assays.

METHODS

Patients with clinically diagnosed FH underwent genetic analysis of *LDLR*, and if negative, sequential testing of *APOB* and *PCSK9*. We analyzed cosegregation of hypercholesterolemia with novel *PCSK9* variants. GOF status was determined by *in silico* analyses and validated by *in vitro* functionality assays.

RESULTS

Among 1,055 persons with clinical FH we identified non-synonymous *PCSK9* variants in 27 (2.6%) patients and 7 of these carried one of four previously reported GOF variants. In the remaining 20 FH patients we identified 7 novel *PCSK9* variants. The G516V variant (c.1547G>T) was found in 5 index patients and cascade screening identified 15 additional carriers. LDL-c levels were higher in these 15 carriers compared to the 27 non-carriers (6.1 ± 1.9 vs 3.2 ± 0.91 mmol/L ($p < 0.001$). *In vitro* studies demonstrated the pathogenicity of the G516V mutation.

CONCLUSIONS

In our study 1.14% of cases with clinical FH were clearly attributable to pathogenic mutations in *PCSK9*. Pathogenicity is established beyond doubt for the novel G516V variant.

BACKGROUND

Familial hypercholesterolemia (FH) is a common genetic disorder characterized by autosomal dominant inheritance of hypercholesterolemia, tendon xanthomata and severely increased risk of cardiovascular disease (CVD) compared to non-FH individuals.[1-6] Most commonly a causal mutation is found in the low-density-lipoprotein receptor (*LDLR*) or apolipoprotein B (*APOB*) genes.[7] In a small proportion (< 1%) of patients proprotein convertase subtilisin kexin type 9 (*PCSK9*) gain of function (GOF) mutations are causal.[8,9]

PCSK9 GOF mutations are of interest not only for establishing an unequivocal molecular FH diagnosis, which enables genetic cascade screening, but also for improving our understanding of PCSK9 biology by allowing further study of the impact specific mutation have on PCSK9 functionality. Inhibition of PCSK9 in treatment of hypercholesterolemia has been shown to decrease CVD risk.[10,11] Identification and characterization of *PCSK9* mutations may reveal novel information regarding the way PCSK9 acts and the roles played by various epitopes, and ultimately allow for more precise targeting of inhibitory therapies.

During the last decade the public-sector lipid clinic at Groote Schuur Hospital (GSH) in Cape Town, South Africa identified seven new sequence variants of unknown significance in *PCSK9* as well as four known GOF *PCSK9* mutations in patients with phenotypic FH. Two to four of these seven novel variants are predicted to be damaging based on *in silico* analysis (Supplemental Material, Table 1), but sensitivity of the *in silico* prediction tools can be disappointing when comparing predicted pathogenicity of *in silico* models with pathogenicity based on the observed biochemical and clinical phenotype in FH cohorts identified by genetic cascade screening.[12] Given that index patients had presented with phenotypic FH, we hypothesized that the majority of these novel *PCSK9* mutations were likely to be GOF. We therefore evaluated the functional impact of these seven putatively GOF *PCSK9* variants using genetic cascade screening and *in vitro* analyses. More specifically, the aim was to establish pathogenicity by segregation analysis, showing that these mutations cosegregated in an autosomal dominant fashion with the FH phenotype. In addition, we performed *in vitro* analyses of likely pathogenic mutations to clarify the molecular mechanisms leading to PCSK9 GOF.

METHODS

PATIENT SELECTION AND ELIGIBILITY CRITERIA

The project was approved by the Human Research Ethics Committee of the University of Cape Town Health Sciences Faculty. We only included participants (index cases) who had provided written informed consent for genetic analysis and further research at their initial consultation. Participants with a clinical diagnosis of FH between 1980 and 2010 underwent genetic analysis of the *LDLR***.** If no causal *LDLR* variant was found, *APOB* and *PCSK9* were analyzed. Approximately 2,500 patients with clinical FH have been identified at the GSH lipid clinic. All patients with potential FH were reviewed by one of two expert clinicians (ADM and DJB) who evaluated patients clinically, including checking the tendons for xanthomata, and then made the diagnosis of FH using elements from both the Simon Broome and Dutch Lipid Clinics Network criteria.[1] DNA analyses had been completed in 1,055 patients by the year 2010, utilizing a strategy of screening for common founder mutations by restriction digests followed by High Resolution Melting (HRM) screening using the Light Scanner 32 (Idaho Technologies). Amplicons with variants were subjected either to restriction digests or sequencing, but MLPA to exclude large insertions or deletions was not available in Cape Town. Exons 26 and 29 of *APOB* were sequenced in case no variant was found in *LDLR*. Hereafter, *PCSK9* mutations were sought through exon by exon screening of *PCSK9*.

Clinical data was extracted from the hospital records as recently described by Van Delden and colleagues.[13] We compared the clinical phenotypes of patients with *LDLR* mutations, *APOB* mutations, known pathogenic *PCSK9* mutations and novel *PCSK9* variants of unknown significance.

In the index patients with novel *PCSK9* variants, large deletions or insertions in the *LDLR* gene were excluded by next generation sequencing at the Amsterdam University Medical Center, The Netherlands (Supplemental Material, Methods, Part 1).

If the *PCSK9* variant remained the most likely cause of FH, patients and their relatives were invited to participate in a cascade screening project. We included all consenting first-degree relatives and included traceable second-degree relatives if no first-degree relatives were available. We excluded subjects who were younger than 12 years. All participants gave written informed consent to the separate cascade screening protocol that was also approved by the University of Cape Town Faculty of Health Sciences Human Research Ethic Committee.

OUTCOME MEASURES

The primary aim was to determine the pathogenicity of the novel *PCSK9* mutations utilizing

lipid-based segregation analysis. We defined an unequivocal difference in low-density lipoprotein-cholesterol (LDL-c) as a level more than 1.0 mmol/L higher in carriers than in non-carriers. This 1.0 mmol/L threshold has previously been used to establish definite pathogenicity of *LDLR* and *APOB* variants,[14] with subsequent validation of CVD risk.[12]

We also assessed the effect of the *PCSK9* variants on CVD risk, based on CVD event rates in index patients with *PCSK9* variants compared to index patients with either *LDLR* or *APOB* mutations. In addition, we measured PCSK9 plasma levels as previously described.[15] Total PCSK9 concentrations were determined using the Quantikine SPC900 ELISA (R&D Systems, Lille, France).

IN VITRO FUNCTIONALITY ANALYSIS OF *PCSK9* MUTATION OF INTEREST

PCSK9 variants functionality assays in HEK 293 cells

The synthesis, secretion and impact of the *PCSK9* mutations on LDLR cell surface expression and LDL particle cellular uptake was studied, as described previously.[16-18] Briefly, plasmids allowing expression of wild-type and the two PCSK9 variants D374Y and G516V were transiently transfected into HEK293 cells. LDLR cell surface expression was measured by flow cytometry 48 hours post transfection. LDL particle uptake was determined by flow cytometry 48 hours after transfection with the different plasmids and after 4 hours of incubation with FITC-LDL (Supplemental Material, Methods, Part 2).

PCSK9 variant functionality assays in HepG2 cells

Stably transfected HEK293cells were grown to subconfluernce (80% coverage), the medium was harvested and PCSK9 was purified. Expression of PCSK9 was detected in cell lysates and media of stably transfected HEK293 cells by SDS-PAGE. Each purified PCSK9 variant was added to the culture medium of HepG2 cells and LDL particle uptake was determined.[17-20] (Supplemental Material, Methods, Part 3).

Solid-phase immunoassay for PCSK9-LDLR ectodomain binding

This method, using previously described techniques, is shown in Supplemental Material, Methods Part 4.[17]

LDLR expression in lymphocytes in mutation carriers and controls

Lymphocyte LDLR expression profiles were performed, as described before.[21] To assess whether the FH phenotype in G516V carriers could be due to potentially undetected *LDLR* genetic defects, a series of LDLR cell surface expression experiments was undertaken in primary lymphocytes isolated from a subset of patients with PCSK9-G516V as well as non-affected family members, as described previously (Supplemental Methods, Part 5).[21,22]

STATISTICAL ANALYSES

We compared CVD event-free survival Kaplan Meier curves from carriers and controls between the index patients with *PCSK9* mutations versus those with *LDLR* mutations or *APOB* mutations by Kaplan Meier analysis with log rank test (unadjusted) and subsequently with Cox-proportional hazard analysis, with adjustment for male gender, age, diabetes, hypertension, smoking status and body mass index. Segregation analysis of mutations with the lipid profile was used to analyze the link between mutations and FH, as described in a similar segregation project on FH genetic cascade screening.[23] Dichotomous variables were compared utilizing the Chi-Square test. Differences in continuous variables with a normal distribution were compared between mutation carriers and unaffected relatives with a two-sided independent Student's *t*-test.

To account for large differences in age, gender and BMI distributions, the LDL-c concentrations between carriers and non-carriers of a specific mutation, were analyzed using multiple linear regression. We estimated the mean LDL-c using the generalized estimating equation method to account for correlations within families and adjusted for the confounders age, gender and BMI.

Due to the fact that during genetic cascade testing not all first degree relatives can be screened the chance of finding mutation carriers is less than 50%.[24] As reported in a previous project to determine pathogenicity of FH mutations,[12,14] we estimated that ~35% of screened relatives would be mutation-positive. With a sample size of 18 carriers and 32 unaffected relatives and an assumed LDL-c standard deviation of 1.1 mmol/L, there is sufficient power (80%) to detect a more than 1.0 mmol/L difference in mean LDL-c between the two groups (two sided, with $\alpha = 0.05$ and $\beta = 0.20$). We did not anticipate identifying 50 relatives for each mutation, and therefore, we determined that a mean difference of more than 1.0 mmol/L in a group of relatives smaller than 50 would be enough to categorize that variant as pathogenic. Plasma PCSK9 levels were compared using a two-sided independent Student's *t*-test. All analyses were performed with SPSS software (version 25). The family trees were drawn using PROGENY software version 10.4.2.0.

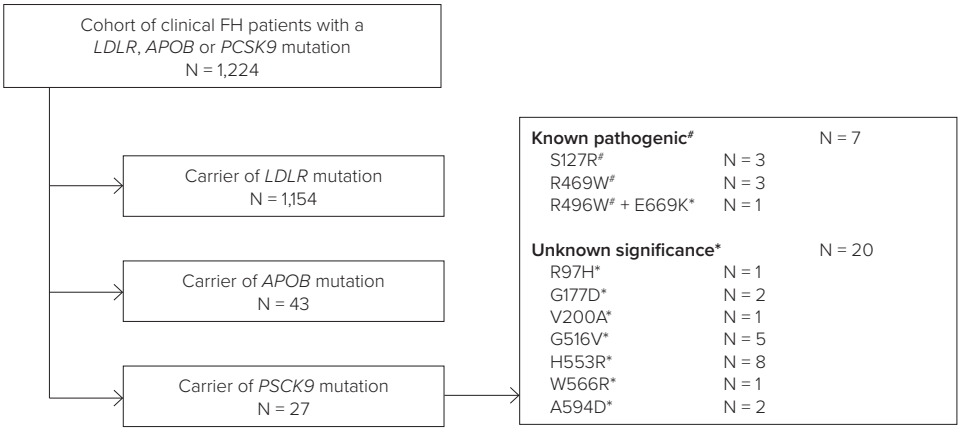
RESULTS

PATHOGENIC VARIANTS IN *LDLR*, *APOB* AND *PCSK9*

By 2010 genetic testing had been performed in 1,055 patients with clinical FH. Pathogenic *LDLR* mutation were identified in 661 (63%) and *APOB* mutations in 14 (1.3%) patients. No mutation was found in 357 (34%) patients. A non-synonymous *PCSK9* mutation was identified

in 27 patients (2.6%). Subsequent opportunistic screening in new clinical FH index cases seen after 2010 was limited to testing for the most prevalent *LDLR* and *APOB* mutations. Following this further screening the total number of mutation positive FH patients was 1,224 (Figure 1). *LDLR* and *APOB* mutations were identified in 1,154 and 43 patients, respectively. The number of patients with a *PCSK9* mutation remained at 27 as ongoing systematic screening was not possible.

Figure 1. Flow diagram of assumed pathogenic FH mutations found in the three FH genes in Groote Schuur Hospital in Cape Town during the last three decades



Seven patients were found to be heterozygous carriers of one of three previously published GOF *PCSK9* mutations.[8,9] Three patients carried the S127R mutation (c.381T >A)[8,16] and three the R469W mutation (c.1405C>T).[9,25,26] One patient carried two variants: the pathogenic R496W (c.1486C>T) mutation and the novel E669K (c.2005G>A) variant.[9] This female had clear phenotypic FH, but we were unable to trace and study her relatives (Supplemental Material, Clinical phenotype novel *PCSK9* variants, part 1). The seven patients with at least one known GOF *PCSK9* mutation had non-significantly higher untreated LDL-c levels (mean ± standard deviation) of 8.84 ± 2.84 mmol/L than carriers of *LDLR* mutations (7.88 ± 1.93 mmol/L) or *APOB* mutations (6.66 ± 1.85 mmol/L), respectively (Supplemental Material, Table 2). The event free survival (95% confidence interval) was not significantly different between carriers of *PCSK9*, *LDLR* and *APOB* mutations, but a trend with known pathogenic GOF *PCSK9* mutations showing worse event free survival was observed at 49 (35-62), 55 (53-56) and 60 (56-64) years, respectively (Supplemental Material, Figure 1).

NOVEL *PCSK9* VARIANTS OF UNCERTAIN SIGNIFICANCE

The remaining twenty FH patients were heterozygous carriers of one of seven *PCSK9* variants of uncertain significance. These non-synonymous variants were: R97H (c.290G>A), Gly177D (c.530G>A), V200A (c.599T>C), G516V (c.1547G>T), H553R (c.1658A>G), W556R (c.1696T>C) and A594D (c.1781C>A).

These seven variants of unknown significance result in single amino acid substitutions, either within the prodomain (R97H), the catalytic domain (G177D, V200A) or the C-terminal domain (G516V, H553R, W566R, A594D) of the PCSK9 protein. We analyzed the potential consequence of these variants with the *in silico* prediction program Polyphen and only the G177D, V200A and G516V variants were classified as "probably damaging", which was only confirmed for the G177D and G516V variants using the Provean predictor. In addition, only the amino acids at position 200 (V) and 516 (G) are highly conserved among species (Supplemental Material, Table 1).

In the cohort of 20 FH patients that carried one of seven *PCSK9* variants of uncertain significance no *LDLR* insertions or deletions were identified upon next generation sequencing, suggesting that these *PCSK9* variants were the most likely explanation for the FH phenotype.

These 20 patients had a mean event free survival of 57 (51-63) years, which was not statistically significantly different from 1,197 heterozygous carriers of a pathogenic *LDLR* or *APOB* mutation (Supplemental Material, Figure 1). Mean LDL-c in these 20 patients was numerically lower than the mean LDL-c in patients with *APOB* and *LDLR* mutations, but none of the differences was statistically significant (Supplemental Material: Table 2 and Figure 1). These observations based on clinical data from index patients carrying one of the seven different *PCSK9* variants of uncertain significance do not permit conclusions on pathogenicity due to the small sample size.

We next performed cascade screening in families with *PCSK9* mutations of uncertain significance. Unfortunately, neither the index patient who was a heterozygous carrier of the V200A variant nor her family could be traced. The female index patient had a clear FH phenotype (Supplemental Material, Clinical phenotype novel PCSK9 variants, Part 2). A total of 50 carriers and 65 non carriers of *PCSK9* variants were identified upon cascade screening within the families of the other six index cases with *PCSK9* variants. The clinical characteristics are shown in Table 1. The mean LDL-c level in carriers of the six *PCSK9* variants (4.4 ± 1.9 mmol/L) was significantly higher compared to controls (3.4 ± 1.1 mmol/L, p = 0.001). Moreover, tendon xanthomas were more prevalent among carriers of the six

PCSK9 variants (15 (30%)) compared to controls (6 (9%), p = 0.013)). The most extensive cascade screening was possible in the families of index cases carrying the G516V variant where data was collected from 42 persons. In the H566R families, G177D families, and R97H family 23, 19 and 14 individuals were enrolled in the current study, respectively. For the single families with the A594D and W566R variants we were able to test eight and seven persons, respectively (Table 2). Analyzing LDL-c differences using generalized estimating equations revealed that the differences between carriers and non-carriers exceeded 1.0 mmol/l only for the G516V mutation. For the other variants the segregation analysis was equivocal, or suggestive of non-pathogenicity (Table 3 and Supplemental Material, Clinical phenotype novel *PCSK9* variants, Parts 3-7).

Table 1. Clinical characteristics of index patients with *PCSK9* mutations of unknown significance and relatives studied with genetic cascade screening

	Index patients			**Tested relatives**		
	All	H553R	G516V	*PCSK9* mutation		
				carrier	control	P-value
Number of persons	20	8	5	50	65	
Male gender (%)	4 (20)	2 (25)	1 (20)	23 (46)	28 (43)	NS
Age ± SD *years*	53 ± 17	51 ± 20	46 ± 23	43 ± 16	46 ± 20	NS
BMI ± SD *kg/m2*	30 ± 6.4	33 ± 6.9	26 ± 3.6	30 ± 7.0	29 ± 7.0	NS
Height ± SD *cm*	161 ± 9.3	160 ± 9.1	164 ± 13	164 ± 11	163 ± 13	NS
Weight ± SD *kg*	77 ± 19	85 ± 25	70 ± 12	79 ± 21	79 ± 21	NS
Hypertension (%)	10 (50)	4 (50)	1 (20)	19 (38)	23 (35)	NS
Diabetes mellitus (%)	4 (20)	0 (0)	1 (20)	15 (30)	10 (15)	0.060
Smoker never (%)	8 (40)	3 (38)	3 (60)	20 (40)	32 (49)	NS
Symptomatic CVD (%)	11 (55)	4 (50)	1 (20)	11 (22)	8 (12)	NS
Blood pressure systolic ± SD *mmHg*	150 ± 32	165 ± 28	146 ± 31	124 ± 17	123 ± 15	NS
Blood pressure diastolic ± SD *mmHg*	88 ± 12	91 ± 14	86 ± 16	76 ± 11	76 ± 11	NS
Tendon xanthoma (%)	17 (85)	6 (75)	4 (80)	15 (30)	6 (9)	0.007
TC pre-treatment ± SD *mmol/L*	8.9 ± 1.5	9.0 ± 1.6	8.3 ± 1.0	7.4 ± 1.5	5.8 ± 1.0	NS
LDL-c pre-treatment ± SD *mmol/L*	6.5 ± 1.3	6.3 ± 1.4	6.3 ± 0.55	4.4 ± 1.9	3.4 ± 1.1	0.001
Lipid lowering treatment (%)	6 (30)	1 (13)	3 (60)	22 (44)	18 (27)	0.069
TC at study visit ± SD *mmol/L*	7.7 ± 2.0	8.4 ± 1.6	5.2 ± 0.67	5.4 ± 1.5	5.0 ± 1.1	NS
LDL-c at study visit ± SD *mmol/L*	5.4 ± 1.7	6.3 ± 1.4	3.3 ± 0.23	3.4 ± 1.3	3.0 ± 0.96	NS
HDL-c at study visit ± SD *mmol/L*	1.4 ± 0.50	1.4 ± 0.64	1.3 ± 0.23	1.3 ± 0.36	1.3 ± 0.40	NS
Triglycerides at study visit ± SD *mmol/L*	2.3 ± 2.0	1.8 ± 1.0	1.3 ± 0.43	1.5 ± 0.97	1.3 ± 0.82	NS
PCSK9 plasma levels ± SD ng/ml				248 ± 89	253 ± 78	NS

SD: standard deviation, BMI: body mass index, CVD: cardiovascular disease, TC: total cholesterol, LDL-c: low-density lipoprotein cholesterol, HDL-c: high-density lipoprotein cholesterol, PCSK9: proprotein convertase subtilisin kexin type 9, Lp(a): lipoprotein (a)

Plasma PCSK9 levels were measured in 111 participants. The PCSK9 levels were similar for the 49 carriers (248 ± 89 ng/mL) and the 62 unaffected controls (253 ± 62 ng/ml, p = 0.38) (Table 1). Irrespective of the family tested, the levels of circulating PCSK9 were

Table 2. Characteristics of the relatives of index patients studied with genetic cascade screening for presence of *PCSK9* mutations of unknown significance

	Type of *PCSK9* mutation of unknown significance residing in their family tested for											
	R97H		G177D		G516V		H553R		W566R		A594D	
	Carrier	Control	Carrier	Control	Carrier	Control	Carrier	Control	Carrier	Control	Carrier	Control
Number of persons	9	5	11	8	15	27	8	15	2	5	5	3
Male gender (%)	5 (56)*[a]	0 (0)	5 (45)	4 (50)	6 (40)	16 (59)	3 (38)	6 (40)	1 (50)	1 (20)	3 (60)	1 (33)
Age ± SD *years*	42 ± 16	45 ± 20	34 ± 17	36 ± 23	49 ± 17	57 ± 16	50 ± 9.5	44 ± 12	58 ± 2.4	50 ± 8.2	33 ± 13*[j]	16 ± 3.1
BMI ± SD *kg/m2*	27 ± 5.8	28 ± 8.9	27 ± 9.1	29 ± 11	31 ± 5.5	31 ± 4.9	31 ± 6.1	28 ± 5.6	28 ± 5.3	34 ± 3.5	31 ± 9.7	23 ± 8.6
Height ± SD *cm*	160 ± 13	152 ± 2.6	167 ± 7.1	165 ± 8.2	164 ± 12	169 ± 10	163 ± 9.7	164 ± 8.1	161 ± 11	160 ± 8.3	164 ± 16	162 ± 10
Weight ± SD *kg*	70 ± 12	65 ± 20	77 ± 28	80 ± 29	85 ± 22	88 ± 13	84 ± 18	75 ± 15	72 ± 3.5	88 ± 9.0	81 ± 20	61 ± 21
Hypertension (%)	5 (56)	1 (20)	3 (27)	2 (25)	5 (33)	11 (41)	5 (63)	6 (40)	1 (50)	3 (60)	0 (0)	0 (0)
Diabetes mellitus (%)	3 (33)	0 (0)	1 (9.1)	0 (0)	5 (33)	9 (33)	2 (25)	1 (6.7	1 (50)	0 (0)	3 (60)	0 (0)
Smoker never (%)	3 (33)	2 (40)	8 (73)	5 (63)	6 (40)	12 (44)	2 (25)*[e]	5 (33)	0 (0) *[j]	4 (80)	1 (20)	2 (67)
Symptomatic CVD (%)	2 (22)	1 (20)	2 (18)	1 (13	2 (13)	7 (26)	2 (25)	2 (13)	0 (0)	0 (0)	0 (0)	0 (0)
Blood pressure systolic ± SD *mmHg*	128 ± 11	124 ± 15	110 ± 13	116 ± 13	131 ± 16	127 ± 14	135 ± 16*[f]	118 ± 15	125 ± 7.0	137 ± 7.5	105 ± 16	105 ± 13
Blood pressure diastolic ± SD *mmHg*	77 ± 12	78 ± 8.4	69 ± 11	68 ± 13	78 ± 7.9	78 ± 12	83 ± 9.6	76 ± 9.7	75 ± 7.0	82 ± 7.5	68 ± 15	63 ± 5.8
Tendon xanthoma (%)	3 (33)	1 (20)	3 (27)	2 (25)	6 (40)*[a]	2 (7.4)	2 (25)*[i]	0 (0)	1 (50)	1 (20)	0 (0)	0 (0)
TC pre-treat ± SD *mmol/L*	6.2 ± 1.2*[b]	4.8 ± 0.1	5.6 ± 0.5	5.5 ± 1.1	8.3 ± 1.3*[c]	5.8 ± 1.2	7.4 ± 0.8*[b]	6.0 ± 0.59	-	6.1	4.3 ± 0.4	3.3 ± 0.55
LDL-c pre-treat ± SD *mmol/L*	3.7 ± 1.4	3.1 ± 0.66	3.6 ± 1.6	3.2 ± 0.95	6.1 ± 1.9*[c]	3.2 ± 0.91	4.9 ± 1.4	4.2 ± 1.3	3.8 ± 0.81	3.7 ± 1.3	2.4 ± 0.56	1.8 ± 0.37
Lipid lowering treatment (%)	3 (33)	1 (20)	2 (18)	2 (25)	10 (67)*[a]	9 (33)	6 (75)[g]	5 (33)	1 (50)	1 (20)	0 (0)	0 (0)
TC pre-treatment ± SD *mmol/L*	4.7 ± 1.05	4.8 ± 0.88	5.0 ± 0.84	4.6 ± 0.63	6.5 ± 2.0*[c]	4.7 ± 0.55	5.6 ± 1.0	5.8 ± 1.0	5.0 ± 0.15	5.3 ± 2.0	4.3 ± 0.4	3.3 ± 0.55
LDL-c pre-treat ± SD *mmol/L*	3.0 ± 1.0	3.1 ± 0.66	3.1 ± 0.97	2.7 ± 0.55	4.3 ± 1.7*[c]	2.7 ± 0.55	3.6 ± 0.97	3.7 ± 1.2	3.1 ± 0.06	3.5 ± 1.3	2.4 ± 0.56	1.8 ± 0.38
Lipid lowering treatment (%)	1.2 ± 0.29	1.3 ± 0.19	1.3 ± 0.35	1.2 ± 0.32	1.4 ± 0.47	1.2 ± 0.35	1.1 ± 0.2*[h]	1.6 ± 0.55	1.2 ± 0.26	1.0 ± 0.19	1.1 ± 0.19	1.2 ± 0.24
TC study visit ± SD *mmol/L*	1.3 ± 0.62	0.97 ± 0.6	1.2 ± 0.95	1.4 ± 0.96	1.6 ± 1.2	1.7 ± 1.08	1.9 ± 0.8*[i]	1.2 ± 0.52	1.4 ± 1.0	1.6 ± 1.5	1.89 ± 1.1	0.54 ± 0.1
PCSK9 plasma levels ± SD *ng/ml*	300 ± 72	254 ± 40	266 ± 91	234 ± 58	218 ± 45[d]	239 ± 81	305 ± 86	299 ± 83	84 ± 17*[k]	241 ± 84	162 ± 45	211 ± 48
Lp(a) (interquartile range) *mg/l*	48 (25-155)	83 (31-165)	5 (5-31)	25 (14-63)	54 (41-83)	37 (16-65)	70 (14-183)	106 (80-162)	49 (16-82)	47 (26-117)	10 (7-52)	65 (5-101)

SD: standard deviation, BMI: body mass index, CVD: cardiovascular disease, TC: total cholesterol, LDL-c: low-density lipoprotein cholesterol, PCSK9: proprotein convertase subtilisin kexin type 9, Lp(a): lipoprotein (a)

* = significant *p*-value; a = 0.038;b = 0.009;c < 0.001;d = 0.066;e = 0.009;f = 0.025;g = 0.057;h = 0.005;i = 0.013; j = 0.030; k = 0.070;l = 0.004

similar or slightly lower in carriers of the *PCSK9* mutations of unknown significance than in non-carriers, ruling out the possibility that their FH phenotype might have resulted from higher plasma PCSK9 levels (Supplemental Material, PCSK9 plasma levels).

Table 3. Calculated mean pre-treatment LDL-c, corrected for age, gender and BMI

	Novel *PCSK9* variant	
	Present	Absent
R97H	3.68 ± 1.39	3.11 ± 0.66
G177D	3.57 ± 1.56	3.22 ± 0.95
G516V	6.12 ± 1,94	3.24 ± 0.91
H553R	4.86 ± 1.38	4.24 ± 1.28
W566R	3.77 ± 0.81	3.73 ± 1.35
A594D	2.42 ± 0.56	1.85 ± 0.37

LDL-c: low-density lipoprotein cholesterol, PCSK9: proprotein convertase subtilisin kexin type 9

LDL-c in mmol/L ± standard deviation

G516V: A TRUE GAIN OF FUNCTION *PCSK9* VARIANT

The *in silico* predictions and LDL-c level differences between carriers and controls indicate that PCSK9-G516V is likely to be GOF. The G516V mutation was found in 5 index patients and cascade testing revealed another 15 carriers. The LDL-c plasma levels were higher and tendon xanthoma were more prevalent in these 15 carriers as compared to their 27 unaffected relatives, with LDL-c plasma levels of 6.1 ± 1.9 vs 3.2 ± 0.91 mmol/L, ($p < 0.001$) and xanthoma prevalence of 40% vs 7% ($p = 0.038$) (Table 2). The largest family tree with the most persons identified with the G516V mutation is depicted in Figure 2. The other family tree of a large family where the G516V mutation was found is shown in Supplemental Material, Figure 2.

To definitely ascertain that the LDLR is expressed normally in PCSK9-G516V carriers, we assessed LDLR expression at the surface of their lymphocytes.[18,24,27] LDLR expression from five PCSK9-G516V carriers did not differ from that found in one unaffected relative (Supplemental Material, Figure 3). Mevastatin increased, whereas recombinant PCSK9 reduced the abundance of the LDLR at the surface of lymphocytes, in a similar fashion for mutation carriers and the non-carrier relative, and fully in line with LDLR expression observed in previous studies.[22,23,28]

To establish a causative association between the novel G516V mutant and the clinical phenotype, we generated expression vectors for PCSK9-G516V, PCSK9-D374Y (a well-established *PCSK9* GOF variant), and PCSK9-wild type (WT) to stably transfect HEK293 cells. The levels of expression, autocatalytic cleavage, and secretion for WT, D374Y and G516V

variants were similar (Supplemental Material, Figure 4). Compared with mock-transfected cells, HEK293 cells expressing PCSK9-WT showed a 39± 4% reduction of LDLR cell surface expression. In HEK293 cells expressing the PCSK9-D374Y variant the LDLR cell surface expression was even lower (-75 ± 5%, $p < 0.05$ vs. wild-type). In HEK293 cells expressing the novel PCSK9-G516V variant LDLR cell surface expression was also reduced (-58 ± 4%, $p < 0.05$ vs. wild-type) (Supplemental Material, Figure 5A). Likewise, compared with mock-transfected cells, fluorescent LDL uptake was reduced in cells expressing PCSK9-WT (-37 ± 4%), and further reduced in cells expressing either PCSK9-D374Y (-86 ± 5%, $p < 0.05$ vs. wild-type) or PCSK9-G516V (-53 ± 5%, $p < 0.05$ vs. wild-type) (Supplemental Material, Figure 5B).

Using recombinant WT, D374Y and G516V PCSK9 isolated and purified from culture media of HEK293 cells, we evaluated the effects of those PCSK9 variants on LDLR cell surface expression and fluorescent LDL particle uptake in HepG2 human hepatoma cells. Compared with mock treated cells, HepG2 cells incubated with PCSK9-WT had 24 ± 6% reduced LDLR cell surface expression, and those treated with PCSK9-D374Y or PCSK9-G516V had further reduced LDLR expression at their surface (-68 ± 6% and -49 ± 3%, respectively, $p < 0.05$ vs. wild-type) (Supplemental Material, Figure 5A). Likewise, compared with mock treated cells, the uptake of fluorescent LDL was reduced in HepG2 cells treated with wild-type PCSK9 (-24 ± 7%), and further reduced in HepG2 cells incubated with PCSK9-D374Y or PCSK9-G516V (-68 ± 10% and -41 ± 3%, respectively, $p < 0.05$ vs. wild-type) (Supplemental Material, Figure 5B).

We next tested the binding affinity of wild-type PCSK9, PCSK9-D374Y and PCSK9-G516V to the extracellular domain of the LDLR at pH 7.2 (found at the cell surface) and at pH 5.5 (found in endosomes).[17] The half maximal effective concentration (EC_{50}) for wild type PCSK9 at pH 7.2 and 5.5 was 101.6 ± 10.1 nM and 16.33 ± 2.80 nM, respectively and in agreement with previous reports.[28] The PCSK9-D374Y variant displayed higher affinities for the LDLR than wild-type PCSK9, shown by the strikingly reduced EC_{50} of 19.3 ± 9.4 nM and 7.4 ± 1.7 nM, at pH 7.2 and 5.5, respectively. The PCSK9-G516V variant also showed higher affinities for the LDLR than wild-type PCSK9, with respective EC_{50} of 52.1 ± 4.4 nM and 10.1 ± 0.4 nM (Table 4).

Taken together with the clinical phenotype these in-vitro studies confirm that PCSK9-G516V qualifies as a genuine GOF mutation.

Figure 2. Family tree: Association of features of FH in the largest family with the G516V mutation in *PCSK9*

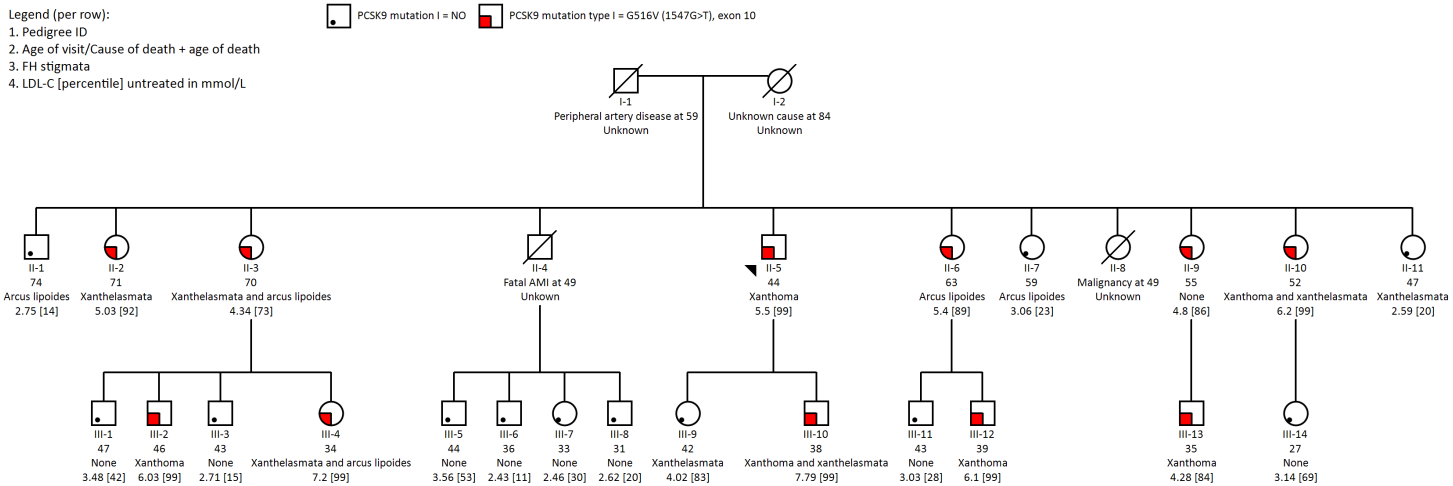


Table 4. EC50 values for the binding of PCSK9 variants to the LDLR determined by solid-phase immunoassay at pH 7.2 and 5.5

	EC50 (nM)	
	pH 7.2	pH 5.5
Wild-Type	101.6 ± 10.1	16.33 ± 2.8
D374Y	19.3 ± 9.4*	7.4 ± 1.7*
G516V	52.1 ± 4.4*	10.1 ± 0.4*

EC50: half maximal effective concentration in nanomolar, * = significantly different from wild type

DISCUSSION

In this study we evaluated the prevalence and clinical consequences of novel *PCSK9* variants in a large cohort of FH patients. The prevalence of heterozygosity for *PCSK9* variants was shown to be 2.6% and we identified a total of seven novel variants. One of these novel variants, the G516V (c.1547G>T) was found to be pathogenic beyond doubt by both segregation analysis and *in vitro* studies. Heterozygous carriers of this variant were found to be characterized by almost twofold higher LDL-c levels compared to family controls. Furthermore, FH stigmata were more prevalent in carriers than in their unaffected relatives. Our studies suggested that five other *PCSK9* variants of unknown significance, where we were able to perform cascade screening, did not have a large impact on LDL-c metabolism in families.

The exact underlying mechanism by which some of the earlier described *PCSK9* variants result in increased LDL-c levels is not fully elucidated.[29] In the current study, however, we unequivocally showed that the G516V variant translates to a proprotein with increased affinity for the LDLR, and thus likely prevents the dissociation of the LDLR from LDL-c particles following internalization of the LDLR-LDL complex in the hepatocyte. This variant at position 516 is located within the M1 module of PCSK9 C terminal domain. Hypothetically, PCSK9-G516V may result in an increased affinity for cyclase-associated protein 1, the protein that, once bound to PCSK9 mediates caveolae-dependent endocytosis and lysosomal degradation of the LDLR.[30] This particular characteristic has been shown for the PCSK9-A514T variant, which, by virtue of this effect, is considered a gain of function (GOF) variant.[31]

The G516V mutation was identified in five different families in the Cape Town area, all with mixed ethnic ancestry commonly labelled as ‘Coloured’ in South Africa. South Africa is populated by people from diverse populations and regional admixtures. The ‘Coloured’ population is descended not only from the Khoisan the original inhabitants of this part of South Africa, but also from European, Malay, southeast Asian and black Africans.[32] This population comprises approximately 9% of South Africans, but is most concentrated around

Cape Town. We could not identify a common ancestor amongst the families with the G516V mutation, as we were not able to conduct detailed genealogical studies reaching back multiple generations.

Our study has several limitations. First, we had incomplete family tracing, most markedly for the V200A (c.599T>C) variant in *PCSK9***.** Based on *in silico* analyses this variant is a likely pathogenic variant, but we could not trace neither the index nor her relatives. We were fortunate that large families with the G516V mutation (Figure 2 and Supplemental Material, Figure 2), the R97H variant and H553R variant agreed to participate (Supplemental Material, Clinical phenotype novel *PCSK9* variants, Part 3 and Parts 5). The sample size was small for some *PCSK9* variants, such as W556R (c.1696T>C) and A594D (c.1781C>A), and we therefore cannot draw firm conclusions on pathogenicity on those variants based on our observations. In total we were able to further evaluate the clinical impact of the 7 newly identified *PCSK9* variants in 19 of the 20 carriers. The novel variant was unequivocally pathogenic (G516V) in only 25% of carriers, emphasizing the importance of further evaluating novel variants before assuming pathogenicity.

However, pathogenic *PCSK9* mutations were prevalent enough in the Cape Town region to justify genetic screening for *PCSK9* mutations in case no mutation is found in *LDLR* or *APOB*. In fact, proven pathogenic *PCSK9* mutations were almost as prevalent as *APOB* mutations in the Cape Town region, with 12 patients found to carry a genuine pathogenic *PCSK9* mutation versus 14 carrying mutations in *APOB*. Among FH patients with an identified pathogenic FH mutation the prevalence of *PCSK9* mutations was 1.7%, i.e. 12 out of 688 FH patients.

A second limitation is that the study physicians (RH and MH) may have over-diagnosed tendon xanthomata during cascade screening. The study physicians were not aware of lipid levels and genetic information at the time of physical examination. Seven percent of the relatives not carrying the pathogenic G516V mutation were considered to have tendon xanthomata (Table 2). A potential solution would have been to measure Achilles tendons by an imaging techniques, e.g. by ultrasound,[33] for more objective confirmation, but this was not feasible as most relatives were evaluated in their homes.

IMPLICATIONS OF FINDINGS

In a large cohort of patients with clinical FH, *PCSK9* mutations were found in 2.6% of cases but pathogenicity was established only for one novel mutation (G516V) by clinical and laboratory studies. Analyzing *PCSK9* contributes to the molecular diagnosis in South African FH patients without *LDLR* or *APOB* mutations.

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5

A DEEP INTRONIC VARIANT IN *LDLR* IN FAMILIAL HYPERCHOLESTEROLEMIA: TIME TO WIDEN THE SCOPE?

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ABSTRACT

AIM

Familial hypercholesterolemia (FH) is an inherited disorder characterized by high plasma low density lipoprotein cholesterol (LDL-c) levels. The vast majority of FH patients carry a mutation in the coding region of *LDLR*, *APOB* or *PCSK9*. We set out to identify the culprit genetic defect in a large family with clinical FH, in whom no mutation was identified in the coding regions of these FH genes.

METHODS

Whole genome sequencing was performed in five affected and four unaffected individuals from a family with an unexplained autosomal dominant FH trait. The effect on splicing of the identified novel intronic *LDLR* mutation was ascertained by cDNA sequencing. The prevalence of the novel variant was assessed in 1,245 FH patients without a FH causing mutation identified by Sanger sequencing and in 2,154 patients referred for FH analysis by next-generation sequencing (covering the intronic region).

RESULTS

A novel deep intronic variant in *LDLR* (c.2140+103G>T) was found to cosegregate with high LDL-c in five patients, but not in four unaffected family members. The variant was shown to result in a 97 nucleotides insertion leading to a frameshift and premature stop codon in exon 15 of *LDLR*. The prevalence of the intronic variant was 0.24% (3/1245) in a cohort of FH patients without a known FH causing mutation and 0.23% (5/2154) in a population of FH patients referred for analysis by next-generation sequencing. Cosegregation analysis of a second family showed full penetrance of the novel variant with the FH phenotype over three generations.

CONCLUSIONS

The c.2140+103G>T mutation in *LDLR* is a novel intronic variant identified in FH that cosegregates with the FH phenotype. Our findings underline the need to analyze the intronic regions of *LDLR* in patients with FH, especially those in whom no mutation is found in the coding regions of *LDLR*, *APOB*, or *PCSK9*.

INTRODUCTION

Familial hypercholesterolemia (FH), a common inherited dominant disorder with a prevalence of 1:250, is characterized by high plasma levels of low density lipoprotein cholesterol (LDL-c).[1] Lifelong exposure to elevated LDL-c levels leads to an increased risk for cardiovascular disease (CVD), with an estimated odds ratio (OR) of 2.2-25.8.[2,3] Patients are diagnosed with FH based on clinical criteria and subsequent genetic analysis is confirmative in a large proportion of FH patients. The Dutch Lipid Network Criteria (DLNC), which encompasses data about family and medical history, the presence of tendon xanthoma, corneal arcus, and LDL-c levels are widely used in clinic. For molecular confirmation the genes encoding low-density lipoprotein receptor (*LDLR*, FH1), apolipoprotein B (*APOB*, FH2), or proprotein convertase subtilisin/kexin type 9 (*PCSK9*, FH3) are analyzed for the presence of pathogenic mutations. However, no mutations are found in the coding regions of these genes in approximately 5-10% of the patients with a high clinical FH score,[4] and these are commonly referred to as 'FH4'.

Variants in the non-coding region of the *LDLR* gene have been found in a small number of FH patients.[5] These variants result in either absent or decreased levels of the LDLR protein by virtue of their effect on splicing of *LDLR* mRNA. Adequate splicing of mRNA is dependent on the recognition of the exon-intron boundaries by the spliceosome[6] and involves recognition of particular signal sequences of nucleotides in pre-messenger RNA (pre-mRNA). Mutations in the signal sequences can abolish or weaken splice sites, or activate cryptic splice sites. Depending on the position of the variant, this can lead to either abnormal or alternative splicing of the pre-mRNA that might lead to abolished or altered and often non-functional proteins. Most aberrant spliced transcripts enter the nonsense-mediated mRNA decay (NMD) pathway, resulting in elimination of the transcript.[7]

The clinical relevance of deep intronic variants in FH is widely unknown. Here we report a novel deep intronic variant in *LDLR* that leads to abnormal splicing and cosegregates with high LDL-c. Furthermore, we analyzed the prevalence and clinical consequence of this newly identified variant in a large cohort of patients, referred for genetic FH analysis. This novel variant was identified in 3 out of 1,245 FH4 patients, underscoring the need for whole *LDLR* gene sequencing, including introns, in FH4 patients.

METHODS

The data, analytic methods, and study materials will not be made available to other researchers for purposes of reproducing the results or replicating the procedure, since privacy legislation does not allow sharing genetic and clinical data of the participants in this study. The study was approved by the Institutional Review Board at the Academic Medical Center in Amsterdam, and all subjects gave written informed consent prior to participation in this study. Detailed methods are available in the Online Data Supplement.

RESULTS

Whole genome sequencing of DNA derived from nine subjects from family 1 retrieved 190 rare (exonic and intronic) variants, which were present in the affected, but not in the unaffected family members (Table 1 in the Online Data Supplement). Manual exploration of these 190 variants identified a rare heterozygous single base pair substitution in intron 14 of *LDLR* (c.2140+103G>T, Chr19(GRCh37):g.11231301G>T) which was present in all family members with the FH phenotype, but not in the unaffected family members (Figure 1; family 1). This variant was of special interest since it was present in one of the major FH causing genes (i.e. *LDLR*). TaqMan analysis of the DNA of one additional affected family member, who was not included in the original whole genome sequencing analysis (ID 20), confirmed the presence of the newly identified rare variant in *LDLR*.

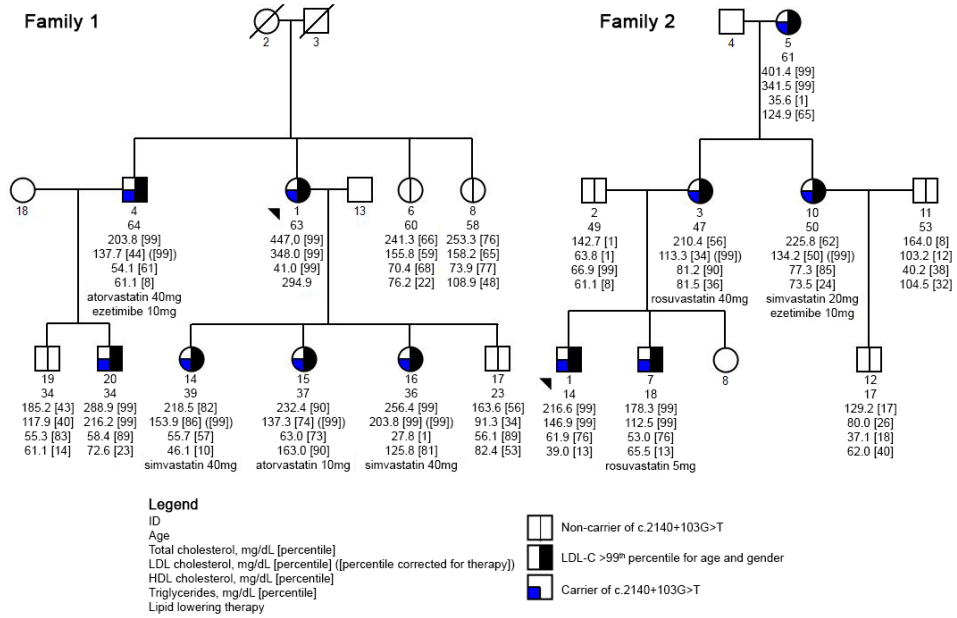
In silico analysis with five splicing prediction algorithms predicted an increase in splicing signal at a potential cryptic splice donor site consensus, five base pairs prior to the identified variant (Table 1). Activation of this cryptic splice site during transcription would in theory result in a 97 bp intronic DNA insertion and a premature stop in exon 15 of *LDLR*, as is shown in Figure 2.

Table 1. In silico splicing predictions

Algorithm (score range)	**Predicted splicing between c.2140+97T and c.2140+98G**	
	Reference sequence	c.2140+103G>T
SpliceSiteFinder-like (0-100)	70.8	75.8
MaxEntScan (0-12)	4.3	8.3
NNSPLICE (0-1)	0.5	0.9
GeneSplicer (0-24)	0	3.3
Human Splicing Finder (0-100)	80.7	82.6

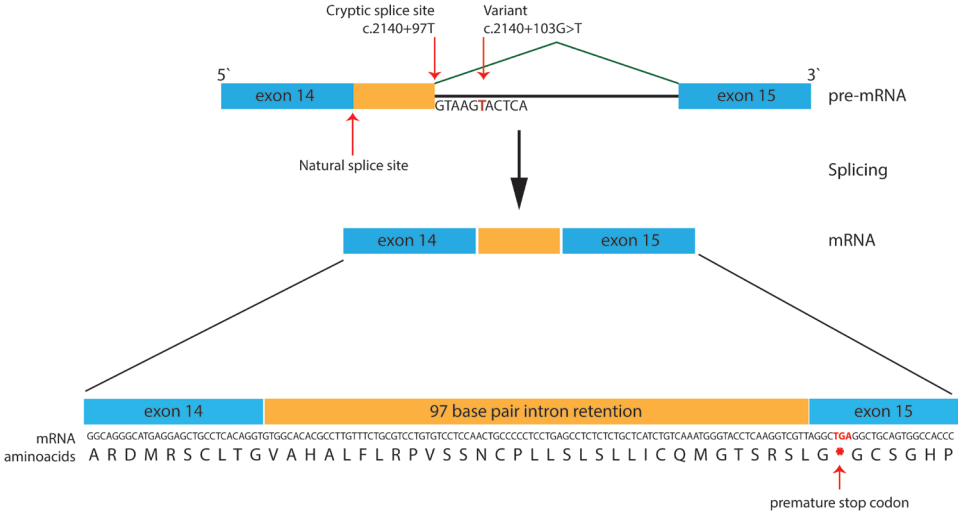
In silico splice prediction in intron 14 between c.2140+97T and c.2140+98G in the presence of the intronic variant c.2140+103G>T and without its presence at the same position (reference sequence). Score ranges of the different algorithms are between brackets.

Figure 1. Pedigree of family 1 and family 2



Hundred percent penetrance of the c.2140+103G>T variant with LDL cholesterol above the 99th percentile for age and sex in two clinical FH families without another FH causing mutation. Males and females are depicted as squares and circles, respectively. LDL(-C) = low density lipoprotein (cholesterol), HDL = high density lipoprotein.

Figure 2. Cryptic splicing of *LDLR* mRNA



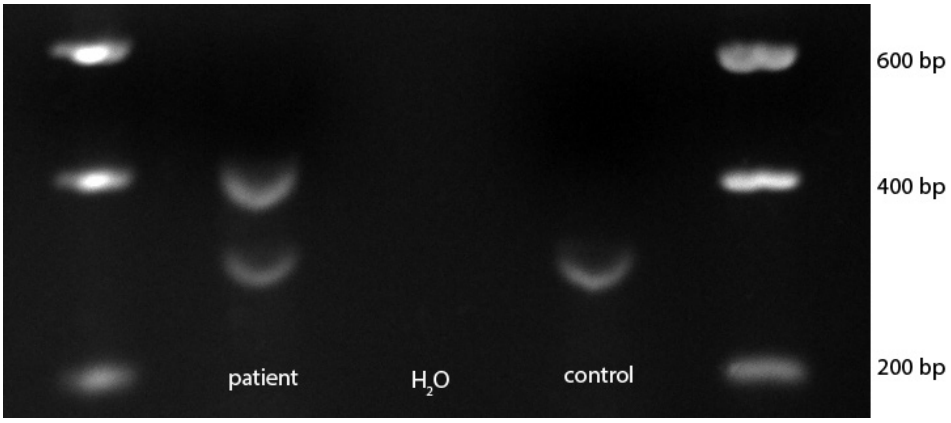
The presence of the intronic variant c.2140+103G>T leads to a cryptic splice site between c.2140+97T and c.2140+98G, resulting in a 97 base pair intron retention in the mRNA after splicing of pre-mRNA. Subsequently, a frame shift leads to a premature stop codon in exon 15 of *LDLR*.

To assess the *in vivo* effect of the variant, cDNA PCR products were generated from RNA of the proband of family 1. A band of 409 base pairs (bp) was identified in addition to the wild type *LDLR* cDNA of 312 bp (Figure 3). Sequencing of the 409 bp band confirmed the 97 bp intron insertion between exon 14 and 15, as predicted with the *in silico* analysis.

Two cohorts of FH patients were examined for the presence of the c.2140+103G>T variant. In the NGS FH cohort, comprising 2,145 patients, we identified five heterozygous carriers (from four families) of the c.2140+103G>T variant, which translates in a prevalence of 0.23% (5/2154). The five patients did not carry any mutation in *LDLR*, *APOB*, or *PCSK9*. In the FH4 cohort, consisting of 1,245 FH patients negative for pathogenic mutations in *LDLR*, *APOB*, and *PCSK9* who had a mean (± standard deviation) LDL-c of 230.3 ± 76.1 mg/dL (Table 2), three additional unrelated carriers of the novel variant were identified, which does result in a very similar prevalence of 0.24% (3/1245) as the first FH cohort. Taken together, a total of eight additional variant carriers (from seven unrelated families) were identified.

We collected plasma and DNA from family members of one of the eight newly identified carriers to examine the segregation of c.2140+103G>T with the FH phenotype in a second family. This family, family 2, also showed 100% penetrance of this variant with the FH phenotype (Figure 1, family 2). While combining the data of family 1 and 2, we observed that the mean LDL-c in carriers of the c.2140+103G>T variant was 252.8 ± 64.5 mg/dL after correction for use of lipid lowering therapies (Table 3), which is above the 99th percentile for age and gender, and significantly ($P < 0.001$) higher than the mean LDL-c of 120.7 ± 35.9 mg/dL in non-carriers in these families. Only the index patient from family 1 had a history of cardiovascular disease (myocardial infarction).

Figure 3. Agarose gel with cDNA PCR products from c.2140+103G>T carrier and control



cDNA PCR products of the proband of family 1 (patient) and a healthy control were run on a 1% agarose gel. PCR products were produced using primers covering the boundary between exon 14 and 15. The patient shows an additional PCR product of ~400 base pairs length, while the control only shows the expected PCR product. bp = base pairs.

Table 2. Characteristics of FH4 cohort

	FH4 cohort (n = 1,245)
Number of females	725 (58.2)
Age (years)	46 ± 19
Total cholesterol (mg/dL)	317.9 ± 72.0
LDL cholesterol (mg/dL)	230.3 ± 76.1
HDL cholesterol (mg/dL)	55.2 ± 30.5
Triglycerides (mg/dL)	159.4 (107.8, 221.4)
Number of subjects with lipid lowering therapy	138 (11.1)

Characteristics of the FH4 cohort consisting of 1,245 subjects with clinical FH but negative for mutations in *LDLR*, *APOB*, and *PCSK9* after Sanger sequencing and Multiplex Ligation-dependent Probe Amplification (MLPA). Data are presented as mean ± SD, except for sex [number (%)],triglycerides [median (IQR)], and subjects on lipid lowering therapy [number (%)]. FH = familial hypercholesterolemia, LDL = low density lipoprotein, HDL = high density lipoprotein.

Table 3. Lipid profiles of c.2140+103G>T carriers and non-carriers of family 1 and 2

	c.2140+103G>T carriers (n = 11)	**Family related** non-carriers (n = 5)	**P-Value**
Sex (number of females (%))	7 (64)	2 (40)	0.38*
Age (years)	42 ± 17	38 ± 20	0.73'
Total cholesterol (mg/dL)	261.8 ± 85.8	194.5 ± 52.3	0.08'
LDL cholesterol (mg/dL)	185.9 ± 85.0	120.7 ± 35.9	0.05'
LDL cholesterol corrected for statin use (mg/dL)	252.8 ± 64.5	120.7 ± 35.9	< 0.001'
HDL cholesterol (mg/dL)	55.4 ± 16.2	58.5 ± 14.6	0.71'
Triglycerides (mg/dL)	73.5 (63.3, 125.3)	76.2 (62.0, 82.4)	0.87‡

Data are presented as mean ± SD, except for sex [number (%)] and triglycerides [median (IQR)]. LDL cholesterol was corrected for the use of lipid lowering medication with correction factors earlier published, depending on the dose and type of lipid lowering medication.[15,16] *Chi-squared test, 'Independent t-test, ‡'Mann-Whitney U test. LDL = low density lipoprotein, HDL = high density lipoprotein.

DISCUSSION

We identified the deepest intronic variant in intron 14 of *LDLR*, c.2140+103G>T known to date, in patients with an FH phenotype. This variant fully segregated with the high LDL-c phenotype in two families. In addition, eight individuals from seven different families were found to be heterozygous carriers of this mutation, with a prevalence of 0.24% in two independent FH cohorts. The c.2140+103G>T variant causes a cryptic splice site in *LDLR*, resulting in a 97 base pairs intron retention between exon 14 and exon 15, which leads to a frameshift and a premature stop codon in exon 15. This deep intronic mutation likely results in a truncated LDLR protein missing the transmembrane and cytosolic domain. Alternatively, the generated mRNA enters the NMD pathway resulting in a smaller amount of newly synthesized LDLR protein.[7,8] Considering the visibility of cDNA containing the intron retention in the electrophoresis gel in Figure 3, one could speculate that the natural splice

site in the affected allele is substantially abolished.

Mutations in the introns of *LDLR* resulting in an FH phenotype have been described before. These intronic variants are mostly located within 20 bp of the exon-intron boundary, and an extensive cosegregation analysis of the mutation is usually not performed.[5]. The variant in our study, however, was identified 103 nucleotides upstream from the intron-exon boundary of exon and intron 14, which is deeper in the intronic region than many of the studies ever described. Moreover, we performed cosegregation analysis, which confirmed the association with high LDL-c phenotype, as we found a 100% penetrance. Lastly, our large cohort of FH patients allowed us to assess the prevalence of this variant. Although the latter was found to be relatively low (prevalence of 0.23-0.24%), we deem the results of the current study to be relevant, as it clearly shows that an exonic centered analysis of genomic data may result in omittance of functional defects in patients with FH. As a matter of fact; eight patients who were screened for a molecular cause of their clinical FH in our two FH cohorts, were considered not to be carrier of a causative mutation. The more elaborate analysis focusing on this deep intronic variant, now teaches us that our initial diagnosis was incorrect. This has a clinical consequence, as this genetic diagnosis forms the basis for further pedigree analysis in many screening programs. We therefore advocate to additionally focus on potential splice site defects in FH patients, in whom no exonic variant is found in the initial molecular screening.

Our data are a confirmation of the relevance of other studies focusing on intronic variants in FH. In their study, Kulseth and coworkers found a carrier of a deep intronic variant in intron 14, c.2140+86C>G, in *LDLR*.[9] The latter variant was found in one patient through extensive mRNA analysis of 30 FH patients with multiple primer combinations covering the whole transcript of *LDLR*. Our current study shows that also deeper intronic variants in *LDLR* can cause FH and that the variant was not restricted to one family.

Interestingly, recently another intronic variant in intron 14, c.2140+5G>A, with a minor allele frequency of 2.2%, was associated with a reduction in non-HDL cholesterol in a genome-wide association study among > 100,000 Icelanders.[10] *In silico* analysis predicted an intronic insertion and a premature stop at approximately the same position as our novel variant, which, in theory, would result in a similar truncated and thus dysfunctional LDLR protein and hence high LDL-c. This is in contrast to the reduced non-HDL-c found for c.2140+5G>A. While we confirmed the insertion of our variant, the *in silico* predicted effects of c.2140+5G>A on insertion and splicing were not confirmed, so its definite effect on the *LDLR* mRNA remains unclear. It might be, that the *in silico* prediction of this variant is not in accordance with the observed association in the GWAS, due to inaccuracy of *in silico* prediction models.

Alternative splicing resulting in abnormal protein sequences is a tightly regulated physiological process. However, genetic variants can disrupt processes involved in splicing machinery. Exonic or intronic variants in the canonical splice consensus can weaken or abolish canonical splice sites and exonic synonymous or intronic variants can create cryptic splice sites. In addition, various genetic variants can also affect enhancer or silencer elements in the gene or can alter the expression or activity of splicing regulatory proteins. Our study fuels the concept that abnormal splicing may play a larger role in pathogenicity than widely appreciated, and might even be a relatively common cause for monogenic disorders.[11]

There is a number of examples of deep intronic mutations that are related to disease phenotypes.[11] Their distance from the exon-intron boundaries can range from a couple of hundred bps (e.g. c.6937+594T>G in the Breast Cancer 2 (*BRCA2*) gene[12]) up to thousands of bps into introns (e.g. 3849+10KbC>T in the Cystic Fibrosis Transmembrane conductance Regulator (*CFTR*) gene[13]) It can be hypothesized that, in resemblance to these two examples, yet unidentified deep intronic variants in the *LDLR* gene might affect splicing and thus explain some FH4 cases. We therefore propose to perform complete *LDLR* gene sequencing, including the promotor and intronic regions, in FH4 patients to elucidate the molecular basis of the FH phenotype.

High-throughput genome-wide techniques in combination with improved *in silico* prediction tools are crucial in determining the prevalence and clinical relevance of intronic variants. The real challenge, however, is not the detection of these variants, but the evaluation of their pathogenicity in a clinical setting. *In silico* prediction tools to ascertain splicing, as we have used here, are not always correct in calling splice sites.[14] As a consequence, mRNA analyses and cosegregation analyses remains necessary, which often is a costly and timely labor for diagnostic laboratories.

The current study is limited by its lack of *in vitro* confirmation of the pathogenicity of c.2140+103G>T, such as the effect on LDLR protein expression and LDL-c uptake. However, we observe a 100% penetrance of this variant in two families over three generations. Moreover, the intronic variant was shown to result in a premature stop codon in exon 15, probably resulting in a truncation of the LDLR protein or reduced LDLR protein synthesis, and lastly, the identification of additional unrelated heterozygous carriers of the mutation among patients with the same FH phenotype strongly suggests that this variant may result in loss of function of the LDLR protein.

In conclusion, c.2140+103G>T is a novel deep intronic mutation in *LDLR* that causes FH. The

current finding suggests that the molecular basis of FH patients with unknown FH causing mutations can at least partly be explained by yet undiscovered (deep) intronic variants. This emphasizes the need to widen the scope from *LDLR* exome sequencing towards whole *LDLR* gene sequencing in patients with a yet unknown genetic cause of FH.

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6

TAKING ONE STEP BACK IN FAMILIAL HYPERCHOLESTEROLEMIA: *STAP1* DOES NOT ALTER PLASMA LDL (LOW-DENSITY LIPOPROTEIN) CHOLESTEROL IN MICE AND HUMANS

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ABSTRACT

OBJECTIVE

STAP1, encoding for Signal Transducing Adaptor Family Member 1, has been reported as a candidate gene associated with Familial Hypercholesterolemia (FH). Unlike established FH genes, expression of *STAP1* is absent in liver but mainly observed in immune cells. In this study, we set out to validate *STAP1* as an FH gene.

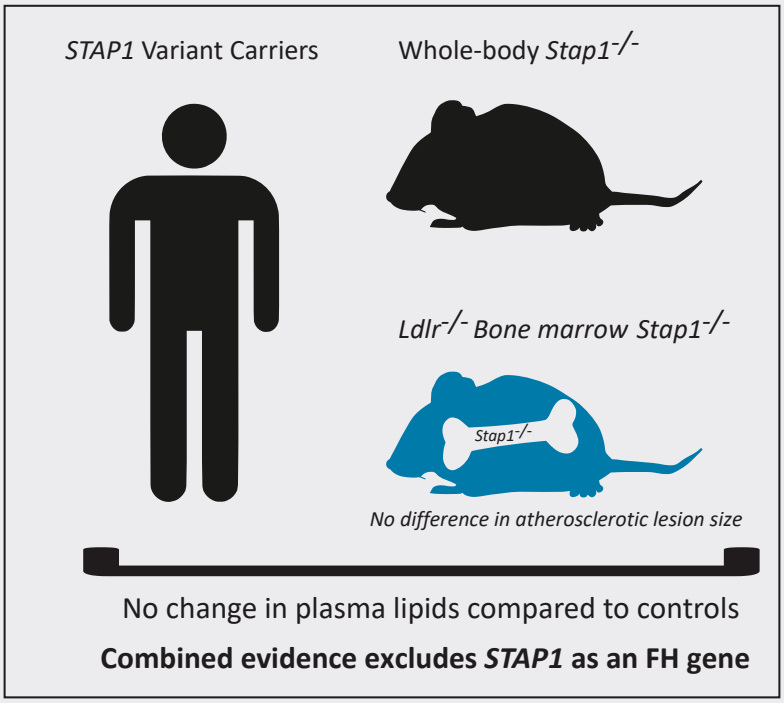
APPROACH AND RESULTS

A whole-body *Stap1* knockout mouse model (*Stap1*$^{-/-}$) was generated and characterized, without showing changes in plasma lipid levels compared to controls. In follow-up studies, bone marrow from *Stap1*$^{-/-}$ mice was transplanted to *Ldlr*$^{-/-}$ mice, which did not show significant changes in plasma lipid levels nor atherosclerotic lesions. To functionally assess whether *STAP1* expression in B cells can affect hepatic function, HepG2 cells were cocultured with peripheral blood mononuclear cells (PBMCs) isolated from heterozygotes carriers of *STAP1* variants and controls. The PBMCs from *STAP1* variant carriers and controls showed similar *LDLR* mRNA and protein levels. Also, LDL uptake by HepG2 cells did not differ upon co-culturing with PBMCs isolated from either *STAP1* variant carriers or controls. In addition, plasma lipid profiles of 39 carriers and 71 family controls showed no differences in plasma LDL-cholesterol, HDL-cholesterol, triglycerides and Lp(a) levels. Similarly, B cell populations did not differ in a group of 10 *STAP1* variant carriers and 10 age- and sex matched controls. Furthermore, recent data from UK Biobank does not show association between *STAP1* rare gene variants and LDL-c.

CONCLUSIONS

Our combined studies in mouse models and carriers of *STAP1* variants indicate that *STAP1* is not an FH gene.

GRAPHICAL ABSTRACT



INTRODUCTION

Familial hypercholesterolemia (FH) is a common genetic disorder characterized by lifelong elevated levels of low-density lipoprotein cholesterol (LDL-c) and increased risk for premature atherosclerotic cardiovascular disease. In approximately 30% of patients with extreme LDL-c (LDL > 4.9 mmol/L according to DLCN score), a genetic cause can be found,[1–3] with 95% accounted for mutations in the genes encoding the LDL receptor (*LDLR*), apolipoprotein B (*APOB*), and proprotein convertase subtilisin/kexin 9 (*PCSK9*).[4–8] Remarkably, DNA sequencing efforts have revealed that at least 30% of patients who exhibit FH features (LDL-c > 4.9 mmol/L, family history of premature atherosclerotic cardiovascular disease, among others), are not found to carry pathogenic gene variants in *LDLR*, *APOB* or *PCSK9*.[1–3] This raises the question whether there are yet to be discovered FH genes, which can explain the substantial proportion of mutation-negative FH patients. Identification of the causal gene(s) in these cases is of importance as it has the potential to improve our understanding of lipid metabolism, can possibly lead to novel targets for lipid-lowering therapies, and has relevant consequences for screening of family members of affected patients.

Several novel candidate genes for FH have been proposed in recent years, including *APOE*,[9,10] *STAP1*,[11] *LIPA*,[12,13] *CCDC22*,[14,15] *WASHC5*,[16] *PNPLA5*,[17,18] *ABCG5* and *ABCG8*.[19] Apart from *STAP1*, all these candidate genes have been demonstrated to play roles in established regulatory pathways of cholesterol homeostasis.[5] However, in depth functional studies into how *STAP1* may affect cholesterol homeostasis and how variants in this gene can cause FH are lacking.

Since its discovery,[11] several investigators have studied *STAP1* as a gene responsible for FH: an incomplete association was found between the *STAP1* p.Pro176Ser variant and an FH phenotype[20] while a p.Glu97Asp variant was discovered in only one Spanish FH patient who experienced an acute myocardial infarction.[21] A p.Thr47Ala variant was furthermore found in two family members with a myocardial infarction and elevated plasma LDL-c.[22] In all these studies, the relatively small number of carriers of *STAP1* variants have precluded firm conclusions about a possible causal relationship with hypercholesterolemia, especially because no clear damaging genetic variants or homozygous for loss-of-function variants have yet been described. In addition, in a recent study, investigators reported being unable to find an association between *STAP1* gene variants and lipid traits in the Berlin FH cohort.[23]

STAP1 (signal transducing adaptor family member 1) protein is mainly expressed in immune tissues including thymus, spleen, lymph nodes and bone marrow[24] and particularly in B cells.[24–26] The protein is also detected in ovary, kidney and colon,[25,27] but current data

show that STAP1 is not expressed in hepatocytes. This is remarkable, since the liver plays a crucial role in regulating LDL-c plasma levels by virtue of hepatic very-low density lipoprotein (VLDL) production, a precursor of LDL, and LDLR-mediated LDL uptake. This led us to hypothesize that *STAP1* expression in B cells may affect hepatocyte function.

To study the mechanisms potentially underlying the association between *STAP1* and cholesterol homeostasis, we developed and characterized two mouse models, and investigated possible effects of peripheral blood mononuclear cells (PBMCs) from *STAP1* variant carriers on LDL metabolism in a hepatocarcinoma cell line. We also investigated the characteristics of the B cells of these carriers. The findings of these studies motivated us to re-address the association of *STAP1* gene variants with plasma lipid and lipoproteins in four families. These combined results indicate that *STAP1* is not an FH or LDL-c modulating gene and should not be considered as such for FH genetic screening.

MATERIALS AND METHODS

All data, analytic methods and materials included in this study are available to other researchers upon reasonable request to the corresponding authors.

ANIMAL EXPERIMENTS

All animal experiments were approved by the Institutional Animal Care and Use Committee from the University of Groningen (Groningen, The Netherlands). Animals were housed under standard laboratory conditions with a light cycle of 12 hours and ad libitum food and water.

GENERATION AND GENERAL CHARACTERIZATION OF WHOLE-BODY *STAP1*-/- MICE

Two mouse lines of whole-body S*tap1*$^{-/-}$ were generated using CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/clustered regularly interspaced short palindromic repeat–associated 9) technology as previously described[28] (technical details provided in the Online Data Supplement). Male and female *Stap1*$^{-/-}$ and wild-type littermates (mixed background 50% FvB and 50% C57BL/6J) were group-housed and fed a standard laboratory diet (RMH-B, AB Diets, The Netherlands) until 13 weeks of age. Next, the mice were fed a high-fat-high-cholesterol diet (cholesterol 0.25%, Research Diets, Denmark) for 4 weeks. Blood was taken by orbital punctures under anesthesia with isoflurane, after 4 hours fasting in the morning, prior to the start of the high-fat-high-cholesterol diet and after two weeks on the high-fat-high-cholesterol diet. Termination was performed by heart puncture under isoflurane anesthesia. Blood was collected in tubes with EDTA-K+, and plasma was separated by centrifugation at 2000 rpm for 10 minutes at 4 °C. Organs and plasma were

snap frozen in liquid nitrogen and stored at -80 °C. The processing and analysis of mouse tissues was performed as indicated below.

BONE MARROW TRANSPLANTATION AND DIET-INDUCED ATHEROSCLEROSIS

Stap1$^{-/-}$ mice were backcrossed to C57BL/6J mice for eight generations. Bone marrow (BM) transplantations were carried out as described elsewhere.[29] In brief, 5x10^6 whole BM cells were isolated from either *Stap1*$^{-/-}$ or wild-type littermate control donors and transplanted into lethally irradiated (9Gy) *Ldlr*$^{-/-}$ female recipient mice, which are prone to develop a more severe hyperlipidemic phenotype as well as extensive atherosclerosis than male *Ldlr*$^{-/-}$ mice[30] (for more details see Online Data Supplement). After a recovery period of five weeks, transplanted animals were fed a Western Type Diet (WTD, 0.15% cholesterol, Research Diets D14010701) for 12 weeks. Blood samples for plasma lipid measurement were obtained by orbital puncture under isoflurane anesthesia from 4 hours-fasted mice before the initiation of the WTD and after eight weeks of WTD. Blood samples for flow cytometry analysis of cell populations were taken by tail bleeds at the indicated time points (Figure 2A). The animals were overnight fasted and then sacrificed by heart puncture under isoflurane anesthesia, after which heart, aorta, liver, spleen, thymus and blood were collected for further analyses. The technical details of the flow cytometry analysis for mice are described in the Online Data Supplement.

ATHEROSCLEROTIC LESION ANALYSIS

Atherosclerotic lesion analysis in the *Ldlr*$^{-/-}$*BM* transplanted mice was performed according to the guidelines from the American Heart Association.[31] The heart was isolated and fixed using formaldehyde 4% solution in phosphate buffer (Klinipath BV, Netherland). The hearts were dehydrated and embedded in paraffin, and cut in 4-µm cross-sections throughout the aortic root area. Haematoxylin-eosin staining was performed on the sections and the average from six sections (with 40 µm of separation between them) for each animal was used to determine lesion size. Lesion size was quantified, in a blinded fashion, by morphometric analysis of the valves using Aperio ImageScope Software Version 12.4.0.5043 (Leica Biosystems Pathology, USA).

PROTEIN ANALYSES BY TARGETED QUANTITATIVE PROTEOMICS

Tissue homogenates were prepared at 10% w/v in NP-40 buffer supplemented with complete protease (Roche) and phosphatase inhibitors 2 and 3 (Sigma-Aldrich), for posterior protein analysis by mass-spectrometry.

Murine STAP1 protein was quantified in various tissues using known concentrations of isotopically labeled peptide standards (^{13}C-labeled lysines and arginines), derived from

synthetic protein concatamers (PolyQuant GmbH, Germany) using the targeted proteomics workflow as described previously for other targets.[32] Briefly, homogenized tissues (50 µg protein) were subjected to in-gel digestion, where the proteins were digested by trypsin (1:100 g/g, Promega) after reduction with 10 mM dithiothreitol and alkylation with 55 mM iodoacetamide, followed by solid-phase extraction (SPE C18-Aq 50 mg/1 mL, Gracepure, Thermo Fisher Scientific) for sample clean-up.

Liquid chromatography on a nano-ultra high performance liquid chromatography (abbreviated as UHPLC) system (Ultimate UHPLC focused; Dionex, Thermo Fisher Scientific) was performed to separate the peptides. The target peptide (amino acid sequence NYSITIR for murine STAP1) was analyzed by a triple quadrupole mass spectrometer equipped with a nano-electrospray ion source (TSQ Vantage; ThermoScientific) and the data were analyzed using Skyline.[33] For the liquid chromatography-mass spectrometer measurements, an amount of the digested peptides equivalent to a total protein amount of 1 µg total protein starting material was injected together with up to 0.64 fmol of isotopically labeled concatamer-derived standard peptides for STAP1 (QconCAT technology, PolyQuant GmbH Germany). The concentrations of the endogenous peptides were calculated from the known concentrations of the standards and expressed in fmol/µg of total protein.

LIPID MEASUREMENTS

Total cholesterol levels were measured with a colorimetric assay (11489232, Roche Molecular, Biochemicals) with cholesterol standard FS (DiaSys Diagnostic Systems) as reference. Triglyceride levels were measured using Trig/GB kit (Roche Molecular Biochemicals) with Roche Precimat Glycerol standard (Roche Molecular Biochemicals) as reference.

FAST-PERFORMANCE LIQUID CHROMATOGRAPHY IN MICE

As part of the initial characterization of the whole-body S*tap1*$^{-/-}$ FVB mice, cholesterol in the main lipoprotein classes was determined using fast-performance liquid chromatography. The system contained a PU-980 ternary pump with an LG-980-02 linear degasser, FP-920 fluorescence and UV-975 UV/VIS detectors (Jasco, Tokyo, Japan). An extra PU-2080i Plus pump (Jasco, Tokyo Japan) was used for in-line cholesterol PAP or triglyceride enzymatic reagent (Roche, Basel, Switzerland) addition at a flow rate of 0.1 ml/min. The plasma from individual mice was run over a Superose 6 HR 10/30 column (GE Healthcare Hoevelaken, The Netherlands) using TBS pH 7.4, as eluent at a flow rate of 0.31 ml/min. Quantitative analysis of the chromatograms was carried out with ChromNav chromatographic software, version 1.0 (Jasco, Tokyo, Japan). The plots for individual FPLC profiles were generated with R version 3.6.1 (2019-07-05) and RStudio[34] using ggplot2_3.2.1, RColorBrewer_1.1-2, dplyr_0.8.3, and tidyr_0.8.3.

For the bone marrow transplantation (BMT) study, FPLC profiles were obtained using pooled plasma samples (350uL) from 12 animals of the corresponding genotype, collected prior to starting WTD diet and after eight weeks. These fast-performance liquid chromatography profiles were run using two Superose6 columns (Pharmacia LKB Biotechnology), after which individual fractions (n = 50) were analyzed for cholesterol using the aforementioned colorimetric kit.

SELECTION OF *STAP1* VARIANT CARRIERS

We contacted and invited all carriers of *STAP1* gene variants (p.Glu97Asp, p.Leu69Ser, p.Ile71Thr, or p.Asp207Asn) originally described by Fouchier et al.[11] to participate. As described previously, these individuals did not carry mutations in *LDLR*, *APOB* or *PCSK9* as assessed by Sanger sequencing and Multiplex Ligation-dependent Probe Amplification for *LDLR*.[11] As controls, we used age- and sex matched unaffected family controls. The study was approved by the Institutional Review Board at the Academic Medical Center in Amsterdam, and all subjects gave written informed consent before participation in this study. Pathogenicity of the *STAP1* variants was assessed with Polymorphism Phenotyping v2[35] and SIFT[36] (Sorting Intolerant From Tolerant; https://sift.bii.a-star.edu.sg/).

PLASMA LIPID AND IMMUNE CELL PROFILING IN PATIENTS

Blood was sampled after an overnight fast and plasma was isolated as described.[11] Plasma levels of TC, LDL-c, HDL (high-density lipoprotein), triglycerides and lipoprotein(a) (Lp[a]) were measured using commercially available assays (Wako Chemicals, Neusss, Germany; DiaSys Diagnostic Systems, Holzheim, Germany; Roche Diagnostics, Almere, The Netherlands), on a Vitalab Selectra E analyzer (Vital Scientific, Dieren, The Netherlands). LDL-c levels were calculated by the Friedewald formula.[37] LDL-c concentrations in humans were corrected for the use of lipid-lowering drugs.[38,39]

IMMUNOLOGICAL PROFILING IN PATIENTS

White blood cell counts and blood cell types were determined using a flow cytometry (Sysmex, Görlitz, Germany) in a subgroup of 10 *STAP1* variant carriers and 10 age- and sex matched controls. IgM and IgG were measured using immunoturbidimetry (Roche diagnostics).

PBMCs were isolated from whole blood, sampled in EDTA-coated tubes. This blood was diluted 1:1 with PBS + 2 mmol/L EDTA after which 30 ml of this mixture was layered upon 15 ml Lymphoprep (STEMCELL Technologies Inc., Vancouver, Canada), centrifuged at 944 g for 20 minutes at RT with slow acceleration and no brake. The PBMC-containing interphases was collected, washed three times with cold PBS + 2 mmol/L EDTA and centrifuged at 563

g for 10 minutes at 4˚C. Cells were counted and sample volume was adjusted with cold PBS + 1% BSA to 1 million PBMC's per 100 µl. A proportion of the PBMCs were stored in TriPure Isolation Reagent (Roche Applied Sciences, Almere, The Netherlands) at -80 ˚C for RNA isolation and gene expression analysis. Three million PBMC's were incubated for 30 minutes at 4˚C protected from light with antibodies against CD3, CD19, CD24, CD27, IgD, and CD43 with or without an antibody against CD38 (see Major Resources in the Online Data Supplement for the information about the antibodies). Subsequently, the PBMCs were washed twice with cold PBS + 1% BSA and centrifuged at 281 g for 5 minutes at 4˚C. The final pellet was resuspended in 200 µl PBS + 1% BSA and subjected to flow cytometry analysis on the BD LSRFortessa flow cytometer and analyzed with FlowJo (FlowJo, LCC). The selection of the different B cell subtypes is adapted from Meeuwsen *et al* [40]. (Figure VI in the Online Data Supplement). In short, non-B lymphocytes are $CD19^-$, naïve B cells are $CD19^+/CD27^-/IgD^+$, transitional B cells are $CD19^+/CD24^{++}/CD38^{++}$, non–class-switched memory B cells are $CD19^+/CD27^+/IgD^+$, class-switched memory B cells are $CD19^+/CD27^+/IgD^-/IgM^-/CD20^+/CD38^{+/-}$, and plasmablasts and plasma cells are $CD19^+/CD27^+/IgD^-/IgM^-/CD20^-/CD38^{++}$.

CELL LINES

The human hepatoma cell-line HepG2 was purchased from ATCC (Manassas, VA) and maintained in DMEM with 4.5 g/l glucose, GlutaMAX, and pyruvate (Gibco-Invitrogen, Breda, Netherlands) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 IU/mL penicillin (Gibco) and 100 µg/mL streptomycin (Gibco). The human B cell precursor leukemia cell-lines Kasumi-2 and Nalm6 were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH., Leibniz, Germany) and maintained RPMI 1640 with GlutaMAX and HEPES (Gibco) supplemented with 10% FBS, 100 IU/mL penicillin and 100 µg/mL streptomycin.

COCULTURE EXPERIMENTS

For cocultures, 125,000 HepG2 cells per well were plated in 24-well plates, allowed to proliferate for ~70 h, washed with PBS and subsequently cultured in coculture medium (DMEM with 4.5 g/l glucose, GlutaMAX, and pyruvate (Gibco) supplemented with 10% lipoprotein-depleted human serum, 100 IU/mL penicillin, 100 µg/mL streptomycin, 5 µM simvastatin (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 10 µM mevalonic acid (Sigma-Aldrich)). PBMCs were isolated from whole blood and resuspended in the coculture medium at a concentration of 1.7 million cells/ml. Of this suspension, 350 µl was added to a 6.5 mm diameter transwell insert with a 0.4 um pore size (Corning, Corning, NY) that were placed on top of the HepG2 cells in the 24-well plate. After 24 hours of coculture, HepG2 cells were either collected for gene expression analysis, used for LDL uptake studies, or

analyzed for LDLR protein expression. Using a similar setup, HepG2 cells were cocultured with B cell precursor acute lymphoblastic leukemia cells Kasumi-2 and Nalm6 instead of isolated human PBMCs.

GENE EXPRESSION ANALYSIS

Total RNA from HepG2 after 24 hours of coculture and isolated PBMCs was isolated using Tripure Isolation Reagent (Roche) according to manufacturer's instructions. Reverse transcription was performed using a cDNA synthesis kit (SensiFAST cDNA synthesis kit, Bioline, London, UK) according to the manufacturer's instructions. Quantitative RT-PCR was performed using SensiFAST SYBRgreen (Bioloine) with a CFX384 Real-Time PCR System (Bio-Rad Laboratories, Hercules, CA). Sequences of the used primers are listed in Table IV in the Online Data Supplement. The expression of each gene was expressed in arbitrary units after normalization to the average expression level of the housekeeping genes 18S ribosomal RNA (RN18S), hypoxanthine phosphoribosyltransferase 1 (HPRT1), and ribosomal protein, large, P0 (RPLP0) using the $2^{-\Delta\Delta Ct}$ method.[41]

LDLR FLOW CYTOMETRY ANALYSIS

After 24 hours of coculture, HepG2 cells were washed with PBS, detached from the plates with Accutase (Sigma-Aldrich) and washed twice with ice-cold PBS with 1% BSA and centrifuged at 12,000 rpm for 4 minutes at 4˚C. Next, the cells were incubated for 30 minutes on ice with 50 µl 40-fold diluted APC-conjugated anti-human LDLR (Cat. No. FAB2148A, R&D Biosciences, Minneapolis, MN), washed twice with ice-cold PBS with 1% BSA and centrifuged at 12,000 rpm for 4 minutes at 4˚C, resuspended in ice-cold PBS with 1% BSA and measured on a BD FACSCANTO II (BD Biosciences, USA) and analyzed using FlowJo (BD Life Sciences).

LDL UPTAKE STUDIES

LDL with a density of 1.019-1.063 g/ml was isolated from plasma of a healthy, normolipidemic donor through gradient ultracentrifugation after which it was fluorescently labeled with DyLight 488 NHS-Ester (ThermoFisher Scientific) for one hour according to the manufacturer's protocol and dialyzed against PBS overnight.[42]

After 24 hours of coculture (HepG2 and PBMCs or HepG2 and B cell precursor acute lymphoblastic leukemia cells), 4 µg DyLight apoB-labelled LDL per well was added. Thirty minutes later, HepG2 cells were washed twice with ice-cold PBS + 0.2% BSA after which they were lysed on ice for 30 minutes with ice-cold RIPA buffer (Pierce, Rockford, IL) supplemented with protease inhibitors (Complete, Roche). The lysates were centrifuged at 13,523 g for 15 minutes at 4˚C. The fluorescence at 488 nm in the supernatant was

determined and compared to cells that were not incubated with labeled LDL.

STATISTICAL ANALYSIS

Statistical analyses were performed with GraphPad Prism (Version 8, GraphPad Software, Inc.) or R (version 3.6.1 2019-07-05) and R studio (2018 version 1.2.1335). An unpaired parametric Student's *t*-test for normally distributed data or a Mann-Whitney test for not-normally distributed data was performed when two different groups with were compared. When more than two groups were compared, Kruskal-Wallis test or two-way ANOVA was performed with Tukey's post hoc test or Sidaks correction for multiple comparisons. $P < 0.05$ was considered significant.

RESULTS

GENERATION, VALIDATION AND INITIAL CHARACTERIZATION OF *STAP1*$^{-/-}$ MICE

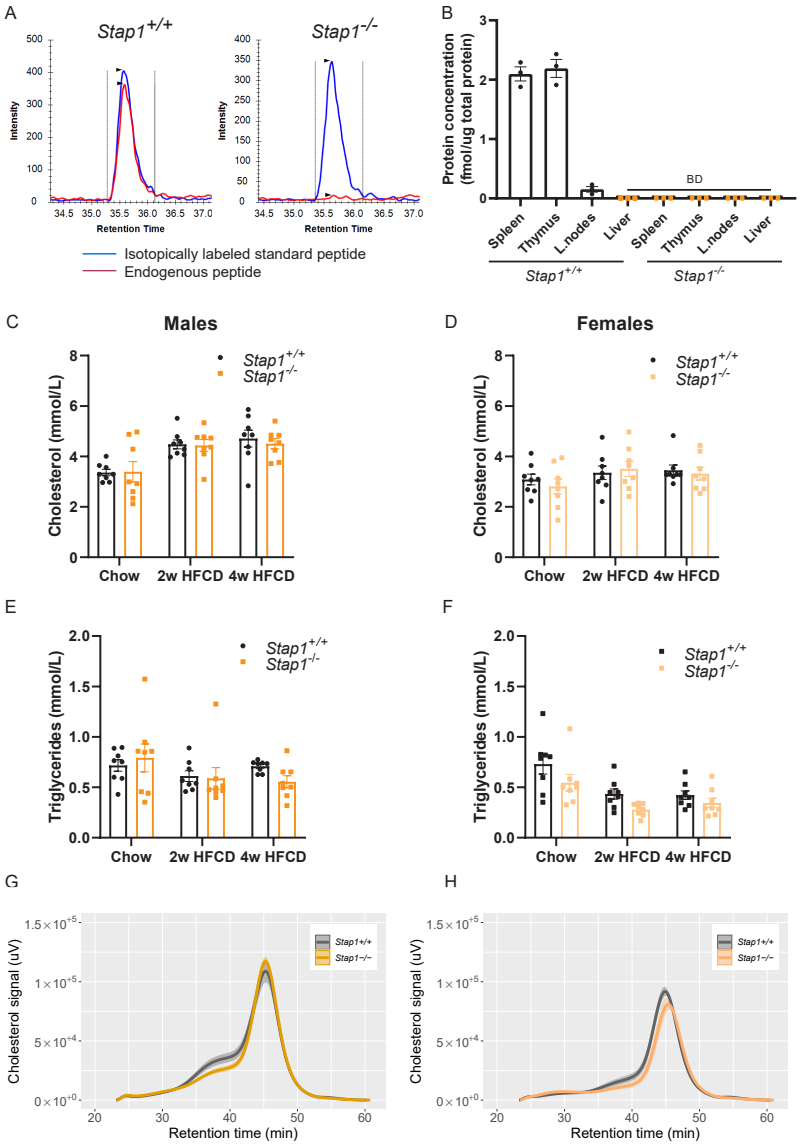
Two *Stap1* knockout (*Stap1*$^{-/-}$) mouse lines were generated by CRISPR/Cas9-mediated editing of exon 3 (Figures IA and IB in the Online Data Supplement). Mouse line A has a deletion of 5 base pairs (Del5bp) and mouse line B carriers a 14bp deletion (Del14bp). Both defects introduced premature stop codons as illustrated in Figure IC in the Online Data Supplement. *Stap1*$^{-/-}$ mice were born at the expected Mendelian ratios without any overt phenotype. Both lines were characterized but only data from mouse line A is shown and discussed here. Confirmatory data from mouse line B is shown in Figure II in the Online Data Supplement.

Using targeted proteomics, we confirmed that in wild-type mice, *STAP1* is mainly expressed in spleen, thymus and lymph nodes while it is below the detection limit in the liver (Figure 1A and 1B). Protein expression of STAP1 was not detected in *Stap1*$^{-/-}$ mice confirming that a premature stop codon at positions Ser81X (due to Leu76fs) and Gly78X (due to Cys75fs) results in a loss of protein in our mouse lines (Figure 1B; Figure I in the Online Data Supplement).

STAP1$^{-/-}$ MICE PRESENT NO ALTERATIONS IN PLASMA LIPID LEVELS

Compared to wild-type littermates, *Stap1*$^{-/-}$ male and female mice did not show differences in TC or triglyceride plasma levels on a standard laboratory diet and after four weeks on a high-fat/ high-cholesterol diet (Figure 1C through 1F; similar data for Line B in Figures IIA through IID in the Online Data Supplement). In addition, plasma lipoprotein profiles of *Stap1*$^{-/-}$ mice did not show significant differences compared to wild-type littermates (Figure 1E and 1F).

Figure 1. Characterization of whole-body *Stap1*$^{-/-}$ (mouse line A) on a standard laboratory diet and after 2 and 4 weeks on high-fat-high-cholesterol diet (HFCD)



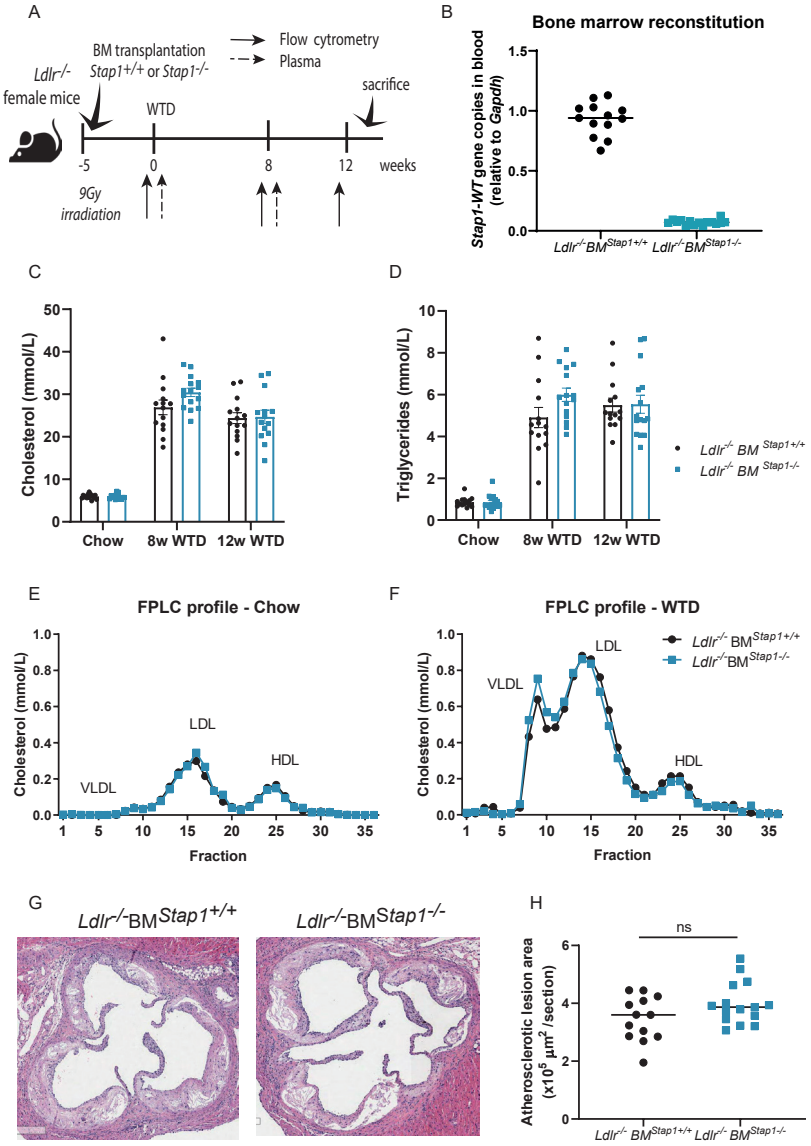
(A) Quantification of STAP1 protein in spleen using a mass-spectrometry-based targeted proteomics assay. The blue peak indicates the stable (heavy) isotope-labeled standard, and the red peak represents the endogenous peptide. (B) STAP1 protein expression profile per tissue for *Stap1*$^{+/+}$ and *Stap1*$^{-/-}$ mice determined by targeted proteomics (n = 3 per genotype). All tissues of *Stap1*$^{-/-}$ mice present STAP1 peptide levels below the detection limit (BD). (C-D) Total cholesterol plasma levels in male (C) and female (D) *Stap1*$^{+/+}$ and *Stap1*$^{-/-}$ male mice on a standard laboratory diet and after 2 and 4 weeks on high-fat cholesterol diet (HFCD). (E-F)Triglyceride plasma levels for *Stap1*$^{+/+}$ and *Stap1*$^{-/-}$ male (E) and female (F) mice on a standard laboratory diet and after 2 and 4 weeks on HFCD. (Figures C-F: Two-way ANOVA with Sidak's multiple comparisons test, *p < 0.05, **p < 0.01, n = 8 animals per genotype). (G-H) FPLC profiles for plasma cholesterol of individual mice for *Stap1*$^{+/+}$ and *Stap1*$^{-/-}$ males (G) and females (H) at termination after 4 weeks on HFCD. The dark line indicates the mean and the light shades indicate SEM, n = 7-8 per genotype. Data shown as mean ± SEM.

IRRADIATED FEMALE *LDLR*$^{-/-}$ MICE TRANSPLANTED WITH BM OF *STAP1*-/- DONORS DO NOT SHOW CHANGES IN PLASMA LIPID LEVELS OR ATHEROSCLEROSIS COMPARED TO CONTROLS

In contrast to humans, wild-type mice carry plasma cholesterol mainly in HDL while presenting low levels of LDL-c. Since STAP1 is mainly expressed in immune cells (B cells),[25] we used BMT to evaluate the effect of STAP1 deficiency, specifically in hematopoietic cells, on plasma lipids and atherosclerosis. The BMT study was performed in *Ldlr*$^{-/-}$ mice which carry cholesterol mainly in (V)LDL and better resemble the human lipoprotein phenotype. This study allowed to experimentally test the hypothesis proposed by Fouchier *et al.,* that STAP1 expression in B cells can affect plasma cholesterol levels in a mouse model with a human-like lipoprotein profile.

A BMT study into *Ldlr*$^{-/-}$ recipients was performed as illustrated in Figure 2A. Transplantation of BM from *Stap1*$^{-/-}$ into *Ldlr*$^{-/-}$ mice (*Ldlr*$^{-/-}$BM$^{Stap1-/-}$) nearly annihilated the presence of *Stap1* wild-type sequence in blood, resulting in 92% of bone marrow reconstitution. The absence of STAP1 protein in spleen was confirmed by mass-spectrometry after sacrifice, indicating long-term downregulation of STAP1 (Figure IIIA in the Online Data Supplement). As in the whole-body *Stap1*$^{-/-}$ mice, no differences in plasma cholesterol or triglycerides concentrations were observed on a standard laboratory diet nor after 8 or 12 weeks of WTD (Figures 2C and 2D). The absence of changes in blood lipids and lipoproteins was corroborated by unchanged lipoprotein profiles (Figure 2E and 2F). No difference in atherosclerotic lesion area was observed in the aortic root of these mice (Figure 3G and 3H), indicating that ablation of *Stap1* in the hematopoietic system does not affect atherosclerotic lesion size. Also, no differences in body weight were observed in these animals (Figure IIB in the Online Data Supplement).

Figure 2. Bone marrow (BM) deficiency of *Stap1* in *Ldlr*$^{-/-}$ female mice does not induce changes in plasma lipids and does not affect the development of atherosclerosis plaques



(A) Experimental design to evaluate BM *STAP1* deficiency on lipid metabolism and atherosclerosis in *LDLR*$^{-/-}$ mice. Samples for flow cytometry analysis and plasma lipids were taken on separate days; **(B)** Relative number of copies of *STAP1* WT gene in total blood after bone marrow transplantation assessed by qPCR.**(C)** Plasma cholesterol and **(D)** triglyceride levels of *LDLR*$^{-/-}$ transplanted with bone marrow from *STAP1*$^{-/-}$ compared to those that received *STAP1*$^{+/+}$ bone marrow. (Figures C-D, Two-way ANOVA with Sidaks correction for multiple comparisons test, n = 13-16 animals per genotype). **(E)** FPLC profile of pool plasma samples of *LDLR*$^{-/-}$*BM*$^{STAP1-/-}$ and *LDLR*$^{-/-}$*BM*$^{STAP1+/+}$ on a standard laboratory diet. **(F)** FPLC profile of pooled plasma samples from *LDLR*$^{-/-}$BM$^{STAP1-/-}$ and *LDLR*$^{-/-}$BM$^{STAP1+/+}$ animals after 8 weeks on WTD. **(G)** Representative example for Hematoxilyn-Eosin staining of hearts showing cardiac valves with atherosclerosis for *LDLR*$^{-/-}$*BM*$^{STAP1-/-}$ and *LDLR*$^{-/-}$*BM*$^{STAP1+/+}$. **(H)** Quantification of atherosclerotic lesion area in *LDLR*$^{-/-}$BM$^{Stap1-/-}$ and *LDLR*$^{-/-}$*BM*$^{STAP1+/+}$. (Figure H: Student ***t*** test). Data shown as mean ± SEM.

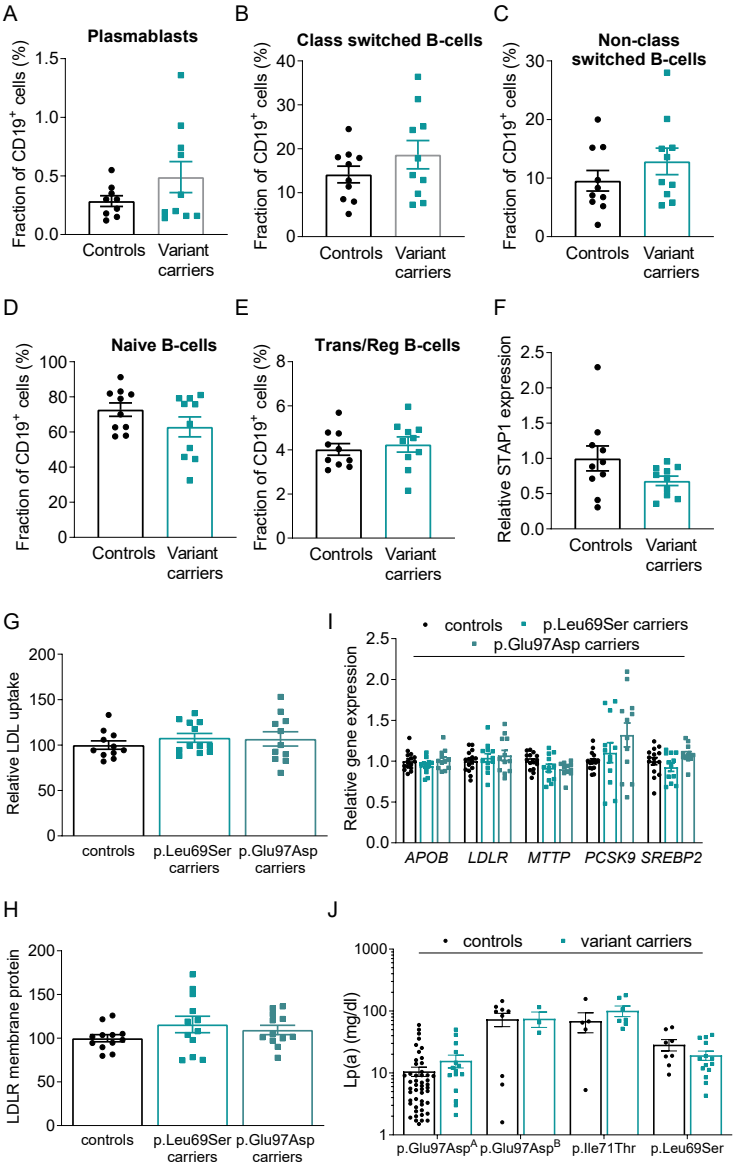
STAP1 DEPLETION IN BM CAUSES MINOR CHANGES IN LYMPHOCYTES AND MONOCYTES IN MICE

As BM transplantation induces stress and inflammation, possibly triggering phenotypic differences in the immune system, we also assessed the main immune cell populations in peripheral blood during the BMT study. On a standard laboratory diet as well as after starting WTD, we observed a very small increase in lymphocytes and B cells in the *Ldlr*$^{-/-}$BM$^{Stap1-/-}$ mice compared to *Ldlr*$^{-/-}$*BM*$^{Stap1+/+}$ (Figure VA-through VC in the Online Data Supplement). For monocytes, no differences were observed on a standard laboratory diet, but WTD induced a 30% decrease of the percentage of monocytes in the *Ldlr*$^{-/-}$*BM*$^{Stap1-/-}$ animals compared to controls (Figure VD in the Online Data Supplement). This difference appeared to specifically involve the Ly6C^{low} subpopulation (Figure VE and VF in the Online Data Supplement). We do not have explanations for the changes in immune cell populations. We assume, however, that their biological relevance for the phenotypes of interest in this study is negligible since no differences were observed in terms of plasma lipid levels or atherosclerosis development. Therefore, we did not further investigate these differences.

VARIANTS IN *STAP1* ARE NOT ASSOCIATED WITH CHANGES IN BLOOD-DERIVED HUMAN (B) CELL POPULATIONS

Since our mouse studies did not show an effect of *STAP1* deficiency on plasma LDL-c concentrations, we decided to more closely study the effects of *STAP1* variants in humans. As STAP1 is predominantly expressed in B cells,[26] we first studied B cell populations in 10 carriers of *STAP1* variants (4 p.Leu69Ser, 5 p.Glu97Asp, and 1 p.Asp207Asn carriers) and 10 age- and sex matched family controls. Table 1 shows that plasma lipids, liver enzymes, IgM and IgG concentrations as wells as white blood cell counts did not differ between the groups. γGT (gamma-glutamyl transferase) was the only blood parameter in which a significant difference was observed between *STAP1* variant carriers and controls. Although this might signal differences in liver function, the lack of correlation with other hepatic enzymes and the absence of a clear plasma cholesterol phenotype suggest a limited biological relevance of this observation. Subsequent fluorescence-activated cell sorting analyses did not reveal differences amongst these groups (Figure 3A trough 3E). *STAP1* mRNA expression appeared lower in PBMCs from carriers compared to controls, but this difference did not reach significance (Figure 3F).

Figure 3. Characterization of blood-derived cells from 10 selected carriers of *STAP1* gene variants and age- and sex matched family controls (Table 1)



(A-E) Relative amount of different B cell subtypes in *STAP1* variant carriers and controls: plasmablasts (A), class-switched B cells (B), non-class switched B cells (C), naïve B cells (D), and transitional and regulatory (Trans/Reg) B cells (E), depicted as percentage of the total CD19$^+$ cells. Data shown as mean ± SEM, n = 10 per group. (F) Relative *STAP1* mRNA expression in PBMCs from *STAP1* variant carriers and family controls, normalized to *RN18S*, *HPRT1*, and *RPLP0* with data from controls set to '1'. Mann-Whitney test was used in Figure A-F. (G-I) PBMCs isolated from either *STAP1* variant carriers or controls were cocultured for 24 h with HepG2 cells. (G) Relative uptake of DyLight labeled LDL by the HepG2 cells after co-culturing. Uptake is corrected for cellular protein content and data from HepG2 cells cocultured with control PMBCs set at 100% (n = 12-15). (H) Relative LDLR protein on the surface of the HepG2 cells after co-culturing as determined by FACS analysis. Data are corrected

for the number of cells, and data from HepG2 cells cocultured with control PMBCs was set at 100% (n = 12-15, Mann-Whitney test was used in Figures A-F). (I) Relative mRNA expression in the HepG2 cells after coculturing. Expression is normalized to *RN18S, HPRT1,* and *RPLP0* with data from HepG2 cells cocultured with control PMBCs defined as '1' (n = 12-15). (J) Comparison of plasma Lp(a) concentrations between *STAP1* variant carriers and their control family members in four different families (two families in which a p.Glu97Asp variant was found, one family with a p.Ile71Thr variant, and one family with a p.Leu69Ser variant). Values shown as mean ± SEM; One-day ANOVA and Kruskal-Wallis test were used in Figures G-J. * $p < 0.05$.

HEMATOPOIETIC CELLS OF CARRIERS OF *STAP1* VARIANTS DO NOT AFFECT LDL METABOLISM EX VIVO

STAP1 is not expressed in the main organ controlling LDL homeostasis, the liver, but is abundantly expressed in B cells. We therefore investigated whether B cells from carriers of a *STAP1* variant can affect hepatic LDL homeostasis by coculturing hematopoietic cells collected from *STAP1* variant carriers and controls with HepG2 cells. We used hematopoietic cells from STAP1 p.Leu69Ser or p.Glu97Asp variant carriers since these two variants are predicted to negatively affect STAP1 protein function, based on two predictive algoritms[35,36] (Table I in in the Online Data Supplement). Hematopoietic cells of *STAP1* variant carriers did not affect mRNA expression of genes encoding for proteins controlling VLDL secretion, such as *APOB* and *MTTP* (Figure 3I). Moreover, no differences in *LDLR*, *PCSK9* and *SREBP2* mRNA expression were found (Figure 3I). In line, cell surface LDLR expression and LDL uptake by HepG2 cells were not different between cocultures of hematopoietic cells from carriers of *STAP1* gen variant and controls (Figures 3G and 3H). Finally, HepG2 cells were cocultured with two different B cells precursor leukemia cell lines Kasumi-2 and Nalm6, which have previously been reported to have low and high *STAP1* mRNA expression, respectively.[43] We could confirm this (Figure VIIA in the Online Data Supplement) but did not observe significant changes in the expression of *APOB, LDLR*, *MTTP, PCSK9, SREBP2* mRNA in HepG2 cells upon co-culturing with these two cell lines (Figure VIIB in the Online Data Supplement). In line, there was no effect on cell surface LDLR protein or LDL uptake (Figure VII and VIID in the Online Data Supplement).

VARIANTS IN *STAP1* ARE NOT ASSOCIATED WITH ELEVATED PLASMA LIPIDS IN HUMANS

The lack of any effect of the *STAP1* variants studied on B cell population and ex vivo LDL homeostasis prompted us to reassess plasma lipid levels in carriers of *STAP1* gene variants and controls. For this, we compared lipid profiles in newly collected plasma of 39 carriers of *STAP1* gene variants carriers with those of 71 family controls. Levels of TC and LDL-c were not different between groups, which was also true for HDL-c and triglyceride levels (Table 2). Also, when stratifying for the three different *STAP1* gene variants and controls, no differences were observed. Finally, we found overall higher mean Lp(a) levels in pooled carriers versus controls, largely due to increased Lp(a) levels in the family carrying the

p.Ile71Thr *STAP1* variant (Table 2). However, this was not statistically different when Lp(a) levels were compared within the respective family, suggesting genetic susceptibility for elevated Lp(a) in this specific family (Figure 3J).

Table 1. Characteristics of 10 carriers of *STAP1* gene variants and 10 age- and sex matched family controls

	***STAP1* controls**	***STAP1* variant carriers**	**p-value**
Males (n)	6	6	1.000
Age (years)	58 ± 14	60 ± 15	0.835
Subjects on lipid-lowering therapy (n)	4	6	0.178
TC (mmol/L)	4.8 ± 1.2	5.1 ± 0.9	0.557
LDL-c (mmol/L)	3.1 ± 0.9	3.1 ± 0.9	0.962
HDL-c (mmol/L)	1.3 ± 0.2	1.4 ± 0.3	0.614
TG (mmol/L)	0.9 ± 0.4	1.4 ± 1.0	0.210
Lp(a) (mg/dL)	143 ± 226	232 ± 167	0.066
Bilirubin (µmol/L)	9.6 ± 3.1	12.8 ± 8.5	0.280
ASAT (U/L)	25 ± 6	28 ± 7	0.332
ALAT (U/L)	23 ± 7	28 ± 11	0.206
AF (U/L)	69.5 ± 12.7	68.9 ± 22.1	0.941
ɣGT (U/L)	23 ± 12	52 ± 39	0.035*
IgG (g/L)	10.8 ± 2.4	9.9 ± 3.0	0.487
IgM (g/L)	0.8 ± 0.4	0.8 ± 0.6	0.966
Leucocytes (10^9/L)	5.7 ± 0.9	6.2 ± 2.2	0.445
Neutrophils (10^9/L)	3.1 ± 0.7	3.8 ± 1.7	0.251
Lymphocytes (10^9/L)	1.8 ± 0.2	1.7 ± 0.5	0.435
Monocytes (10^9/L)	0.5 ± 0.1	0.5 ± 0.3	0.731
Eosinophils (10^9/L)	0.15 ± 0.08	0.16 ± 0.08	0.687
Basophils (10^9/L)	0.05 ± 0.02	0.05 ± 0.02	1.000

TC: total cholesterol, TG: triglycerides, ASAT: aspartate-aminotransferase, ALAT: alanine-aminotransferase, AF: alkalic phosphatase, ɣGT: gamma-glutamyltransferase, IgG: immunoglobulin G, IgM: immunoglobulin M. LDL-c concentrations were calculated by the Friedewald formula[37] . Values are mean ± SD or median with interquartile range (TG and Lp(a)). *p < 0.05.

Table 2. Plasma lipid parameters of *STAP1* variant carriers and family controls

	Family controls	***STAP1* variant carriers**			
		All	p.Glu97Asp	p.Ile71Thr	p.Leu69Ser
No. of subjects	71	39	18	7	14
Males (%)	46	49	56	43	43
Age (years)	48.2 ± 16.7	44.7 ± 18.8	39.6 ± 17.7	40.2 ± 21.9	53.8 ± 18.5
BMI (kg/m^2)	25.8 ± 4.4	24.1 ± 3.2	24.5 ± 3.7	NA	23.4 ± 2.9
TC (mmol/L)	5.5 ± 0.9	5.7 ± 1.3	5.5 ± 1.5	5.5 ± 0.5	6.0 ± 1.4
LDL-c (mmol/L)	3.5 ± 0.8	3.6 ± 1.1	3.5 ± 1.3	3.5 ± 0.5	3.8 ± 1.1
LDL-c corrected (a) (mmol/L)	3.9 ± 1.2	3.6 ± 1.7	3.9 ± 1.6	3.7 ± 0.5	3.3 ± 2.2
HDL-cholesterol (mmol/L)	1.3 ± 0.3	1.3 ± 0.2	1.3 ± 0.2	1.3 ± 0.2	1.3 ± 0.3
TG (mmol/L)	1.4 (1.1-1.9)	1.3 (1.0-2.2)	1.2 (1.1-2.0)	1.3 (0.9-2.0)	1.4 (1.1-2.7)
Lp(a) (mg/dL)	8.9 (4.3-29.7)	17.1 (10.2-47.6)*	12.6 (9.3-38.7)	72.4 (66.1-135.3)*	15.6 (11.4-27.7)

TC: total cholesterol, TG: triglycerides, NA: not assessed. LDL-c concentrations were calculated by the Friedewald formula [37]. (a) Off treatment LDL-c levels are calculated based on type and dose of lipid lowering therapy.[38,39] Values are mean ± SD or median with interquartile range (TG and Lp(a)). *p < 0.05 vs. family controls.

DISCUSSION

In 2014, *STAP1* was reported as a novel FH candidate gene.[11] This finding was intriguing especially because *STAP1* is mainly expressed in immune tissues and absent in liver - the main organ involved in lipoprotein metabolism.[25,27] Thus far, functional validation studies have not been reported and possible mechanisms by which *STAP1* could influence plasma lipid levels are not known. In experimental mouse studies as well as studies with PBMCs of carriers of *STAP1* gene variants, we were unable to find a role for STAP1 in controlling plasma LDL-c concentration. Following these negative findings, our combined studies exclude *STAP1* as an FH gene.

In line with our current findings, supportive evidence for *STAP1* as an FH gene has not grown in the five years following its identification in two FH families by Fouchier *et al.*, despite the inclusion of the gene in sequencing panels for the screening of patients with hypercholesterolemia. Three additional studies reported *STAP1* variants in individual FH patients.[20–22] However, none of the *STAP1* gene variants published thus far rendered clear-cut-loss-of-function effects (e.g. out frame deletions/insertions, and nonsense variants leading to premature protein truncation) and did not show clear segregation with high LDL-c levels in small families, hindering the interpretation of these limited findings. Moreover, recent large genome-wide association studies have not provided support for *STAP1* as a lipid gene.[44,45] Finally, a recent study reported no association between lipid traits in carriers and noncarriers of *STAP1* gene variants in a Berlin FH cohort and a population-based cohort from South Tyrol. [23]

A recent genome-wide rare variant analysis, based on exome sequencing data from more than 50,000 UK Biobank participants, also aligned with our findings.[46] In this dataset, 150 rare variants (minor allele frequency < 0.1%) affecting coding regions of *STAP1* were found in 37,889 individuals. Carriers of these variants did not present with statistically significant changes in LDL-c values (summary statistics: $\beta = 0.049193$, SE = 0.080952, $P = 0.54$). Of the variants included in our present study, only *STAP1* p.Ile71Thr and p.Pro176Ser were found in the UKBB dataset and did not show association with LDL-c levels (Table II in the Online Data Supplement).

Retrospectively, LDL-c levels in carriers of *STAP1* gene variants in the original publication were only 11% higher compared to controls[11] - an effect considerably smaller than observed in carriers of causal mutations in *LDLR*, *APOB* and *PCSK9*. To detect a statistically significant difference of 11% in LDL-c levels, power calculations reveal that one needs around 100 subjects per group. Forty *STAP1* variant carriers were studied in the original report by

Fouchier *et al.* [11], suggesting that the statistically significant association that was initially identified was likely a spurious finding.

In addition, the employed FH classification was not stringent: FH was defined as TC or LDL-c levels above the 95th percentile for age- and sex, leaving room for polygenic contributions or elevated Lp(a) levels.[2,3,47–49] Furthermore, the family in which the lead *STAP1* variant (p.Glu97Asp) was identified also included several phenocopies (same phenotype but not carrying the variant) and one case of non-penetrance (no phenotype despite carrying the variant)[11] which may have brought about false-positive findings.

One of the limitations of reassessing the association between *STAP1* gene variation and plasma lipid levels is that we were unable to include carriers of all known *STAP1* variants. On the other hand, our observations in 10 carriers of *STAP1* variants predicted to be damaging were all negative, as well as *ex vivo* studies into a possible role of immune cells in controlling LDL homeostasis. Thus, our study also highlights that *in silico* predictions of the effect of gene variation at the protein level should be interpreted with care.

Our findings have practical implications for the molecular diagnosis of FH as *STAP1* is currently included in targeted sequencing panels for of FH: we propose to exclude *STAP1* from these panels. Furthermore, our findings are relevant to patients in whom *STAP1* gene variants have been identified with respect to screening family members as well as for studies aiming to find novel FH candidate genes. Clearly, our findings emphasize the importance of in-depth validation studies which is particularly important for the field of lipoprotein metabolism where so many novel genes have been proposed as novel candidate genes for plasma lipid regulation without functional follow-up.

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PART II

THERAPY IN FAMILIAL HYPERCHOLESTEROLEMIA

7

PREVENTION OF CARDIOVASCULAR DISEASE IN PATIENTS WITH FAMILIAL HYPERCHOLESTEROLAEMIA: THE ROLE OF PCSK9 INHIBITORS

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ABSTRACT

Familial hypercholesterolaemia (FH) is an autosomal dominant inherited disorder characterized by elevated low-density lipoprotein cholesterol (LDL-c) levels and consequently an increased risk of atherosclerotic cardiovascular disease (ASCVD). FH is relatively common, but is often underdiagnosed and undertreated. Cardiologists are likely to encounter many individuals with FH; however, patients presenting with premature ASCVD are rarely screened for FH and fasting lipid levels are infrequently documented. Given that individuals with FH and ASCVD are at a particularly high risk of subsequent cardiac events, this is a missed opportunity for preventive therapy. Furthermore, because there is a 50% chance that first-degree relatives of individuals with FH will also be affected by the disorder, the underdiagnosis of FH among patients with ASCVD is a barrier to cascade screening and the prevention of ASCVD in affected relatives. Targeted screening of patients with ASCVD is an effective strategy to identify new FH index cases. Statins are the standard treatment for individuals with FH; however, LDL-c targets are not achieved in a large proportion of patients despite treatment. Proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors have been shown to reduce LDL-c levels considerably in individuals with FH who are concurrently receiving the maximal tolerated statin dose. The clinical benefit of PCSK9 inhibitors must, however, also be considered in terms of their cost-effectiveness. Increased awareness of FH is required among healthcare professionals, particularly cardiologists and primary care physicians, in order to start early preventive measures and to reduce the mortality and morbidity associated with FH and ASCVD.

INTRODUCTION

Familial hypercholesterolaemia (FH) is an autosomal dominant inherited lipid disorder that, in most cases, is caused by mutations occurring in one (or more) of three genes: the low-density lipoprotein (LDL) receptor gene (*LDLR*), the apolipoprotein B gene (*APOB*) and the proprotein convertase subtilisin/kexin type 9 gene (*PCSK9*).[1] Mutations in these genes lead to impaired LDL metabolism and elevation of plasma LDL-cholesterol (LDL-c). FH can also be caused by mutations in other genes, including signal transducing adaptor family member 1 (*STAP1*),[2] however mutations in such genes are rare. Regardless of the underlying cause, patients with FH have elevated LDL-c levels and are therefore at increased risk of premature atherosclerotic cardiovascular disease (ASCVD). ASCVD most commonly manifests as coronary heart disease (CHD),[3] but patients may also present with stroke.[4]

Recent studies have shown that the prevalence of heterozygous FH (HeFH) is 1 in 200–250 people[7–9] and that of homozygous FH (HoFH) is 1 in 160,000–300,000 people.[5] Although HeFH is not uncommon, it is often underdiagnosed and undertreated. This leads to substantial mortality and morbidity;[6] approximately 50% of men and 30% of women with FH will develop CHD before the age of 50 years if the disorder is left untreated.[3] ASCVD in patients with FH is therefore an important public health challenge. Patients with confirmed FH should receive high-intensity lipid-lowering therapy (LLT), which has been shown to improve their life expectancy[7] and quality of life markedly.[8]

Owing to the high prevalence of ASCVD in patients with FH, it is important that individuals presenting with ASCVD are assessed for FH.[9] However, screening for FH among this patient population remains low,[10] which leads to suboptimal management of patients with FH and ASCVD. Furthermore, given the autosomal dominant inheritance of FH, family members of individuals diagnosed with FH should be screened in a cascade approach to identify affected relatives. As the prevalence of FH is much greater in those presenting with ASCVD than in the general population,[10,11] patients with ASCVD represent a key target population for FH screening.[10] It is, therefore, vital that cardiologists and other medical professionals are aware of current guidelines and consensus statements on the diagnosis and treatment of patients with FH.[5,6,8] Understanding of the molecular pathology and genetic basis of FH is vital to support screening so that LLT is initiated in individuals with FH in order to prevent ASCVD events.[12]

CARDIOVASCULAR DISEASE RISK IN PATIENTS WITH FH

Patients with FH are up to 16 times more likely to develop ASCVD than the overall population.[13-16] In a population-based cohort study comprising 69,016 individuals from Denmark, it was estimated that 33% of patients with FH had CHD.[13] Patients with FH typically develop premature ASCVD, with ASCVD events often occurring in patients with HeFH before 55 years of age in men and before 60 years of age in women.[6] A recent cohort study of CHD risk in patients with FH, which included 65,565 people and a follow-up of 78,985–308,378 person-years, found that CHD risk was accelerated in patients with FH by 10–20 years in men and 20–30 years in women.[17] ASCVD is a leading cause of death in those with FH;[18] in an 21-year cohort study of 5,518 patients with FH, ASCVD was the most common cause of death, accounting for 42% of the 189 deaths occurring during the study period.[19]

Given the relative rarity of HoFH (estimated prevalence is 1 in 160,000–300,000 people[5]), little is known about the exact ASCVD risk and associated mortality in these patients. Sjouke et al. found that 29% of 49 patients with HoFH had ASCVD.[20] Patients with HoFH develop ASCVD much younger than those with HeFH, often before 20 years of age. In a study of 149 patients with HoFH, Raal et al. found that, in those who were untreated, the age (mean ± SD) at first non-fatal major adverse cardiac event (MACE) was 12.8 ± 5.9 years and the age of ASCVD-related death was 17.7 ± 10.1 years.[21]

CARDIOVASCULAR DISEASE RISK STRATIFICATION

Not all patients with FH develop atherosclerosis and ASCVD to the same extent.[22] ASCVD risk depends mainly on plasma LDL-c levels.[23] The underlying genetic mutation, patient comorbidities and lifestyle factors influence LDL-c levels and ASCVD risk.[22] Stratification of patients by their individual ASCVD risk factors may help to identify those who would benefit from high-intensity LLT.[24]

Mutation of a known FH causing gene can be found in approximately 80% of patients with definite FH.[22] Among patients with an identifiable genetic mutation, the most common cause of FH is mutations in *LDLR*, affecting over 90% of these patients.[6] These fall into six classes: class 1 mutations are null mutations that result in no detectable LDLR protein; class 2 mutations disrupt the transport of LDLR from the endoplasmic reticulum to the Golgi apparatus; class 3 mutations lead to the expression of non-functional LDLR; class 4 mutations result in LDLR–LDL complexes that cannot cluster in coated pits; class 5 mutations lead to inefficient recycling of LDLR; and class 6 mutations disrupt the targeting of the receptor to the basolateral membrane. [8]

Null mutations in *LDLR* are consistently found to be associated with the most severe forms of FH (in terms of both LDL-c levels and ASCVD risk).[20,25,26] In a study of 1,088 patients with premature myocardial infarction (MI), it was found that, compared with the general population, those with a class 1 mutation in *LDLR* had a 13-fold increased risk of MI, while those with other classes of *LDLR* mutations had a 4.2-fold increased risk.[14] Furthermore, *LDLR* mutations overall confer a more severe phenotype than *APOB* mutations. In a study of CHD risk in patients with FH and their unaffected relatives, individuals with any class of *LDLR* mutation had an 8.5-fold increased risk of CHD, whereas those with an *APOB* mutation had a 2.7-fold increased risk compared with unaffected relatives.[27]

Mutations in *APOB* occur in 5–10% of patients with FH.[1] However, the frequency of mutation in this gene varies by country and has not been found to occur in Finland, Spain, Russia and Japan.[28] The most frequent FH causing mutation in this gene is the R3500Q (Arg3500Gln) mutation.[1] Patients carrying this mutation have been shown to have significantly increased LDL-c levels and a seven times increased risk of ischemic heart disease compared with the general population.[29] Not all mutations in *APOB* are associated with FH, for example patients with the R3531C (Arg3531Cys) mutation have been shown not to have an increased risk of ischemic heart disease compared with the general population.[29]

Mutations in *PCSK9* are relatively rare, occurring in fewer than 1% of patients with HeFH,[30] which makes it difficult to obtain sufficient data to assess the magnitude of the ASCVD risk specifically associated with mutations in this gene.[31] More than 20 different mutations have been identified in *PCSK9* and all of these have different effects on lipid levels and ASCVD risk.[6,32] In a study of 130 patients with FH without mutations in *LDLR* or *APOB*, it was found that different mutations in *PCSK9* cause variable phenotypes, and that the type and severity of hyperlipidemia and level of ASCVD risk could vary among individuals from the same family.[32] Furthermore, one particular mutation in *PCSK9* has been shown to be associated with a very severe phenotype; in a retrospective analysis of 49 patients with FH, over a 30-year follow-up period, individuals carrying the D374Y (Asp374Tyr) *PCSK9* mutation were affected by premature CHD more than 10 years earlier than those with severe mutations in *LDLR*.[33]

The degree of elevation of LDL-c is the main factor driving ASCVD risk in patients with FH, and those with LDL-c levels greater than 10 mmol/L are at particularly high risk.[5,24] In addition to LDL-c, lipoprotein a (Lp[a]) has been recently identified as a possible independent risk factor for ASCVD, both in the general population and in patients with FH. In a cross-sectional analysis of 1,960 patients with FH and 957 relatives without FH, patients with FH had higher plasma levels of Lp(a) than in their unaffected relatives. In individuals with FH, ASCVD-free survival was significantly lower in patients who had Lp(a) levels above 50 mg/dL than

in those with Lp(a) levels below 50 mg/dL.[25] Furthermore, in a recent prospective cohort study of 46,200 individuals, patients with FH and high Lp(a) levels had the highest risk of MI, compared with individuals without FH and lipoprotein(a) concentrations of 50 mg/dL or less, hazard ratios for MI were 1.4 for those without FH and Lp(a) levels above 50 mg/dL, 3.2 for those with FH and Lp(a) levels of 50 mg/dL or less and 5.3 in those with FH and Lp(a) levels above 50 mg/dL.[34]

Other ASCVD risk factors that apply to the general population also play a role in ASCVD risk in patients with FH, but their predictive value differs from that of the general population.[22] These factors include diabetes mellitus, obesity, hypertension, smoking, renal insufficiency, low high-density lipoprotein cholesterol (HDL-c) levels, and a family history of premature ASCVD.[25,35-38] A family history of premature ASCVD in a patient with FH probably reflects the autosomal dominant inheritance of the disorder. Thus, it is important to take a family history of premature ASCVD events to gain a full picture of ASCVD risk. Similar to the general population, male sex increases the risk of premature ASCVD in the FH population: men with FH have been shown to develop ASCVD approximately seven years earlier than women with FH.[35,39] This difference in ASCVD risk is probably driven by the cardioprotective effects of oestrogen.[40] In addition, high levels of testosterone may be linked to premature ASCVD,[41] but the relative contribution of this factor has not been established in patients with FH. Clinical characteristics specific to patients with FH, such as the presence of tendon xanthomas, do not appear to be independently associated with ASCVD risk in individuals with FH.[38]

Subclinical atherosclerosis in the coronary arteries is an independent risk factor for ASCVD in the general population.[42] This is also the case in patients with FH; a recent prospective study of 101 patients with HeFH, in whom 21 MACEs occurred during a median follow-up of 941 days, found that an increased coronary atherosclerotic plaque score was independently associated with coronary events.[43] Non-invasive imaging techniques, such as ultrasonography and computed tomography, can be used to determine the extent of subclinical atherosclerosis.[44] Use of such tests could help to identify patients with FH and advanced atherosclerosis who may be at high risk of ASCVD.[24]

Risk calculators, such as the US Framingham Risk Score and the European SCORE (Systematic Coronary Risk Evaluation), are not suitable for those with FH because these patients are at considerably higher risk of ASCVD due to lifelong exposure to elevated plasma LDL-c levels.[6] Evidence of the suitability of existing criteria for assessing the ASCVD risk in patients with FH is limited. Therefore, the International Atherosclerosis Society Severe Familial Hypercholesterolemia Panel has provided a consensus statement, based

on expert opinion, that suggests the following criteria to identify patients with severe FH who are at high risk of ASCVD: patients with LDL-c levels above 10 mmol/L (> 400 mg/dL) at diagnosis, or greater than 8 mmol/L (> 310 mg/dL) or 5 mmol/L (> 190 mg/dL) at diagnosis if another one or two risk factors, respectively, are present (i.e. age > 40 years, smoking, male sex, high Lp(a) [> 75 nmol/L], low HDL-c [< 1 mmol/L], hypertension, diabetes mellitus, impaired renal function, body mass index (BMI) > 30 kg/m^2, family history of premature ASCVD). Patients with advanced subclinical atherosclerosis or those who have previously experienced a cardiovascular event should also be considered as having severe FH and being at high risk of ASCVD.[24]

UNDERDIAGNOSIS AND UNDERTREATMENT

FH is common among patients presenting with ASCVD.[9] The European Action on Secondary and Primary Prevention by Intervention to Reduce Events (EUROASPIRE) IV cohort study of 7,998 individuals with CHD across 24 European countries found that, among 7,044 evaluable patients, 8.3% had potential FH (defined as a score of ≥ 6 using a modified version of the Make Early Diagnosis to Prevent Early Deaths [MEDPED]/World Health Organization [WHO] criteria and the Dutch Lipid Clinic Network [DLCN] diagnostic criteria).[10] Rates of potential FH in patients with CHD varied considerably across European regions, ranging from as low as 3.4% in the Finnish centers to 20.8% in Bosnia and Herzegovina. These large regional differences in FH prevalence may relate to genetic founder effects.[45-47] In addition, the types of centers participating in the study in each region may have impacted on the reported FH prevalence.[10] Lifestyle factors, such as variations in lipid intake across regions,[48] may also lead to misdiagnoses of FH in some countries.

The EUROASPIRE IV study found that FH prevalence in individuals with CHD was inversely related to age; the prevalence of potential FH was eight times greater in patients younger than 50 years than in those older than 70 years.[10] This association with age may partly be explained by the fact that patients with FH die earlier resulting in a decline of the prevalence of potential FH by age.[10] Furthermore, CHD occurred prematurely in 78% and 73% of men and women with potential FH, respectively, compared with 33% in men and 37% in women without FH.[10] Similarly, in a smaller cohort study of 4,778 patients with acute coronary syndromes (ACS), FH prevalence (defined by the Simon Broome Register and DLCN diagnostic criteria) inversely correlated with age of ACS onset.[9] These recent studies reflect the results of seminal work from Genest et al. in 1992, who found that more than half of patients with premature CHD had a familial lipoprotein disorder.[11]

Prevention of ASCVD in individuals with FH is failing, partly because of underdiagnosis in this patient population Yudi et al. conducted a retrospective analysis of 210 patients admitted to hospital in Australia for premature coronary artery disease (events occurring in male patients aged ≤ 55 years and female patients aged ≤ 60 years) in a 12-month period, which found that only 96 patients (46%) had their fasting lipid levels recorded following a hospital admission for premature coronary artery disease.[49] Among individuals for whom lipids were measured 3 (1%) were found to have probable FH and 50 (24%) had possible FH, as assessed using the DLCN criteria.[49] In a Norwegian registry study of 5,538 patients with genotype-verified FH, 1,411 patients were hospitalized over a 15-year period; ischemic heart disease was reported in the hospitalization of 90% of these patients. However, the diagnosis of FH was registered in only 46% of the patients at discharge.[50]

The underdiagnosis of FH among individuals with ASCVD has led to inadequate administration of therapy to prevent further ASCVD events. In the Australian study by Yudi et al., 23% of individuals with retrospectively diagnosed possible or probable FH were discharged from hospital without LLT.[49] Furthermore, data from the EUROASPIRE IV study showed that only 55% of patients with CHD and potential FH received high-intensity statin therapy,[10] and data from the Danish study by Benn et al. showed that only 48% of patients with clinically defined FH received LLT.[13] Even in patients who are receiving LLT, the therapy may not be sufficient to reduce ASCVD risk adequately; a recent observational study, conducted in Europe, China, Canada, Russia, Africa, and the Middle East, which included 54,811 patients receiving statin therapy found that 60.1% of patients with probable FH had CHD, compared with 38.8% of those in the total study population.[51] Taken together, the results of these studies suggest that patients with ASCVD are receiving suboptimal treatment. Given that appropriate treatment can reduce the risk of ASCVD, the issues of underdiagnosis and undertreatment of FH and ASCVD require attention.[52] Until recently, however, there were few available agents with the potency and tolerability needed to treat FH adequately.

In many patients with FH, the disease only becomes evident after the first major cardiovascular event and cardiologists are, therefore, frequently the first to diagnose a patient with FH.[53] A lack of awareness among cardiologists of the relatively high prevalence of FH in patients with ASCVD may account for the low level of FH screening in these individuals. In a survey conducted among American College of Cardiology CardioSurve members in 2011, the majority of whom had over 10 years of experience in cardiovascular clinical practice, most (~80%) were unaware of the true prevalence of FH. Although more than 95% of cardiologists surveyed agreed that patients with FH are at a moderate/high risk of future ASCVD events, only 10% reported feeling very or extremely confident about their understanding of FH.[54] Furthermore, fewer than 30% of cardiologists recognized FH

when they were shown a case brought by the National Lipid Association.[54] The survey also revealed a lack of understanding of the genetic causes of FH; 60% of cardiologists were unaware of the fact that, given the autosomal dominant mode of inheritance, there is a 50% chance that first-degree relatives of a patient with FH will also have the disorder.[54] Increased awareness of FH among cardiologists is required to improve the diagnosis of this condition in patients with ASCVD and to facilitate initiation of appropriate treatment earlier in the disease course. Moreover, increasing the understanding of the genetic basis of FH may support cardiologist-led initiation of screening and ASCVD prevention by referral of a patient's relatives to primary care physicians or lipid specialists.

SCREENING

As a result of the prevalence of FH, a systematic approach to screening is warranted. Cascade screening, whereby first- second- and third-degree relatives of an established index case are assessed for FH via genetic testing and LDL-c measurement, has been shown to be a cost-effective approach.[55] Targeted screening in selected groups that have a high prevalence of FH, such as patients presenting with premature ASCVD, is an efficient method of identifying new FH index cases.[56] Universal screening, in which a population is systematically screened, could be applied to FH via cholesterol measurement or genotyping of children. Although this approach has not been used in FH, universal screening has been successful in detecting other disorders such as phenylketonuria and cystic fibrosis.[57] Universal genotyping may not identify patients whose FH is caused by novel mutations or polygenic mutations and may be most effective in populations in which genetic founder effects restrict the number of prevalent mutations.[56] Data suggest, however, that it may be prudent to initiate cholesterol screening in children, in whom elevated levels of LDL-c alone are strongly diagnostic of FH.[58] In addition, screening of children has been shown to be an effective method for the diagnosis of affected parents and siblings through a cascade approach.[59] Furthermore, identifying FH in childhood enables treatment to be initiated early, which could result in improved long-term outcomes for patients, although research is needed to ascertain the exact age to begin treatment and the long-term safety of LLTs.[60]

Unfortunately, screening programs are not conducted on a large scale in most countries. Slovenia initiated universal genetic screening for FH among 5-year old children in 2009.[61] A national cascade screening programs has been conducted in the Netherlands,[62] and several countries, including Spain, the UK, and Norway, have regional screening programmes.[63,64] In addition, a pilot screening program has recently started in Croatia (I Pećin, personal communication). It is important to note that the effectiveness of screening program varies

and there is disparity in detection levels between countries, ranging from 20% of patients with confirmed FH in Spain to 36% in the Netherlands and 39% in Slovenia.[60,61,63] To improve detection levels in Spain, a national cascade screening approach has recently been advocated.[65] Further improvements, in both the number of countries with screening program and the effectiveness of established screening programmes, are required to tackle the ASCVD burden associated with FH (Online Figure 1).

Lack of awareness of FH prevalence may contribute to the absence of nationwide screening program in many countries. It is also likely that the initial cost of implementing such program acts as a barrier to their establishment and use; however, screening for FH has been shown to be cost-effective. In an Australian study it was estimated that genetic cascade screening for FH would reduce the 10-year incidence of CHD from 50% to 25% among people with FH, leading to a gain of 29 quality-adjusted life-years for every 100 individuals screened.[66] Understanding the long-term benefits in terms of quality of life and healthcare resource utilization associated with early diagnosis of FH and prevention of ASCVD may act as an incentive for screening program to be initiated. Further support for genetic cascade screening in FH recently came from a study analyzing FH severity in patients diagnosed with FH as part of the Netherlands cascade screening program. This study showed that the deleterious effect of FH, both in terms of LDL-c levels and ASCVD risk is the same in people distantly related to the index patient compared with those who are more closely related, suggesting that FH severity is mainly determined by the underlying mutation.[67]

Electronic screening of patient medical records may be a further cost-effective approach to increase the rate of diagnosis of FH in primary care.[68,69] For example, Troeung et al. retrospectively screened the primary care medical records of 3,708 patients using the TARB-Ex electronic screening tool, which extracts routine clinical information from electronic medical records to derive a DLCN criteria score and identify patients at risk of FH, who may therefore require clinical investigation.[68] The records of patients with potential FH (DLCN score ≥ 5) identified by TARB-Ex were then reviewed by a primary care physician and a lipid specialist; patients subsequently considered to be at high risk of FH were recalled for clinical assessment. The TARB-Ex identified 32 patients at risk of FH compared with 22 identified by a physician-led manual review of medical records, which was considered the 'gold standard' for FH screening in this study. Sensitivity was 95.5%, specificity was 96.7%, negative predictive accuracy was 99.7%, and positive predictive accuracy was 65.6%. Ten patients were recalled for clinical examination, seven of whom attended. Six of these patients were diagnosed with phenotypic FH according to clinical criteria and one patient was referred for FH genetic testing. Electronic screening with TARB-Ex was completed in 10 minutes, compared with 60 hours for manual record review.

International and national patient registries collect data on individuals with FH in a systematic and standardized manner. This information can be useful for understanding the epidemiology of the disorder and risk factors associated with the development of ASCVD, as well as for recruiting for clinical trials, improving healthcare services, facilitating patient education and identifying gaps in knowledge.[70] Through cascade screening, such registries also support the cost-effective identification of additional patients with FH.[70,71] Examples of FH registries include international registries such as the European Atherosclerosis Society (EAS)-FH Studies Collaboration, the 10 Countries Project and the ScreenPro FH program, the HoFH International Clinical Collaborators (HICC) registry, and national registries such as the DLCN, Spanish FH Foundation, Lipid TransPort Disorders Italian Genetic Network (LIPIGEN, Italy), CASCADE FH Registry™ (USA), SWEDEHEART (Sweden), the Czech MEDPED database, and the Portuguese FH Study.[63,72-74] Several countries, including Austria, Greece, and Poland, have recently established new national FH registries, with the aim of increasing awareness of FH and stimulating the initiation of nationwide screening programmes.[63] Furthermore, the international EAS-FH Studies Collaboration (FHSC), which aims to disseminate information on the detection and management of FH, is a first step towards creating a global consensus on best practice in FH diagnosis and treatment.[63]

DIAGNOSIS

For screening program to be effective, physicians need to be aware of the diagnostic criteria for FH. Several clinical criteria algorithms are used to diagnose FH (Table 1). The DLCN criteria are widely accepted and can be used to estimate the likelihood of FH.[75] The Simon Broome Register diagnostic criteria[18] and the MEDPED/WHO criteria[76,77] are also used. In routine clinical practice, however, some data needed for the diagnostic algorithms may be inaccurate or incomplete (e.g. detailed family history of ASCVD, xanthomas).[78] It is important to note that diagnostic criteria are likely to differ by geographical region because certain clinical presentations of FH vary across different patient populations; for example, xanthelasmas have been shown to occur in 32% of patients with FH in Finland compared with 8% of patients in Norway.[79] In addition, with the increased use of LLTs among the general population, some characteristics of FH may be masked preventing diagnosis of FH in the assessed individual as well as in affected family members. In recognition of this issue, Haralambos et al. recently developed modified FH diagnostic criteria based on the DLCN criteria that additionally provide a LDL-c correction factor to estimate pre-treatment LDL-c levels in patients receiving LLT.[79] It goes without saying that secondary causes of hypercholesterolaemia, such as nephrotic syndrome, hypothyroidism, diabetes mellitus, or medication, must be excluded prior to applying any of the above algorithms.[1,80]

Table 1. FH diagnostic criteria

Criteria	Points
(a) Dutch Lipid Clinic Network diagnostic criteria	
Family history	
First-degree relative with premature[a] ASCVD OR	1
First-degree relative with LDL-c ≥ 95th percentile for age and sex	1
First-degree relative with tendon xanthomas and/or arcus cornealis OR	2
Children ≤ 18 years old with LDL-c ≥ 95th percentile for age and sex	2
Clinical history	
Patient with premature ASCVD	2
Patient with premature[a] cerebral or peripheral vascular disease	1
Physical examination	
Tendinous xanthomas	6
Arcus cornealis in patients ≤ 45 years old	4
LDL-c level, mmol/L (mg/dL)	
≥ 8.5 (330)	8
6.5–8.4 (250–329)	5
5.0–6.4 (190–249)	3
4.0–4.9 (155–189)	1
DNA analysis	
Functional mutation in *LDLR*, *APOB* or *PCSK9* gene	8
Diagnosis (point total): definite FH, > 8 points; probable FH, 6–8 points; possible FH, 3–5 points, unlikely FH, < 3 points	
(b) Simon Broome Register diagnostic criteria	
Diagnosis of definite FH	
Functional mutation in *LDLR*, *APOB* or *PCSK9* gene	
OR	
Adult: cholesterol > 7.5 mmol/dL or LDL-c > 4.9 mmol/dL	
Child:[b] cholesterol > 6.7 mmol/dL or LDL-c > 4.0 mmol/dL	
PLUS	
Tendon xanthomas in patient of first- or second-degree relative	
Diagnosis of probable FH	
Adult: cholesterol > 7.5 mmol/dL or LDL-c > 4.9 mmol/dL	
Child:[b] cholesterol > 6.7 mmol/dL or LDL-c > 4.0 mmol/dL	
PLUS	
Family history of ASCVD: < 60 years of age in a first-degree relative or < 50 years of age in a second-degree relative	
OR	
Family history of raised total cholesterol level: > 7.5 mmol/dL in an adult first- or second-degree relative or > 6.7 mmol/dL in a child[b] or sibling	

(c) MEDPED diagnostic criteria				
Age (years)	Total cholesterol cut-off points, mmol/dL			
	First-degree relative with FH	Second-degree relative with FH	Third-degree relative with FH	General population
< 20	5.7	5.9	6.2	7.0
20–29	6.2	6.5	6.7	7.5
30–39	7.0	7.2	7.5	8.8
≥ 40	7.5	7.8	8.0	9.3
Diagnosis is made if total cholesterol levels exceed cut-off points				

APOB: apolipoprotein B gene; ASCVD: atherosclerotic cardiovascular disease; FH: familial hypercholesterolaemia; LDL-c: low-density lipoprotein cholesterol; *LDLR*: low-density lipoprotein receptor gene; *PCSK9*: proprotein convertase subtilisin/kexin type 9 gene.

[a]Premature ASCVD, cerebral or peripheral vascular disease defined as occurring in males aged < 55 years and in females aged < 60 years.

[b] < 16 years of age.

Simplified diagnostic criteria would facilitate the identification of patients with FH. The recent publication of the Familial Hypercholesterolaemia Ascertainment Tool (FAMCAT), which uses data collected from primary care records, including total cholesterol levels and family history of FH, may help clinicians to identify patients with a high probability of having FH. To facilitate routine identification of patients with FH, work is underway to integrate the FAMCAT algorithm into the UK primary care computer systems, supported by a user-friendly interface.[81]

In addition, using clinical and genetic data from 64,106 patients who were screened for FH in the Dutch FH screening program, Besseling et al. developed a model to predict the presence of a FH causing mutation based on factors routinely collected in clinical practice, including: age; sex; levels of LDL-c, HDL-c, and triglycerides; history of CVD and age at onset; use of statins; smoking; alcohol; and presence of hypertension. Validation of the model in a separate patient cohort confirmed that the model showed good discrimination of patients at risk of FH. The model will be available as an online calculator to aid physicians in deciding whether or not to refer patients for genetic testing.[69]

Genomic tests to identify pathogenic mutations in *LDLR*, *APOB* and *PCSK9* are also available and may be considered the 'gold standard' for FH diagnosis; however, their use varies widely between countries, probably owing to issues of cost and availability.[6] Therefore, in clinical practice, FH is most commonly diagnosed by clinical examination and laboratory tests, because a high LDL-c level is the main clinical factor contributing to an increased ASCVD risk, and should be treated regardless of the results of mutational analysis. Genetic testing may also have an impact on issues related to life insurance reimbursement, and access to treatment.[82] In the Netherlands, issues of genetic discrimination have been circumvented by the implementation of guidelines to protect patients with FH under the Medical Examination Act (1998).[83,84] When setting up screening program, countries may also need to consider introducing relevant guidelines or laws to mitigate the potential for such genetic discrimination.

LIPID TARGETS FOR PATIENTS WITH FH

The aim of FH treatment is to reduce LDL-c levels to prevent ASCVD. The 2016 joint European Society of Cardiology and EAS guidelines recommend target LDL-c levels of less than 3.5 mmol/L (< 135 mg/dL) in children with FH over 10 years of age, 50% reduction of LDL-c at younger ages, less than 2.6 mmol/L (< 100 mg/dL) in adults with FH, or less than 1.8 mmol/L (< 70 mg/dL) in adults with FH in the presence of ASCVD.[85] LDL-c targets apply

for both HeFH and HoFH; however, with current treatment options, these are very difficult to achieve in children and adults with HoFH.[6]

TREATMENT OPTIONS

DIET AND LIFESTYLE MODIFICATIONS

Diet and lifestyle have an effect on LDL-c levels and ASCVD risk.[86,87] Patients with FH should be counselled regarding lifestyle modifications to reduce fat and cholesterol intake, to avoid tobacco products, and to balance physical activity with caloric intake to maintain a healthy BMI.[1] It is important to note that, although FH cannot be managed by diet and lifestyle changes alone, healthy lifestyle modifications should be used in conjunction with optimized LLT in order achieve LDL-c targets and minimize ASCVD risk.[88]

STATINS AND OTHER LIPID-LOWERING THERAPIES

Statins are the cornerstone of treatment for patients with FH; however, target LDL-c levels are not reached in a large proportion of patients, despite the use of statins and additional LLT.[89] Statins lower LDL-c by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A reductase, which results in upregulation of the LDLR.[90] The 2013 EAS consensus statement recommends that patients with FH receive high-dose statin treatment (atorvastatin 80 mg/day, rosuvastatin 40 mg/day or pitavastatin 4 mg/day) at diagnosis.[6] The efficacy of statins in reducing morbidity and improving survival rates in patients with FH has been demonstrated in retrospective and cohort studies. In a retrospective study, statins were shown to reduce the risk of CHD by 76% in patients with FH who received statins before the onset of CHD.[52] In addition, the results of a cohort study of 3,382 patients with FH indicated that treatment with statins resulted in a 37% reduction in CHD mortality.[91] There is, however, a lack of data from randomized placebo-controlled trials on the clinical benefit of statins in patients with FH. Given the high risk of ASCVD in these patients, such trials are not ethically justifiable. However, the randomized placebo-controlled Lipid Research Clinics Coronary Primary Prevention Trial, which studied a patient population that was likely to be enriched for individuals with FH, may provide some insights into the clinical benefit of statins. The study showed that patients receiving the statin cholestyramine had a 12% reduction in LDL-c and a 19% reduction in CHD risk compared with placebo-treated patients.[92] In addition, a real-world retrospective analysis of 2,447 patients with HeFH found that moderate- to high-intensity statin therapy lowered the risk of ASCVD and death by 44% compared with the risk in patients who had never received statins.[12]

Despite the administration of high-dose potent statins, nearly 80% of patients with HeFH do

not achieve target LDL-c levels.[89] These individuals require additional agents to enable them to reach these targets. Statin intolerance may contribute to an insufficient LDL-c response to therapy. There is, however, a lack of data on the incidence of statin intolerance and it is likely that non-adherence to statin treatment is the main cause of suboptimal outcomes in patients prescribed statins.[93,94]

Ezetimibe decreases cholesterol absorption at the brush border of the small intestine by inhibiting Niemann-Pick C1-like protein (NPC1L1)[95] and can be used in combination with statin therapy when LDL-c targets have not been met with statin monotherapy.[6,85] Ezetimibe is useful in the management of patients with HoFH because the mechanism of action does not rely on *LDLR* expression.[95] The clinical efficacy of ezetimibe was questioned following results from the Ezetimibe and Simvastatin in Hypercholesterolemia Enhances Atherosclerosis Regression (ENHANCE) trial, which showed that addition of ezetimibe to simvastatin led to a reduction in LDL-c, but not to a reduction in carotid intima–media thickness.[96] Recent data from the IMProved Reduction of Outcomes: Vytorin Efficacy International Trial (IMPROVE-IT), however, suggest that the further reduction in cholesterol levels in patients receiving ezetimibe provides clinical benefit, in terms of reducing both LDL-c levels and cardiovascular events.[97] Ezetimibe can also be prescribed as monotherapy to individuals who are intolerant of statins.[98,99]

Bile-acid-binding resins (cholestyramine, colestipol or colesevelam) decrease the absorption of bile acid, resulting in increased conversion of cholesterol to bile acids and enhanced production of LDLR.[95] For patients with FH and established ASCVD, combination therapy with a statin, ezetimibe and a bile-acid-binding resin is recommended.[8,85]

Statins are less effective in patients with HoFH than in those with HeFH due to the severely decreased LDLR function in HoFH patients.[100] The therapy of choice for these patients is LDL apheresis in combination with high-intensity statin treatment, with or without ezetimibe.[5,6] LDL apheresis should also be considered in patients with HeFH who are intolerant to statins or in whom LDL-c levels and ASCVD risk remain high following maximally tolerated LLT.[101] Although apheresis lowers LDL-c levels by 55–70%, the effect is transient and levels rebound to pre-apheresis concentrations within a few days.[102] In addition, apheresis is expensive and time-consuming to administer, and therefore is not widely available.[102]

Lomitapide is a new LLT that inhibits the microsomal triglyceride transfer protein, which functions in the production of LDL. Phase 3 data from a single-arm study in 29 patients with HoFH showed that, at week 26, addition of lomitapide to current LLT reduced LDL-c levels by 50% from baseline.[103]

Mipomersen is an antisense oligonucleotide that inhibits ApoB protein synthesis. In phase 3 trials, compared with placebo, addition of mipomersen to maximally tolerated statin therapy (with or without other LLT) has been shown to reduce LDL-c levels significantly in patients with HeFH and CHD,[104] and in patients with HoFH.[105] Mipomersen is approved in the USA in combination with LLTs for the treatment of patients with HoFH.[106] Approval has not been granted in Europe owing to hepatic and cardiac safety concerns.[107]

Fibrates are agonists of peroxisome proliferator-activated receptor-α, which via regulation of transcription factors regulates various steps in lipid and lipoprotein metabolism.[85] In combination with the maximum tolerated dose of statins, fibrates should be considered in patients with FH who have elevated triglycerides and low HDL-c, or in those with serum triglycerides levels greater than 5.7 mmol/L (> 500 mg/dL).[6] However, it must be noted that the lipid-lowering effect of fibrates is minimal, and with more potent therapies now available, they are unlikely to be a standard treatment choice for patients with FH.

Overall, current data suggest that the treatment of patients with FH is often not optimal. In a population-based cohort study, individuals with FH who were not treated with LLT had a 13-fold increased risk of CHD compared with those without FH. This risk was reduced, to 10-fold, when LLTs were used.[13] This highlights the need to monitor response to therapy and to ensure that patients with FH are receiving treatment of sufficient intensity. A 2-year prospective study of 325 patients with FH showed that high-intensity statin therapy (high-dose atorvastatin) was more effective at reducing LDL-c levels and decreasing carotid intima–media thickness than lower-intensity statin therapy (simvastatin).[108] Furthermore, pharmacokinetic alterations have been reported when statins are co-administered with drugs metabolized through the cytochrome P450, 3A4 or 2C9 pathways (through which most statins are metabolized). These changes could lead to reduced efficacy and an increase in adverse events (AEs), making statins unsuitable for patients who require certain concomitant medications.[109,110] Novel treatments are required to prevent cardiovascular events effectively in patients with FH, particularly in individuals with established ASCVD or homozygous mutations, and in those who are intolerant to statin therapy.

PCSK9 INHIBITORS

Monoclonal antibodies that inhibit PCSK9 are a promising treatment modality for patients with FH.[111] PCSK9 is a serine protease secreted by hepatocytes that binds to the LDLR and promotes its degradation.[112] Monoclonal antibodies to PCSK9 prevent its interaction with the LDLR and thereby restore LDLR recycling and LDL-c uptake.[113] A hint towards a beneficial effect of PCSK9 inhibition was derived from studies in patients carrying non-sense mutations in *PCSK9* that were found to be associated with low LDL-c levels and a reduced

risk of CHD.[114] Two antibodies to PCSK9, evolocumab and alirocumab, were approved in 2015 in the USA, Canada and Europe for the treatment of patients with FH in whom target LDL-c levels are not achieved with available therapies.[115-119] Recently, the global clinical development program for a third PCSK9 antibody, bococizumab, was discontinued owing to an unanticipated attenuation of efficacy over time associated with higher immunogenicity and a higher rate of injection-site reactions than seen with other agents in this class.[120] RNA interference (RNAi) inhibition of proprotein convertase subtilisin/kexin type 9 (PCSK9) is also being investigated.[121] ALN-PCSsc is a first-in-class RNAi that acts by switching off PCSK9 synthesis in the liver.[121] Promising efficacy and safety results have been reported in a phase 1 study in healthy volunteers and in a recent phase 2 study in patients at high risk of ASCVD who had elevated LDL-c levels;[122,123] however, phase 3 data in patients with FH are required to evaluate the role of this therapy in FH.

Evolocumab and alirocumab have shown efficacy in randomized controlled trials in reducing LDL-c levels in patients with FH at high risk of developing ASCVD. Evolocumab has been evaluated in a broad patient population, including individuals whose LDL-c levels were not controlled by statin therapy, patients with HoFH and those with severe atherosclerosis. In the Reduction of LDL-c with PCSK9 Inhibition in Heterozygous Familial Hypercholesterolemia Disorder (RUTHERFORD) phase 3 trial, 331 patients with HeFH whose LDL-c levels were not adequately controlled with LLT received evolocumab (140 mg every 2 weeks or 420 mg monthly) in addition to current therapy. Following 12 weeks of treatment, LDL-c levels were reduced by 60% in patients receiving evolocumab. These patients also experienced a 30% reduction in Lp(a) levels compared with those receiving placebo.[124] This might have a large clinical impact because elevated Lp(a) is an independent risk factor for ASCVD in patients with FH.[25,34] Evolocumab has also shown efficacy in patients with HoFH. In the phase 3 Trial Evaluating PCSK9 Antibody in Subjects with LDL Receptor Abnormalities (TESLA) study involving 50 patients who received LLT but did not undergo apheresis, 12 weeks of treatment with evolocumab 420 mg monthly led to a significant mean LDL-c reduction of 31% compared with placebo.[125]

Alirocumab has shown efficacy in reducing LDL-c and Lp(a) levels in patients with HeFH. In the multicenter, placebo-controlled randomized phase 3 trials ODYSSEY FH I and II, 735 patients with HeFH, with or without a history of ASCVD, whose LDL-c levels were not controlled by the maximum tolerated dose of statins, were randomly allocated to receive alirocumab 75 mg every 2 weeks, increasing to 150 mg every 2 weeks if LDL-c levels remained above 70 mg/dL, or placebo. After 24 weeks, alirocumab treatment led to a 49% reduction in LDL-c levels, with 40% of patients requiring the 150 mg dose.[126]

PCSK9 inhibitors have also shown promising results in treating patients with FH whose LDL-c levels are difficult to control and for whom regular LDL-c apheresis is required. In a small study of three patients with FH and CHD, switching from LDL-c apheresis to evolocumab maintained LDL-c lowering.[127] After apheresis, HDL-c levels increased and remained constant on evolocumab treatment. Evolocumab was also associated with a non-significant trend towards improved patient quality of life.[127] The ODYSSEY ESCAPE study assessed the efficacy of alirocumab in 62 patients with severe HeFH who had been receiving regular LDL-c apheresis for a mean of 7 years; 46% of patient receiving alirocumab and 62% of those receiving placebo were also receiving a statin therapy. Treatment with alirocumab 150 mg every 2 weeks significantly reduced the frequency of required apheresis treatments by 75% ($p < 0.0001$) from week 7 to week 18. Apheresis was no longer required in 63% of patients receiving alirocumab compared with 0% of patients receiving placebo.[128]

As well as a strong efficacy profile, PCSK9 inhibitors have been shown to be well tolerated. In the phase 3 RUTHERFORD trial, the rates of AEs with evolocumab were similar to those seen with placebo. The most common AEs in patients receiving evolocumab were nasopharyngitis and muscle-related AEs, occurring in 9% and 5% of patients, respectively, compared with 5% and 1% of those receiving placebo, respectively.[124] Injection-site reactions occurred at similar frequencies in patients receiving evolocumab (6%) and in those receiving placebo (4%). No patients discontinued treatment owing to an AE.[124] As with evolocumab, similar AEs were associated with alirocumab. In the phase 3 ODYSSEY FH I and II trials, the most common AEs were injection-site reactions and nasopharyngitis. Injection-site reactions occurred in 12% and 11% of patients receiving alirocumab in ODYSSEY FH I and FH II, respectively, compared with 11% and 7% of those receiving placebo, respectively. Nasopharyngitis occurred in 11% and 13% of those receiving alirocumab in FH I and FH II, respectively, compared with 7% and 22% of those receiving placebo. Few patients discontinued treatment owing to AEs.[126] A recent meta-analysis on the long-term safety of PCSK9 inhibitors suggested that PCSK9 inhibitors are not associated with an increased risk of cumulative severe AEs, musculoskeletal effects or stroke compared with standard of care.[129] A subgroup analysis of larger outcome studies found a two-fold increase in the incidence of neurocognitive events with PCSK9 inhibitors compared with standard of care.[129] However, the results of the non-inferiority EBBINGHAUS cognitive function study, conducted in 1,900 patients enrolled in the Further Cardiovascular Outcomes Research With PCSK9 Inhibition in Subjects With Elevated Risk (FOURIER) study, found that evolocumab did not increase the risk of impairment of cognitive function compared with placebo.[130]

PCSK9 inhibitors have been shown to reduce LDL-c levels, but their impact on long-term disease progression and clinical outcomes is less well established. The recent Global

Assessment of Plaque Regression With a PCSK9 Antibody as Measured by Intravascular Ultrasound (GLAGOV) study of 968 patients with angiographic coronary disease assessed the impact of PCSK9 inhibition on the progression of coronary atherosclerosis. After 78 weeks, compared with patients receiving statins and placebo, those receiving evolocumab and statins had a significantly greater percentage reduction in atheroma volume (−0.95% vs. 0.05%; $p < 0.0001$) and absolute atheroma volume (−5.8 mm^3 vs. −0.9mm^3; $p < 0.001$).[131] Although this study was not conducted in patients with FH, these data suggest that addition of evolocumab to statin therapy could lead to significant regression of atherosclerotic plaques. This could be beneficial for individuals with FH and ASCVD, if the results are replicated in this patient population.

Preliminary long-term efficacy data on PCSK9 inhibitors preventing MACE are also encouraging. In two open-label randomized trials of evolocumab (OSLER-1 and OSLER-2), 4,465 patients, of whom 10% had FH, received standard therapy or evolocumab (140 mg every 2 weeks or 420 mg monthly) plus standard therapy. At 1 year, patients receiving evolocumab had a significant reduction in the rate of MACE compared with individuals receiving standard therapy alone (0.95% vs. 2.18%; $p = 0.003$).[132] Similarly, in the phase 3 ODYSSEY Long Term trial of alirocumab, 2341 patients at high risk of ASCVD, of whom 18% had HeFH, received alirocumab 150 mg every 2 weeks for 78 weeks.[133] In a post hoc analysis, the rate of MACE was lower for patients receiving alirocumab than for those receiving placebo (1.7% vs. 3.3%; $p = 0.02$).

It should be noted, however, that in OSLER 1 and 2 and ODYSSEY Long Term, the MACE event numbers were very low in both the treatment and control groups, and a longer follow-up period is required to confirm the long-term impact of PCSK9 inhibitors on the rate of MACE. The results of such prospective interim analyses give support to, but are not proof of, the efficacy of PCSK9 inhibitors in the prevention of MACE. Recently, the results of the long-term FOURIER trial have shown that at a median follow-up of 2.2 years additional LDL-c lowering with evolocumab (in combination with optimized statin therapy) significantly reduces the risk of cardiovascular events in patients with ASCVD and high LDL-c levels (> 1.8 mmol/L) compared with placebo ($p < 0.001$).[134] The ODYSSEY OUTCOMES trial assessing the extent to which alirocumab reduces the risk of MACE is ongoing.[135,136] Long-term follow up of cardiovascular outcomes in patients receiving the now discontinued PCSK9 inhibitor bococizumab suggests that, compared with placebo, after a median follow-up of 12 months bococizumab significantly reduced MACE in patients with a high risk of MACE (LDL-c > 2.6 mmol/L; $p = 0.02$). However, after a median follow-up of 7 months bococizumab provided no benefit compared with placebo in patients at low risk of MACE (LDL-c level > 1.8 mmol/L; $p = 0.94$).[137]

As with all new treatments, the benefit of PCSK9 inhibitors in patients with FH must be considered in relation to their cost. A recent cost-effectiveness analysis conducted in the USA determined that at their 2015 prices, PCSK9 inhibitors did not meet incremental cost-effectiveness thresholds.[138] However, it must be noted that the pricing structure and the cost-effectiveness model used in this analysis apply specifically to the USA and are not applicable to other regions. Indeed, in Europe the National Institute for Health and Care Excellence has determined that both evolocumab and alirocumab have favorable incremental cost-effectiveness ratios in patients with HeFH.[139,140]

TREATMENT: WHEN TO USE PCSK9 INHIBITORS

Patients with FH and ASCVD, or another major risk factor for ASCVD such as diabetes mellitus with target organ damage or hypertension, or those with severe HeFH should receive statins (preferably atorvastatin or rosuvastatin) at the maximally tolerated dose plus ezetimibe.[141] Patients who have a less-than-anticipated response on maximally tolerated statin therapy (< 50% reduction in LDL-c), should be assessed for adherence by evaluating the number of missed statin doses per month and any barriers to adherence. Patients who are unable to tolerate even a moderate-intensity statin should be evaluated for statin intolerance and considered for referral to a lipid specialist.[142] Physicians should consider adding a PCSK9 inhibitor to the regimen for patients with ASCVD, or a major risk factor for ASCVD, if LDL-c levels are more than 3.6 mmol/L (> 140 mg/dL) or more than 2.6 mmol/L (> 100 mg/dL) with evidence of rapid progression of ASCVD. Patients with severe HeFH without ASCVD should be considered for PCSK9 inhibition therapy if LDL-c levels are more than 5.0 mmol/L (> 200 mg/dL) or more than 4.5 mmol/L (> 175 mg/dL) in the presence of one or more risk factors for ASCVD including diabetes mellitus, elevated lipoprotein levels (> 50 mg/L), hypertension and premature familial ASCVD. Most patients with HoFH should receive maximal LLT including LDL apheresis plus a PCSK9 inhibitor. However, it should be noted that patients with a homozygous null mutation in LDLR should not receive a PCSK9 inhibitor.[141] Treatment options for patients with HeFH and HoFH are presented in Table 2.

Table 2. Summary of treatment options for patients with FH

Treatment	Mechanism of action	HeFH	HoFH
Statins (atorvastatin, fluvastatin, pravastatin, rosuvastatin, simvastatin)	Upregulate LDLR through inhibition of HMG-CoA reductase	A first-line treatment option for patients with HeFH. Patients should receive up to the maximum approved/tolerated dose in order to lower LDL-c levels[6]	A first-line treatment option for patients with HoFH. Patients should receive up to the maximum approved/tolerated dose in order to lower LDL-c levels[6]
Ezetimibe	Inhibits cholesterol absorption in the small intestine	Can be administered in combination with statins for patients not reaching LDL-c levels [6] or as a single agent for those who are intolerant to statins[98]	Can be administered in combination with statins for patients not reaching LDL-c levels [6] or as a single agent for patients who are intolerant to statins[98]
Bile acid sequestrants (colesevelam, colestipol, cholestyramine)	Bind bile components in the gastrointestinal tract leading to increased production of bile, which requires LDL	Can be administered in combination with statins and ezetimibe for patients with a very high risk of CHD/established CHD/ type 2 diabetes mellitus/LDL-c levels > 1.8 mmol/dL or ~70 mg/dL[6]	Can be administered in combination with statins and ezetimibe for patients with a very high risk of CHD/established CHD/ type 2 diabetes mellitus/ LDL-c levels > 1.8 mmol/dL or ~70 mg/dL[6]
Fibrates (bezafibrate, ciprofibrate, fenofibrate, gemfibrozil)	Increase lipid catabolism through activation of peroxisome proliferator activated receptors	Can be administered in combination with other LLTs after first-line therapy has failed; however, combination with statins increases risk of myopathy. Fibrates are not recommended in patients without elevated triglyceride levels[143]	Can be administered in combination with other LLTs after first-line therapy has failed; however, combination with statins increases risk of myopathy. Fibrates are not recommended in patients without elevated triglyceride levels[143]
Apheresis	Physical removal of LDL from the blood	Can be administered in treatment-resistant patients with CHD[6]	A first-line treatment option for patients with HoFH. Apheresis should be initiated as early as possible following diagnosis. Treat every 1–2 weeks[5]
Lomitapide	Inhibits VLDL assembly	X	Recommended in combination with other LLTs, with or without apheresis[5]
Mipomersen	Inhibits synthesis of apolipoprotein B-100 in the liver	X	Recommended in combination with other LLTs in the USA.[106] Not currently approved in Europe[107]
PCSK9 inhibitors (evolocumab, alirocumab)	Block LDLR degradation	Indicated in adults in combination with a statin or statin with other LLTs in patients unable to reach target LDL-c levels with the maximum tolerated statin dose, or alone or in combination with other LLTs in patients who are statin intolerant, or for whom a statin is contraindicated[115, 116]	Evolocumab is indicated in adults and adolescents aged 12 years and over in combination with other LLTs.[115] Alirocumab is not indicated in patients with HoFH[116]

CHD: coronary heart disease; FH: familial hypercholesterolaemia; HeFH: heterozygous familial hypercholesterolaemia; HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzyme A; HoFH: homozygous familial hypercholesterolaemia; LDL: low-density lipoprotein; LDL-c: low-density lipoprotein cholesterol; LDLR: low-density lipoprotein receptor; LLT, lipid-lowering therapies; PCSK9: proprotein convertase subtilisin/kexin type 9; VLDL: very low-density lipoprotein cholesterol.

CONCLUSIONS

FH is common in patients presenting with cardiovascular events, particularly when the events occur at an early age. Screening for FH in individuals with ASCVD is currently inadequate, leading to a missed opportunity to initiate preventive therapies and to reduce the morbidity and mortality associated with FH. Primary care physicians are at the front line of FH screening and thus need to be informed about the prevalence of FH and how to diagnose the disorder so that interventional treatments can be administered before the onset of ASCVD. Cardiologists are likely to encounter a large proportion of patients with FH and with increased awareness and appropriate support, they can make a substantial positive impact on outcomes in these individuals. Ultimately, the care of people with FH requires a multi-disciplinary approach involving primary care physicians, lipid specialists, cardiologists, nutritionists, nurses, pharmacists and patient support groups. Statin therapy reduces LDL-c levels and ASCVD risk, and treatment with high-dose statins should be initiated in patients with FH. For individuals at highest risk of developing ASCVD, additional therapies are required to control their disease adequately. Furthermore, data suggest that patients with potential FH should also be treated because the associated raised LDL-c levels substantially increase the risk of ASCVD.[10] New PCSK9 inhibitors offer an effective and well-tolerated treatment option for patients with FH.

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8

ALIROCUMAB EFFICACY IN PATIENTS WITH DOUBLE HETEROZYGOUS, COMPOUND HETEROZYGOUS, OR HOMOZYGOUS FAMILIAL HYPERCHOLESTEROLEMIA

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ABSTRACT

BACKGROUND

Mutations in the genes for the low-density lipoprotein receptor (*LDLR*), apolipoprotein B *(APOB)*, and proprotein convertase subtilisin/kexin type 9 *(PCSK9)* have been reported to cause heterozygous and homozygous familial hypercholesterolemia (FH).

OBJECTIVE

The objective is to examine the influence of double heterozygous, compound heterozygous, or homozygous mutations underlying FH on the efficacy of alirocumab.

METHODS

Patients from six alirocumab trials with elevated low-density lipoprotein cholesterol (LDL-c) and FH diagnosis were sequenced for mutations in the *LDLR*, *APOB*, *PCSK9*, LDLR adaptor protein 1 (*LDLRAP1*), and signal-transducing adaptor protein 1 *(STAP1)* genes. The efficacy of alirocumab was examined in patients who had double heterozygous, compound heterozygous, or homozygous mutations.

RESULTS

Of 1,191 patients sequenced, 20 patients were double heterozygotes (n = 7), compound heterozygotes (n = 10), or homozygotes (n = 3). Mean baseline LDL-c levels were similar between patients treated with alirocumab (n = 11; 198 mg/dL) vs placebo (n = 9; 189 mg/dL). All patients treated with alirocumab 75/150 or 150 mg every two weeks had an LDL-c reduction of ≥ 15% at either week 12 or 24. At week 12, one patient had an increase of 7.1% in LDL-c, whereas in others, LDL-c was reduced by 21.7% to 63.9% (corresponding to 39–114 mg/dL absolute reduction from baseline). At week 24, LDL-c was reduced in all patients by 8.8% to 65.1% (10–165 mg/dL absolute reduction from baseline). Alirocumab was generally well tolerated in the six trials.

INTRODUCTION

Mutations in the genes for the low-density lipoprotein receptor (*LDLR*), apolipoprotein B (*APOB*), and proprotein convertase subtilisin/kexin type 9 (*PCSK9*) have been reported to cause heterozygous familial hypercholesterolemia (HeFH) and homozygous familial hypercholesterolemia (HoFH), conditions which are characterized by high levels of low-density lipoprotein cholesterol (LDL-c) and increased risk of coronary heart disease.[1-3] Mutations in LDLR adaptor protein 1 (*LDLRAP1*) gene are recessive and cause HoFH.[1] LDL-c levels can vary markedly due to the phenotypic variability of mutations in the LDL-c pathway. Residual LDLR pathway activity correlates with disease severity and response to some lipid-lowering agents.[4,5] For example, the majority of patients who are *LDLR* negative have higher LDL-c levels and poorer clinical prognosis compared with patients who are *LDLR* defective.[6,7]

In general, patients with homozygous (identical mutations in both alleles) *LDLR* negative mutations or with compound heterozygous (different mutations in both alleles of the same gene) *LDLR* negative mutations have the highest mean LDL-c levels overall.[5] This is followed by those with compound heterozygous *LDLR* defective plus *LDLR* negative mutations, those with homozygous *LDLRAP1* or *LDLR* defective mutations, those with homozygous *APOB* or *PCSK9* gain of function (GOF) mutations, those with double heterozygous (mutations in two different genes) mutations, and then those with HeFH.[5,8] However, LDL-c level is the main determinant of cardiovascular disease risk and not the genetic defect per se.[7,9]

We have previously reported the effect of single mutations in genes causative for familial hypercholesterolemia (FH) in 1,191 patients enrolled in one phase 2 and five phase 3 studies of the PCSK9 antibody alirocumab.[10] Here, we focus on the treatment effect of alirocumab in patients with FH who were double heterozygotes, compound heterozygotes, or homozygotes.

METHODS

DNA samples from patients with a diagnosis of FH who were enrolled and provided written consent for participation in six clinical trials, and also provided written consent for the present genotyping analysis, were sequenced for mutations in genes causative for FH (*LDLR, APOB, PCSK9, LDLRAP1,* and signal-transducing adaptor protein 1 (*STAP1*)). The trials included one phase 2 trial (NCT01375764)[11] and five phase 3 clinical trials from the ODYSSEY program (LONG TERM[NCT01507831],[12] HIGH FH [NCT01617655],[13] FH I [NCT01623115], FH

II [NCT01709500],[14] and ALTERNATIVE [NCT01709513][15]). The original diagnosis of FH was performed either by previous genotyping or on clinical presentation. Clinical diagnosis was based on the Simon Broome criteria for definite FH or the World Health Organization/ Dutch Lipid Network criteria (score > 8 points).[16–18] The original genotyping results were not recorded in the trials; hence, patients were sequenced regardless of how they were originally diagnosed. Full details of the genotyping analysis for the present study have been described previously.[10]

The present analysis focuses on those patients who had more than one mutation in one or more of the sequenced genes. No patients from the ALTERNATIVE trial were found to have more than one mutation. Study designs of the other trials were as follows. In the 12-week phase 2 study, patients received 1 of 4 alirocumab doses (150 mg every 2 weeks [Q2W], 150 mg every 4 weeks [Q4W], 200 mg Q4W, 300 mg Q4W) or placebo.[11] In the 78-week phase 3 trials, patients received either alirocumab 150 mg Q2W (LONG TERM and HIGH FH) or an initial alirocumab dose of

75 mg Q2W, which was increased to 150 mg Q2W at week 12 if LDL-c was ≥ 70 mg/dL at week 8 (FH I and FH II); control was placebo in each trial.[12–14] The primary efficacy endpoint in the phase 3 trials was the percentage reduction in LDL-c from baseline to week 24. Safety assessments included treatment-emergent adverse events, which were events occurring from first to last dose and up to 70 days after the last dose (follow-up).

LDL-c levels were calculated using the Friedewald equation[19] except when triglyceride levels exceeded 400 mg/dL, in which case LDL-c was determined by direct measurement using beta quantification.[20] In this post hoc analysis, a clinically meaningful response to alirocumab was defined as a reduction in LDL-c of ≥ 15% at week 12 or 24 (the available timepoints), as described previously.[10] Analysis of lipid and lipoprotein parameters was performed at a central laboratory. Lipoprotein (a) [Lp(a)] levels were analyzed using a validated immunoturbidimetric assay as previously described.[21]

RESULTS

PATIENTS

Of 1,191 patients sequenced, 20 patients were double heterozygous (n = 7), compound heterozygous (n = 10), or homozygous (n = 3) for genes causative of FH and included in the present analysis (Table 1). Six patients were double heterozygotes with mutations in both *APOB* and *LDLR*, of whom three patients were *APOB* defective/*LDLR* negative and the

remaining three *APOB* defective/*LDLR* defective. One patient was double heterozygote with *LDLR* negative and *PCSK9* GOF mutations. Of those who were compound heterozygotes, three were *LDLR* defective/*LDLR* negative, and seven were *LDLR* defective/*LDLR* defective. Of the three patients who were homozygotes, one had *LDLR* defective mutations (further details on this patient are presented in the Online Data Supplement) and two were homozygous for mutations in *LDLRAP1***.** In this analysis, 11 of 20 patients received alirocumab, and the remaining 9 received placebo (Table 1). The mean age at baseline was 49.2 years, and 50% were males. Baseline characteristics of individual patients are presented in Table 1 in the Online Data Supplement. The mean baseline LDL-c level was 198 mg/dL for those treated with alirocumab and 189 mg/dL for those treated with placebo. All patients were receiving concomitant statin, and the majority were receiving additional lipid-lowering therapies at baseline (Table 1 in the Online Data Supplement). Most patients were at very-high cardiovascular risk at baseline. The cardiovascular history of individual patients at baseline is presented in Table 2 in the Online Data Supplement.

Table 1. Distribution of mutations and treatment received by each patient (sequenced cohort)

Patient number	Study	Mutation category	Genotype	Treatment
1	FH I	*APOB* defective/*LDLR* negative	p.Arg3527Gln.c.1846-?_21401?del	Alirocumab 75/150 mg Q2W†
2	HIGH FH	*APOB* defective/*LDLR* negative	p.Arg3527Gln.2390-?_25831?del	Alirocumab 150 mg Q2W
3	FHI	*APOB* defective/*LDLR* defective	p.Arg3527Gln.p.Asp227Glu	Alirocumab 75/150 mg Q2W†
4	FH II	*APOB* defective/*LDLR* defective	p.Arg3527Gln.p.Cys209Tyr	Alirocumab 75/150 mg Q2W†
5	FH II	*LDLR* defective/*LDLR* negative	c.(-16)G > C.p.Trp562*	Alirocumab 75/150 mg Q2W†
6	FH II	*LDLR* defective/*LDLR* negative	c.31311G > A.p.Val462Ile	Alirocumab 75/150 mg Q2W†
7	R727-CL-1003 phase 2	*LDLR* defective/*LDLR* defective	p.Arg81Cys.c.(≥268)G > T	Alirocumab 150 mg Q2W
8	HIGH FH	*LDLR* defective/*LDLR* defective	p.Asp266Asn.p.Gly592Glu	Alirocumab 150 mg Q2W
9	HIGH FH	*LDLR* defective homozygous	p.Asp227Glu.p.Asp227Glu	Alirocumab 150 mg Q2W
10	FH I	*LDLRAP1* negative	c.34411G > A.c.34411G > A	Alirocumab 75/150 mg Q2W†
11	R727-CL-1003 Phase 2	*LDLR* negative/*PCSK9* GOF	p.Cys143.p.Leu22_Leu23dup	Alirocumab 150 mg Q4W
12	FH I	*APOB* defective/*LDLR* negative	p.Arg3527Gln.p.Tyr375Trpfs*7	Placebo
13	FH I	*APOB* defective/*LDLR* defective	p.Arg3527Gln.p.Gly478Arg	Placebo
14	FH I	*LDLR* defective/*LDLR* negative	p.Glu600Asp.c.191-?_10601?del	Placebo
15	FH I	*LDLR* defective/*LDLR* defective	p.Glu408Lys.p.Gln770Arg	Placebo
16	FH I	*LDLR* defective/*LDLR* defective	p.Glu337Lys.p.Asp482Asn	Placebo
17	LONG TERM	*LDLR* defective/*LDLR* defective	p.Asp651Asn.p.Asp221Gly	Placebo
18	LONG TERM	*LDLR* defective/*LDLR* defective	p.Asp700Glu.p.Asp227Glu	Placebo
19	LONG TERM	*LDLR* defective/*LDLR* defective	p.Leu432Val.p.Tyr465Asn.p.Pro685Leu	Placebo
20	LONG TERM	*LDLRAP1* negative	p.Gly24Alafs*32.p.Gly24Alafs*32	Placebo

APOB: apolipoprotein B; GOF: gain of function, LDL-c: low-density lipoprotein cholesterol, LDLR: low-density lipoprotein receptor, LDLRAP1: LDLR adaptor protein 1, PCSK9: proprotein convertase subtilisin/kexin type 9; Q2W, every 2 weeks; Q4W, every 4 weeks. †Alirocumab 75 mg Q2W was increased to 150 mg Q2W at week 12 depending on LDL-c at week 8. ‡Data for all lipid endpoints were not available for patient 2.

INFLUENCE OF DOUBLE HETEROZYGOUS, COMPOUND HETEROZYGOUS, OR HOMOZYGOUS MUTATIONS ON THE EFFICACY OF ALIROCUMAB

Percentage changes from baseline in LDL-c at weeks 12 and 24 for individual patients with available data are shown in Figure 1; absolute changes are shown in Table 3 in the Online Data Supplement. In this analysis, an LDL-c reduction of ≥ 15% at week 12 or 24 was observed in patients who had received alirocumab 75/150 or 150 mg Q2W (Fig. 1). At week 12, an LDL-c reduction of 21.7% to 63.9% (corresponding to 39–114 mg/dL absolute reduction) with alirocumab treatment was observed in all but one patient (patient 10, *LDLRAP1* negative, baseline LDL-c 140 mg/dL, from the FH I study) who had an LDL-c increase of 7.1%; however, this patient had an LDL-c reduction of 34.3% (absolute reduction of 48 mg/dL) from baseline to week 24. LDL-c reduction from baseline to week 24 in other patients was 8.8% to 65.1% (absolute reduction of 10–165 mg/dL).

Furthermore, patient 5 (*LDLR* defective/*LDLR* negative from the FH II study) had an LDL-c reduction of 52.6% (absolute reduction of 60 mg/dL from baseline value of 114 mg/dL) at week 12, compared with a reduction of 8.8% (absolute reduction of 10 mg/dL) at week 24. Patient 9 (*LDLR* defective homozygous from the HIGH FH study) had an LDL-c reduction of 22.9% (absolute reduction of 92 mg/dL from baseline value of 402 mg/dL) at week 12 compared with a reduction of 11.9% (absolute reduction of 48 mg/dL) at week 24.

Overall, alirocumab treatment provided LDL-c reductions of 39.3% to 55.7% and 55.1% to 62.0% in patients with double heterozygous mutations (*APOB* defective/*LDLR* negative and *APOB* defective/*LDLR* defective) at weeks 12 and 24, respectively. The corresponding reductions in patients with compound heterozygous mutations (*LDLR* defective/*LDLR* negative and *LDLR* defective/*LDLR* defective) were 21.7% to 63.9% and 8.8% to 65.1% at weeks 12 and 24, respectively. At week 12, two patients (patients 5 and 6, both *LDLR* defective/*LDLR* negative) achieved an LDL-c level of < 70 mg/dL with alirocumab treatment. In addition, two patients (patient 1 [*APOB* defective/*LDLR* negative] and patient 4 [*APOB* defective/*LDLR* defective]) achieved LDL-c < 100 mg/dL. Overall, the LDL-c levels were maintained in these patients at week 24 except in patient 5 who had an LDL-c level of 104 mg/dL, compared with 54 mg/dL at week 12.

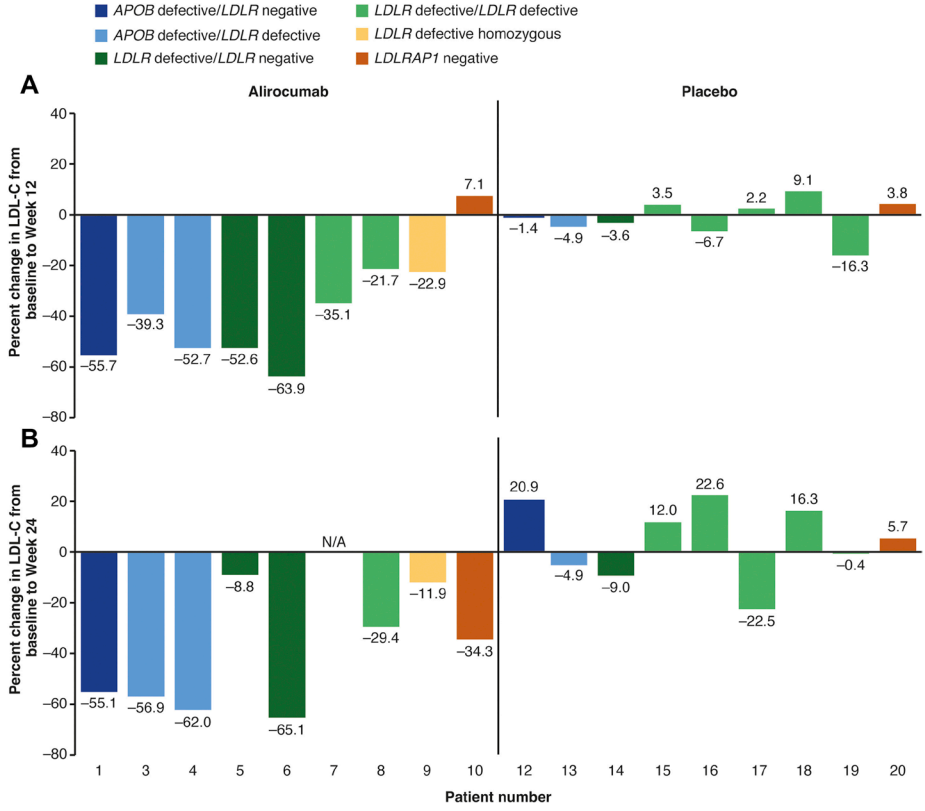
Reductions with alirocumab treatment at weeks 12 and 24 were also observed across the mutation backgrounds in ApoB, Lp(a), non–high-density lipoprotein cholesterol, and triglycerides (Figure 1 through 4 in the Online Data Supplement). The patient with the *PCSK9* GOF and *LDLR* negative mutations (patient 11) received a different administration regimen of alirocumab (150 mg Q4W) in the phase 2 study and is not included in Figure 1 or in Figure 1 through 4 in the Online Data Supplement; an LDL-c reduction of 44.1% (corresponding to

60 mg/dL absolute reduction in LDL-c from baseline value of 136 mg/dL) was observed at week 10, 2 weeks after the last alirocumab dose was administered.

SAFETY

Safety data for all patients sequenced for mutations in genes causative for FH (n = 1,191) have been reported previously.[10] The rates of treatment-emergent adverse events in the overall sequenced cohort were comparable for alirocumab (82.9%) vs comparator (83.3%; comparator included placebo as well as ezetimibe).[10] The incidence of injection-site reactions (mostly mild and transient) was higher for alirocumab (11.4%) vs comparator (8.8%).[10] Given the small population (n = 20) for the present analysis, no further safety analysis was performed for this specific cohort.

Figure 1. Percentage change from baseline in LDL-c at (A) week 12 and (B) week 24 for individual patients



Data were not available for patient 2 (*APOB* defective/*LDLR* negative). Patient 7 was from the 12-week phase 2 study therefore no data were available at week 24. Patient 11 (*PCSK9 GOF and LDLR* negative) received a different alirocumab administration regimen and is not included in this figure. *APOB*: apolipoprotein B, *LDLR*: low-density lipoprotein receptor, *LDLRAP1*: LDLR adaptor protein 1, LDL-c: low-density lipoprotein cholesterol N/A, not available.

DISCUSSION

In the present analysis, we identified 20 patients with double heterozygous, compound heterozygous, and homozygous FH mutations, from six of the alirocumab clinical trials. All patients who received alirocumab 75/150 or 150 mg Q2W (the majority of whom were receiving background statins) in the trials responded to treatment (defined by LDL-c reduction ≥ 15% on at least week 12 or 24). At week 12, alirocumab treatment resulted in LDL-c reductions of 21.7% to 63.9% (absolute reductions of 39 - 114 mg/dL) in all but one patient (patient 10; a 39-year-old female with *LDLRAP1* negative mutations) who had an increase of 7.1% in LDL-c (baseline LDL-c was 140 mg/ dL); however, a reduction of 34.3% (absolute reduction of 48 mg/dL) from baseline to week 24 was observed in this patient, following alirocumab dose increase from 75 mg Q2W to 150 mg Q2W at week 12.

Furthermore, two alirocumab-treated patients showed inconsistent LDL-c reductions at week 12 vs week 24. Patient 5 had an LDL-c reduction of 52.6% and 8.8% at weeks 12 and 24, respectively. The corresponding values for patient 9 were 22.9% and 11.9%, respectively. Although there is no firm explanation for the differences in response between week 12 and week 24 LDL-c reductions in these patients, nonadherence to therapy cannot be excluded.

Reductions of 24% to 30% in LDL-c, regardless of baseline levels, have been reported to provide clinical benefits, including reduced risks of cardiovascular events and deaths.[22–24] With the range of LDL-c reductions observed in this analysis, patients with more than one FH mutation will be expected to have reduced cardiovascular risks with alirocumab treatment. At week 12, although only two and four alirocumab-treated patients achieved risk-specific LDL-c goals of < 70 mg/dL or < 100 mg/dL, respectively, those who did not achieve the LDL-c goals had reductions of 21.7% to 39.3%, equivalent to 39 to 114 mg/dL absolute reductions in LDL-c (despite high baseline LDL-c level of ≥ 180 mg/dL). With these high baseline LDL-c levels, achievement of LDL-c < 70 mg/dL is unlikely, but these patients will be expected to have reduced risk of cardiovascular events and improved survival with the observed reductions in their LDL-c.

The LDLR mediates uptake of low-density lipoprotein (LDL) particles into the liver cell, via interaction with the ApoB component of LDL. PCSK9 binds to the LDLR and prevents the receptor recycling to the cell surface, targeting the LDLR for degradation by endocytosis. Inhibition of PCSK9 with the monoclonal antibody alirocumab reduces LDL-c levels by increasing the level of LDLRs on the liver cell surface, resulting in an increased uptake of LDL particles.[25] Therefore alirocumab's mode of action involves the LDLR, ApoB, and PCSK9 (and likely other proteins such as LDLRAP1, which interacts with the LDLR), and

mutations in genes encoding these proteins could conceivably impact the treatment effect of alirocumab. For example, complete loss of both copies of LDLR may be expected to nullify the effect of a PCSK9 inhibitor. Indeed, another PCSK9 inhibitor showed no effect on LDL-c levels when examined in three patients with *LDLR* negative/negative mutations,[26,27] with similar results seen in a large open-label study.[28] None of the patients examined in our analysis was *LDLR* negative/negative.

In this analysis, alirocumab treatment provided substantial reductions in LDL-c in patients with FH and residual LDLR function (including patients with mutations in both copies of the gene). Double heterozygous mutations in *APOB* and *LDLR* appeared not to influence the efficacy of alirocumab, with reductions in the same range as reported for the overall pooled analysis of FH patients from alirocumab phase 3 trials (mean reductions from baseline to week 24 of 48.8% and 55.0% with alirocumab doses of 75 mg Q2W [with possible dose increase to 150 mg Q2W at week 12] and 150 mg Q2W, respectively).[29]

Published data have shown a mean reduction in LDL-c of 29.6% at week 12 in 20 HoFH patients with *LDLR* defective mutations in one or both alleles, following biweekly treatment with another PCSK9 inhibitor, supporting the efficacy of PCSK9 inhibitors in patients with defective LDLR function.[27] In our study, alirocumab 75/150 or 150 mg Q2W treatment in seven patients with defective LDLR function (patients 3–9) provided LDL-c reductions of 21.7% to 63.9% at week 12, a mean reduction of 41.2%. Of note, this includes patients who also have other mutations including defective APOB function (patients 3 and 4) and negative LDLR function (patients 5 and 6).

Alirocumab treatment resulted in LDL-c reduction in the patient with *LDLR* negative and *PCSK9* GOF mutations, lending further support to previously published results suggesting that *PCSK9* GOF mutations in general do not impair the efficacy of alirocumab[30]; similar findings were observed with another PCSK9 inhibitor.[28]

Previous reports have indicated mean reductions in Lp(a) of approximately 20% with alirocumab treatment.[21] Lp(a) is known to be an independent risk factor for cardiovascular disease.[31] In the present analysis, Lp(a) reductions with alirocumab varied between week 12 and week 24. At week 24, reductions in the range 19.8% to 49.5% were observed across the patients treated with alirocumab, although (for reasons that are unclear) two patients (with *LDLRAP1* and *LDLR* defective homozygous mutations, respectively) did not have an Lp(a) reduction at week 24.

Baseline Lp(a) levels also varied considerably between patients (25–99 mg/dL).

The alirocumab safety profile in the cohort of sequenced patients was comparable between those who received alirocumab or placebo,[10] consistent with pooled safety data from the overall FH populations of alirocumab phase 3 trials.[29]

LIMITATIONS

Limitations of this post hoc analysis include the small number of patients with each mutation type; however, this is inevitable given the rarity of these mutations. Furthermore, patients with a known history of HoFH were excluded in the individual clinical trials, and so very few patients with HoFH were included in the present analysis. However, in general, the data are robust, with low heterogeneity. The analysis was well controlled with a similar group of patients who received placebo during the study. The impact of rare mutation types may be better assessed in specifically designed trials using a placebo-phase approach, whereby each patient acts as their own control, as previously described.[30]

CONCLUSION

A clinically meaningful LDL-c lowering activity was observed in patients receiving alirocumab who are double or compound heterozygous, or homozygous for genes that are causative for FH, such as *LDLR, APOB, PCSK9*, and *LDLRAP1*. LDL-c lowering activity of alirocumab in these mutations is likely to be attributable to the presence of at least one partially functional allele.

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ACHIEVED LDL CHOLESTEROL LEVELS IN PATIENTS WITH HETEROZYGOUS FAMILIAL HYPERCHOLESTEROLEMIA: A MODEL THAT EXPLORES THE EFFICAFY OF CONVENTIONAL AND NOVEL LIPID-LOWERING THERAPY

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ABSTRACT

BACKGROUND

A large proportion of patients with heterozygous familial hypercholesterolemia (heFH) do not reach low-density lipoprotein cholesterol (LDL-c) levels advocated by international guidelines (< 70 mg/dL or < 100 mg/dL).

OBJECTIVE

We set out to model which proportion of patients reach targets using conventional and novel therapies.

METHODS

We performed a cross-sectional analysis in a large cohort of genetically identified heFH patients and calculated the proportion reaching treatment targets in four scenarios: 1) after 50% LDL-c reduction (representing maximal dose statin); 2) after 70% LDL-c reduction (maximal dose statin+ezetimibe) 3) additional 40% LDL-c reduction representing CETPi; 4) 60% LDL-c reduction (PCSK9i), on top of scenario 2. We applied 100% adherence rates and literature based adherence rates from 62-80%.

RESULTS

We included 1,059 heFH patients with, and 9,420 without CHD. With maximal dose statin, 8.3% and 48.1% of patients with and without CHD would reach their recommended LDL-c targets, respectively. This increases to 54.3% and 93.2% when ezetimibe is added. Addition of CETPi increases these numbers to 95.7% and 99.7% while adding PCSK9i would result in 99.8% and 100% goal attainment. Using literature based adherence rates, these numbers decrease to 3.8% and 27.3% for maximal dose statin, 5.8% and 38.9% combined with ezetimibe, 31.4% and 81.2% when adding CETPi, and 40.3% and 87.1% for addition of PCSK9i.

CONCLUSIONS

Less than 10% with and 50% of heFH patients without CHD would reach treatment targets with maximal dose statin, but this substantially increases upon addition of ezetimibe, CETPi or PCSK9i. However, considering recently published adherence data, this might be lower in real life, especially in heFH patients with CHD.

INTRODUCTION

Heterozygous familial hypercholesterolemia (heFH) is a common autosomal dominant genetic disorder affecting approximately 1 in 200-250 persons and is caused by mutations in the low-density lipoprotein (LDL) receptor (*LDLR*), apolipoprotein B (*APOB*) or pro-protein convertase subtilisin/kexin 9 (*PCSK9*) genes.[1,2] Patients with heFH are characterized by high plasma LDL cholesterol (LDL-c) levels and increased risk for premature coronary heart disease (CHD). The hazard ratio (HR) or odds ratio (OR) for CHD is 3.6 to 22.3 times higher in heFH patients compared to non-heFH controls.[3-5] Current clinical guidelines recommend striving for LDL-c levels below 70 mg/dL (1.8 mmol/L) or below 100 mg/dL (2.6 mmol/L) in these patients with and without a history of CHD, respectively.[6]

Several observational studies have shown that these levels are not reached in a large proportion of patients, despite the use of lipid lowering therapy (LLT) (i.e. statin with or without ezetimibe).[7,8] In recent years, additional LDL-c lowering agents were developed to address this unmet clinical need. Inhibitors of cholesteryl ester transfer protein (CETPi) and proprotein convertase subtilisin/kexin type 9 (PCSK9i) have been extensively studied in heFH patients. Anacetrapib, an oral CETP inhibitor, was shown to result in an additional 40% LDL-c reduction compared to placebo in heFH patients who were using maximally tolerated LLT in the REALIZE trial [9] and recently is was stated in a press release that in the REVEAL trial, anacetrapib significantly decreases major coronary events.[10] In a similar trial design in the RUTHERFORD studies and ODYSSEY FH studies, subcutaneous injections of alirocumab or evolocumab, both monoclonal antibodies directed against PCSK9, resulted in an approximately 60% additional LDL-c decrease.[11-13] However, patients enrolled in these clinical trials do not necessarily represent “the general heFH patient” since the in- and exclusion criteria of such studies usually result in selection of patients whose LDL-c levels are higher compared to heFH patients not participating in a clinical trial.[14] In addition, the enrolled populations are heterogeneous, as both genetically and clinically defined heFH patients could participate in these trials. Moreover, adherence to medication in real life has shown to be substantially lower compared to the adherence in clinical trials.[15-18] Therefore, we set out to calculate which fraction of heFH patients identified by cascade screening, would reach the recommended LDL-c levels with maximally conventional LLT (maximal dose statin combined with ezetimibe) and additional CETPi or PCSK9i at different adherence rates.

METHODS

DATA COLLECTION AND STUDY COHORT

The data used in present study were collected during the familial hypercholesterolemia (FH) cascade screening program in the Netherlands which ran from 1994 to 2014. Details have been described previously.[19,20] In short, a cascade started with the identification of a carrier of an FH causing mutation. Subsequently molecular analysis took place in first degree relatives. Blood was drawn in fasting state, and demographic and clinical data of participants were collected by a certified genetic field worker. Lipids and lipoproteins were measured by default in all participants since 2004. In the current study, we included heFH patients with a pathogenic mutation[3] aged 18 or above and of whom a lipid profile and information about the use of lipid lowering therapy was available. Homozygous and compound heterozygous FH patients were excluded. All patients provided written informed consent. This study was approved by the Medical Ethical Committee of the Academic Medical Centre, University of Amsterdam, the Netherlands and the scientific board of the Landelijk Expertisecentrum Familiaire Hypercholesterolemie (LEEFH), the non-profit organization in charge of the data collection.

MEASUREMENT OF LIPID LEVELS

The lipid profile was measured with the LDX-analyzer (Cholestech Corporation, Hayward, CA, USA).[21] Levels of LDL-c were subsequently calculated with the Friedewald formula, unless triglycerides were above 400 mg/dL (4.5 mmol/L).[22] Off-treatment LDL-c levels in patients using LLT at the time of screening were calculated based on type and dose of medication, according to the adjustment coefficients as previously described.[17,23]

MUTATION ANALYSIS

DNA was isolated from 10 ml of freshly collected blood containing EDTA as anticoagulant. The method of mutation analysis has been described previously.[24,25]

CORONARY HEART DISEASE (CHD)

We defined CHD as a history of a non-fatal event of any of the following: myocardial infarction (MI), coronary artery bypass surgery (CABG), or percutaneous transluminal coronary angioplasty (PTCA).

STATISTICAL ANALYSIS

The primary outcome measure was the proportion of patients reaching guideline recommended LDL-c levels, defined as 70 mg/dL and 100 mg/dL for patients with and without CHD, respectively.[26] We addressed this in four scenarios:

- Maximal dose statin. We modelled a 50% reduction from baseline LDL-c levels,

representing maximal dose statin.[27]

- Maximal conventional LLT, where a 70% LDL-c reduction is calculated. The 70% is comprised of the 50% LDL-c lowering induced by a high dose statin combined with 20% reduction by ezetimibe.
- Maximal conventional LLT combined with CETPi. A 40% additional reduction of LDL-c levels was calculated in this model (based on the mean LDL-c reduction found in clinical trials with heFH patients[9]) on top of 70% reduction derived by maximal conventional LLT (which was calculated in scenario 2).[27]
- Maximal conventional LLT combined with PCSK9i. A 60% reduction of LDL-c levels was calculated in this model, (based on the average decrease in LDL-c known from trials with heFH patients[11-13]) again on top of 70% lowering induced by maximal conventional LLT (which was calculated in scenario 2).[27]

Patients were stratified according to CHD history and the first analysis was performed while applying a theoretical adherence rate of 100%. In the second analysis we used an adherence rate of 80% for statin therapy**,** based on earlier data showing that the efficacy of conventional LLT is around 80% of the expected LDL-c lowering found in clinical trials.[15,17,28] Based on this assumption, the LDL-c reduction is 40% (0.8 times 50%). Because real life adherence data for CETPi are lacking, we modeled an 80% adherence rate corresponding to statin therapy, since both are once daily oral medication, which leads to a 32% LDL-c reduction (0.8 times 40%). For PCSK9i an adherence rate of 62% was used, based on real life data from implementation of PCSK9i in clinical practice, resulting in a 37.2% reduction in LDL-c (0.621 times 60%).[29] In addition, we performed the same analysis for severe heFH patients (according to the definition described by Besseling and colleagues) [23] For statistical analyses we used SPSS 23 Windows (IBM Software, NY, USA) and R Statistics 3.0.1.

RESULTS

STUDY POPULATION

A total of 64,171 individuals underwent molecular screening for FH between January 1994 and January 2014. A pathogenic mutation was identified in 26,232 (40.9%) of these subjects, including the index patients. Of these, we excluded 65 (0.2%) homozygous and compound heterozygous patients. Furthermore, a total of 5,961 children below 18 years (22.7%) were excluded. Complete lipid profiles with a known LDL-c were unavailable in 9,727 (37.1%) patients due to the fact that this was not measured standard in all participants before 2004. This resulted in our final study population comprising 10,479 heFH patients of whom 1,059 (10.1%) had a history of CHD and 9,420 (89.9%) who did not. Figure 1 depicts the flowchart

describing the selection of the study population. Demographic and clinical characteristics of included patients are shown in Table 1. The mean age (± SD) of patients with and without CHD was 63.6 (± 12.5) and 44.5 (± 16.4 years), respectively. Cardiovascular risk factors were more prevalent amongst heFH patients with CHD compared to patients without CHD (body mass index (BMI) (mean ± SD: 26.9 ± 4.2 kg/m^2 vs 25.0 ± 4.3 kg/m$^{2)}$; smoking: 43.0% vs 35.5%; diabetes mellitus: 11.6% vs 2.6% and hypertension: 41.6% vs 11.6%, respectively.)

Figure 1. Flow diagram for patient screening and selection for achieved LDL-c Levels in heterozygous familial hypercholesterolemia patients

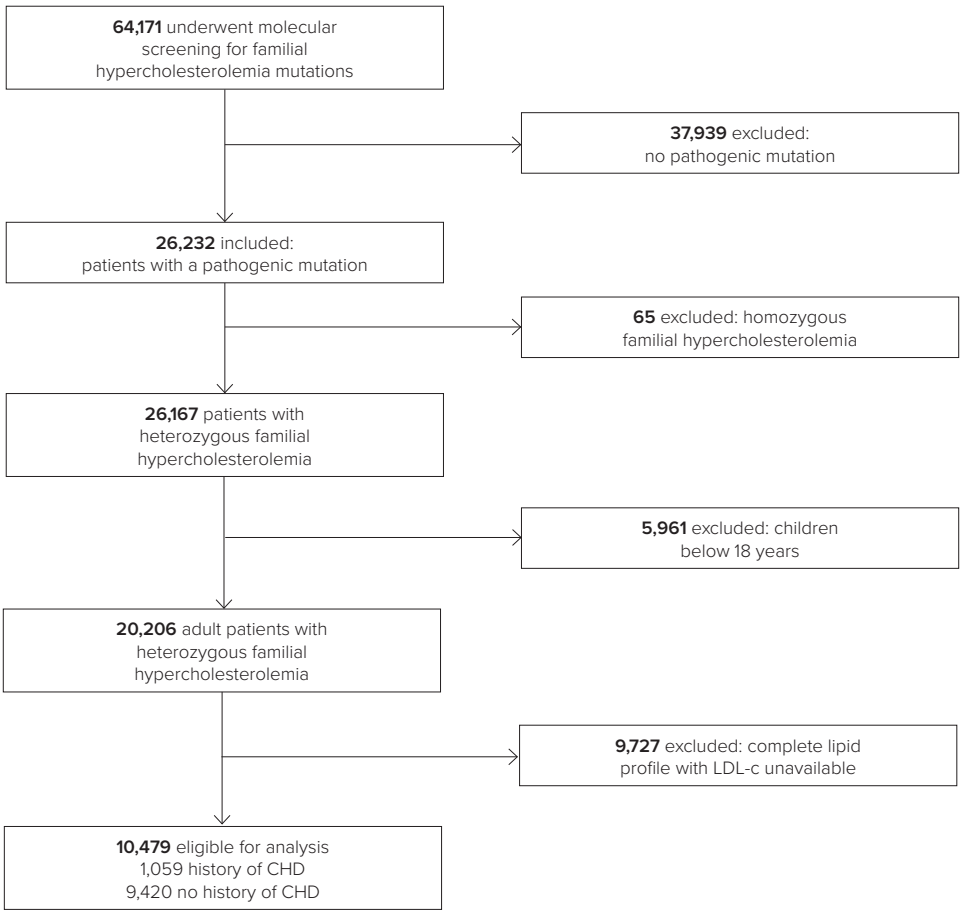


Table 1. Demographic and clinical characteristics of adult heterozygous familial hypercholesterolemia patients

	HeFH patients with CHD*	HeFH patients without CHD*
No. of patients (n, %)	1,059 (10.1%)	9,420 (89.9.0%)
Age (years) - mean (SD)	63.7 (12.5)	44.5 (16.4)
Male gender - no. (%)	693 (65.4%)	4,247 (45.1%)
BMI (kg/m^2) - mean (SD)	26.9 (4.2)	25.0 (4.3)
Current smoking - no. (%)	455 (43.0%)	3,342 (35.5%)
Diabetes mellitus - no. (%)	123 (11.6%)	249 (2.6 %)
Hypertension - no. (%)	441 (41.6%)	1,094 (11.6%)
LDL-cholesterol (mg/dL) - mean (SD)	130.2 (46.9)	164.0 (54.0)
Off treatment recalculated LDL-cholesterol (mg/dL) - mean (SD)†	230.5 (79.8)	212.7 (78.3)
HDL-cholesterol (mg/dL) - mean (SD)	42.1 (13.5)	46.5 (14.3)
Triglycerides (mg/dL) - median (IQR)	115.1 (168.7 – 79.7)	101.0 (149.7 – 69.1)
Untreated n (n %)	78 (7.4%)	5,177 (55.0%)
Treated with lipid lowering therapy at diagnosis – no. (%)	980 (92.6%)	4,243 (45.0 %)
-Low/moderate intensity statins – ezetimibe, no. (%)‡	282 (28.8%)	2,206 (52.0 %)
-Low/moderate intensity statins + ezetimibe, no. (%)‡	122 (12.4 %)	540 (12.7 %)
-High intensity statins – ezetimibe, no. (%)‡	371 (37.9 %)	1,062 (25.0 %)
-High intensity statins + ezetimibe, no. (%)‡	194 (19.8 %)	391 (9.2 %)
-Other lipid lowering therapy, no. (%)‡	11 (1.1 %)	43 (1.0 %)

HeFH: heterozygous familial hypercholesterolemia BMI: body mass index; CHD: coronary heart disease; LDL: low-density lipoprotein; HDL: high-density lipoprotein; no.: number; SD: standard deviation; IQR: interquartile range

* Defined as myocardial infarction, coronary bypass surgery and percutaneous transluminal coronary angioplasty in medical history.

† Off treatment LDL cholesterol levels are recalculated based on type and dose of lipid lowering therapy as described previously.

‡ Percentage of lipid lowering therapy treated patients.

QUANTIFICATION OF PATIENTS ATTAINING LDL-C TREATMENT

In the model where patients would not use any LLT, 0.4% of patients with and 3.1% of patients without CHD would meet the guideline-recommended LDL-c goals. With the assumption of 100% adherence, as well as a theoretical 50% LDL-c reduction based on maximal dose statin, these proportions increased to 8.3% and 48.1%, respectively. The addition of ezetimibe, which would translate in an additional 20% LDL-c lowering (resulting in a total LDL-c reduction of 50% + 20% = 70%) would lead to 54.3% and 93.2% of patients attaining treatment target. An additional 40% LDL-c reduction, mimicking the anticipated effect of CETPi, resulted in 95.7% of patients with and 99.7% of patients without CHD reaching the recommended LDL-c levels. With an anticipated additional 60% LDL-c lowering effect of PCSK9i on top of maximal available conventional LLT, 99.8% of patients with and 100% of patients without CHD would reach their target LDL-c level. When assuming 80% adherence for conventional maximal LLT and CETPi and 62% adherence for PCSK9i, the proportion of patients with and without CHD that would reach their individual LDL-c target level, would be 3.8% and 27.3% with maximal dose statin, 5.8% and 38.9% for maximal conventional LLT, 31.4% and 81.2% with an additional

32% reduction of LDL-c (CETPi) and 40.3% and 87.1% with 37.2% reduction of LDL-c (PCSK9i) reduction on top of LLT. The LDL-c distribution curves in the different scenarios are depicted in Figure 2. In addition, we quantified the proportion of severe FH patients that would theoretically be able to reach LDL-c target levels. As expected, due to high baseline LDL-c levels, 'severe FH' patients are less likely to attain treatment targets. However, the conclusions made from this analysis should be taken with precaution: due to the small number of patients this could not be very accurate since only 135 patients in our dataset were defined as 'severe FH' who suffered from an CHD event. The LDL-c distribution curves in different scenarios are depicted in Figure 1 in the Online Supplementary Data (for clinical characteristics see Table 1 in the Online Supplementary Data).

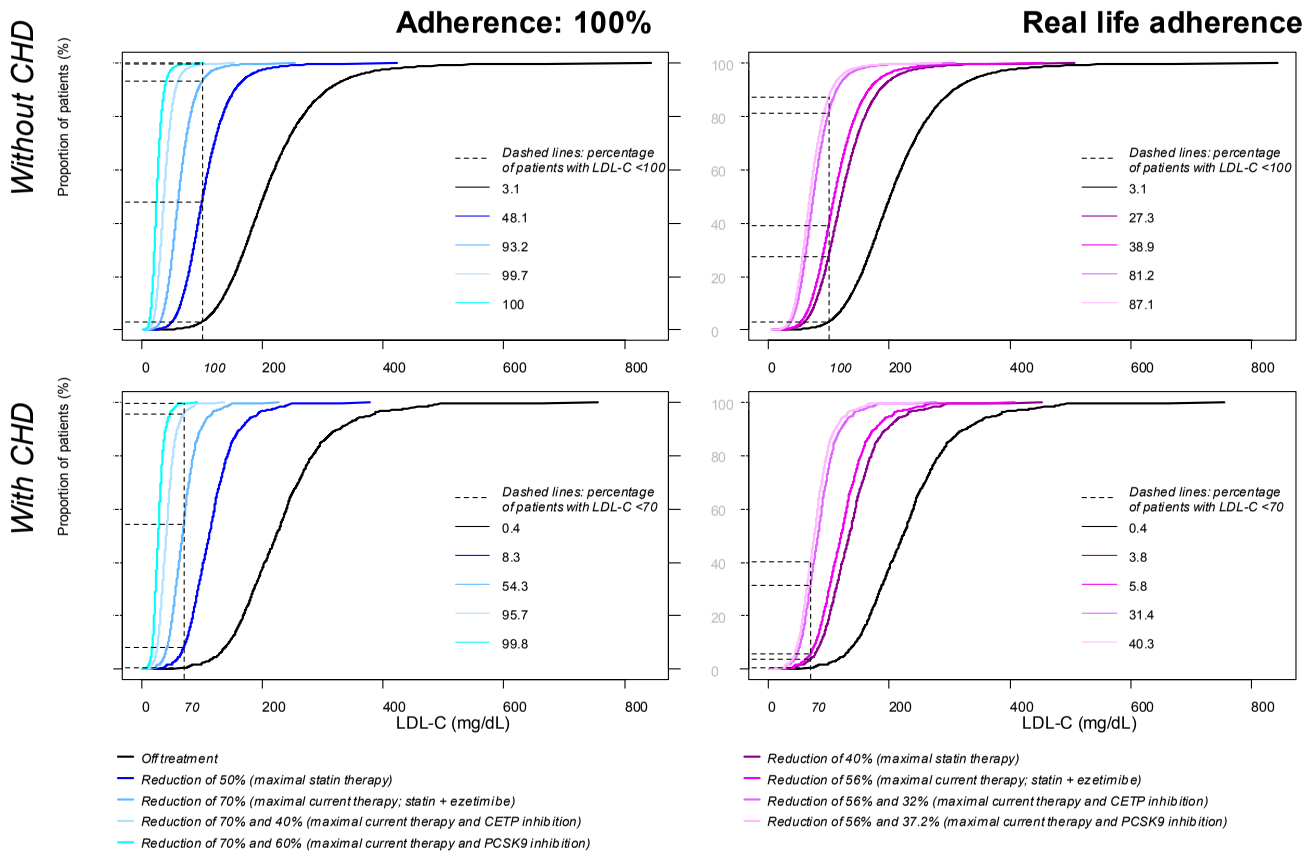
Table 2. Heterozygous familial hypercholesterolemia patients achieving LDL-c target in clinical trials

		Percentage of patients attaining LDL-c target	
PCSK9i (on top of conventional maximal LLT)		70 mg/dL (< 1.8 mmol/L)	100 mg/dL (< 2.6 mmol/L)
RUTHERFORD I (2012)	Evolocumab -350 mg Q4W	44%	70%
	-420 mg Q4W	65%	89%
RUTHERFORD II (2014)	Evolocumab -140 mg Q2W	68%*	-
	-420 mg Q4W	63%*	-
ODYSSEY FH I (2015)	Alirocumab -75/150 mg Q2W	59.8%*	-
ODYSSEY II (2015)	Alirocumab -75/150 mg Q2W	68.2%*	-
CETPi (on top of conventional maximal LLT)			
REALIZE (2015)	Anacetrapib -100 mg	44%	82%

LDL-c: low-density lipoprotein cholesterol, PCSK9i: proprotein convertase subtilisin/kexin type 9 inhibitors; LLT: lipid lowering therapy; CVD: cardiovascular disease; CETPi: cholesteryl ester transfer protein inhibitor. Q4W indicates 4 wk, Q2W indicates 2 wk.

*Patients (%) achieving LDL-c < 70 mg/dL (< 1.8 mmol/L) regardless of prior cardiovascular events.

Figure 2: Proportion of patients on LDL-c target in different treatment regimens stratified by history of CHD, with 100% adherence for all treatment scenario's, or 80% adherence for statin therapy with or without ezetimibe, 80% for CETPi and 62% for PCSK9i



DISCUSSION

In this cross-sectional study in a very large cohort of subjects with genetically defined heFH in the Netherlands, we found that with universal application of maximal dose statin assuming a 100% adherence, half the heFH patients without a CHD history would reach their individual LDL-c target level, but only around 1 in 10 heFH patients with a history of CHD will in theory comply with current guidelines.[30] Assuming a 100% adherence rate, these numbers are considerable increased with the addition of ezetimibe. The figures changes even more dramatically, if novel therapies are added to maximal lipid lowering therapy; addition of inhibitors CETP will in theory increase this proportion to more than 95%, and to more than 99% of patients reaching their LDL-c goals upon administration of PCSK9i. However, when applying adherence data based on recent literature, these proportions substantially decrease leading to 87% of patients in primary prevention and only to 40% in a secondary prevention setting reaching their treatment targets when PCSK9i is added to maximal conventional LLT.

Our results differ from those described in recent clinical trials (depicted in Table 2). In particular, in the RUTHERFORD trial, adding 420 mg evolocumab every four weeks to standard care resulted in 65% and 89% of heFH patients with and without CHD achieving an LDL-c level of less than 70 and 100 mg/dL, respectively.[11] In the RUTHERFORD II trial, the proportion of patients on target were 68% and 63% when evolocumab 140 mg every two weeks and 420 mg every four weeks were administered, respectively.[12] In addition, in ODYSSEY FH I and II, alirocumab treatment resulted in 59.8% and 68.2% of heFH patients attaining LDL-c concentrations below 70 mg/dL.[13] Likewise, in the REALIZE trial, anacetrapib led to a lower proportion of patients achieving LDL-c levels below 70 mg/dL and 100 mg/dL, compared to our study (44% and 82% respectively).[9] These proportions are substantially lower than in our cohort at 100% adherence, which might be explained by the fact that our study population comprises unselected patients identified by cascade screening, where 25% does not have LDL-c levels above the 90th percentile.[31] This contrasts the LDL-c levels allowed for patients in clinical trials, who are selected for higher levels despite lipid lowering therapy. For example, in the RUTHERFORD trials, the baseline LDL-c levels were above 150 mg/dL, even though approximately 90% of the patients were on high intensity statin therapy. The relative differences in LDL-c reduction for CETPi in comparison to PCSK9i, might be attributable to the lower baseline LDL-c levels in REALIZE (128 mg/dL), combined with a less pronounced reduction in LDL-c by anacetrapib in comparison to PCSK9i. Thus, our study suggests that compared to recent clinical trials, the proportion of heFH patients that are able to achieve their target LDL-c levels will potentially be greater in real life when the adherence rate is 100%.

However, we know that adherence to lipid lowering medication in real life is lower in comparison to the adherence in clinical trials.[15,16,32] conversely, discontinuation of statin therapy has been shown to associate with a significant increase in CV morbidity and mortality, underscoring the paramount importance of compliance to lipid lowering medication.[33] When applying estimated true adherence rates in outpatient clinical setting, a substantial proportion of heFH patients without CHD and even a more considerable part of patients with CHD will not reach their treatment targets. Thus, these novel therapies would in theory be very effective in lowering LDL-c, but due to lower adherence rates in real life, goal attainment might still be more challenging. Most heFH patients would greatly benefit when adherence could be increased, especially heFH patients with established CHD. To overcome this adherence problem, PCSK9 specific RNA silencing in the liver may offer an additional advantage compared to antibody therapy achieving efficacy with markedly reduced injection frequency. Thus, the results from the ORION-1 trial have shown to still lower LDL-c effectively at 180 days after a single 300 mg dose of inclisiran.[34] Moreover, a vaccine against PCSK9 recently resulted in significant reduction in plasma lipids in mice.[35] When this, in the future, becomes a realistic therapeutic option in humans, it might be a treatment modality suitable for long term LDL-c management, not dependent of adherence.

LDL-c is the leading CHD risk factor in heFH.[36] In fact, CHD event rate exceeds 30% in 10 years for adult heFH patients with a severe pathogenic *LDLR* mutation.[37] Early institution of therapy does result in a 40% reduction of major adverse cardiac events.[38] International guidelines therefore advocate LDL-c levels less than 100 mg/dL for primary prevention and less than 70 mg/dL for secondary prevention. These treatment targets are based on results from meta-analyses of clinical trials, where a dose dependent reduction of CHD risk is observed.[39,40] However, CETPi appears to form an exception to this principle since the risk reduction seen with CETPi s less than expected based on the effect on LDL-c levels.[41] Mendelian randomization analyses suggest that the causal effect of CETPi on the risk of cardiovascular events is driven by the effect apoB-containing lipoproteins rather than changes in LDL-c or HDL-c.[42] Statins, ezetimibe and PCSK9i have been shown to lower LDL-c and apoB in a concordant way, while CETP inhibitors typically have a more pronounced effect on LDL-c levels compared to apoB levels. Merck has announced that the development of anacetrapib has been discontinued despite the finding of a modest positive effect of anacetrapib on CVD endpoints in the REVEAL trial.[43] However, CETPi remains relevant because it is an oral drug that delivers moderately effective LDL-c lowering. In particular as a monotherapy, it could still be of additional value to the armamentarium of lipid lowering therapies in patients that do not response sufficiently or do not tolerate statin therapy, or who prefer an oral drug over subcutaneous administration by injections. Moreover, other oral LDL-c lowering agents with similar efficacy are currently evaluated in

patients with heFH.[44]

Although LDL-c goals lack validation in patients with heFH, consistent LDL-c lowering is especially important in these patients at high risk for CHD due to their life time exposure to elevated LDL-c. To reach these targets, the therapy of first choice is maximal dose high intensity statin therapy.[26] In our cohort, this therapy only led to 50% attainment of goals or even less when taking into account real life adherence. However, goal attainment will increase when ezetimibe is added to this regimen. Despite maximal conventional LLT, taking into account real life adherence, almost 95% of patients with and over 60% of patients without CHD will not reach their guideline recommended treatment targets, emphasizing the unmet clinical need for additional LDL-c reduction in heFH patients. By quantifying the proportion of patients that will not reach their targets, insight is obtained into what part of the general heFH population at large, would benefit from novel therapies. It is clear from our analysis that such therapies answer to a still highly relevant unmet clinical need.

LIMITATIONS AND STRENGTHS

Some strengths and limitations of our study should be acknowledged. The proportion of patients reaching guideline directed LDL-c levels is only an indication and might be an overestimation, since we could not account for adherence to CETPi. However, CETPi and statins show similarities, such as oral administration. Furthermore, the modeled adherence rate of 62% for PCSK9i in our model might vary between countries. This number that was used in our model, was based on data from the United States, while in a recent published study from the Netherlands it was shown that in clinical practice the adherence for PCSK9i amongst FH patients over a period of 6 weeks was 92%.[45] However, the prolonged adherence for PCSK9i has not been investigated extensively yet, due to the novelty of this treatment agent and can be, for example, influenced by local reimbursement regulations. Also, we applied mean LDL-c reductions while it is known that significant interindividual variability exists regarding LDL-c lowering potency of these medications as shown earlier with statins[46] or with PCSK9i.[11,47]

A major strength of our study is its large sample size. Furthermore, the largely unbiased recruitment of patients in the screening program has created a study population that is likely to be representative for the overall heFH population in our country. The decision to perform the genetic analysis in a participant was based on the presence of a pathogenic mutation in the family, not on clinical suspicion for familial hypercholesterolemia. Therefore, the risk of selection bias is probably minimal.

CONCLUSION

The majority of heFH patients would likely not reach treatment targets, even once high dose statin is prescribed at maximal dose. With maximal adherence, goal attainment can be increased if ezetimibe is added to this regimen. New treatment modalities such as CETPi and PCSK9i can be of additional value when it comes to achieving LDL-c treatment targets. However, issues of availability, accessibility and adherence remain for these novel agents,

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PCSK9 INHIBITORS IN CLINICAL PRACTICE: DELIVERING ON THE PROMISE?

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ABSTRACT

BACKGROUND AND AIMS

In clinical trials, protein convertase subtilisin/kexin type 9 (PCSK9) inhibitors robustly lowered LDL-cholesterol (LDL-c) and had a favorable tolerability and safety profile. Based on these findings, PCSK9 inhibitors are incorporated in updates of clinical treatment guidelines. However, trial results do not necessarily predict the effectiveness under real-world conditions. The aim of the current study is to determine the efficacy and tolerability of PCSK9 inhibitors in routine outpatient care.

METHODS

The cohort comprised all patients who were prescribed evolocumab or alirocumab at the outpatient clinic of a large university hospital in the Netherlands. Eligible patients required additional lipid-lowering despite maximally tolerated statin therapy and ezetimibe, or were statin intolerant. Data were systematically collected during routine outpatient visits.

RESULTS

The study included 238 patients of whom 67.2% had familial hypercholesterolemia (FH) and 42.9% were statin intolerant. The mean LDL-c reduction was 55.0% from a baseline of 4.4 mmol/L. LDL-c goals were attained by 62.3% of patients. Side effects were reported by 15.5% of patients, and 2.5% discontinued treatment. No meaningful differences in efficacy or tolerability were observed between patients with FH or statin intolerance, or across treatment regimens.

CONCLUSIONS

The observed lipid reductions and side effects profile of PCSK9 inhibitors in a routine care setting were comparable to observations in clinical trials.

INTRODUCTION

It has been unequivocally shown that a log-linear relation exists between low-density lipoprotein cholesterol (LDL-c) and the risk of cardiovascular disease (CVD), without a lower threshold below which the strength of the relationship is attenuated.[1] The advent of statin therapy in the 1990s has enabled unparalleled cardiovascular risk reduction and these drugs should be regarded as one of the major advances in contemporary medicine. However, a considerable number of patients are unable to tolerate statins at sufficiently high doses to achieve acceptable lipid levels due to side effects, whereas other patients are unable to reach lipid goals even despite highly dosed statin therapy.[2,3] The clinical consequences of the ensuing undertreatment are particularly detrimental for high-risk patients, such as those with familial hypercholesterolemia (FH). Protein convertase subtilisin/kexin type 9 (PCSK9) inhibitors have recently emerged as a valuable addition to the repertoire of lipid-lowering drugs. Until the introduction of PCSK9 inhibitors, there was little that could be done to effectively treat high-risk patients who needed additional cholesterol reduction beyond addition of ezetimibe, which has only modest effects on LDL-c and clinical outcomes.[4]

Large-scale clinical trials have consistently shown that PCSK9 inhibitors yield an incremental 50-60% reduction in LDL-c when added to statin therapy.[5,6] In the FOURIER clinical outcomes trial, it was recently confirmed that evolocumab reduces cardiovascular event rates in line with expectations based on the observed LDL-c reduction.[7,8] Importantly, no serious adverse effects of PCSK9 inhibition have been observed in clinical trials and discontinuation rates were generally similar between treatment and placebo groups (approximately 5% per year).[5,6]

Based on these clinical trial results, the PCSK9-inhibiting monoclonal antibodies evolocumab and alirocumab have been approved by regulators for use by patients with a high CVD risk who need additional lipid-lowering and by those who are unable to tolerate statin therapy. PCSK9 inhibitors are incorporated in updates of clinical guidelines of major professional societies.[9–12] However, in contrast to the wealth of evidence from clinical trials, there is limited data regarding the efficacy and tolerability of PCSK9 inhibitors in routine patient care. Due to differences between clinical trials and the real world setting, extrapolation of trial results could possibly lead to inflated expectations regarding the efficacy of new therapies (e.g., due to better adherence in trials). Determining whether PCSK9 inhibitors are capable of ‘delivering on their promise’ is of crucial importance, particularly given their high costs. Hence, the objective of the current report is to describe the efficacy and tolerability in a cohort of patients prescribed PCSK9 inhibiting monoclonal antibodies in routine care.

METHODS

All patients who used alirocumab or evolocumab up to June, 2017 were identified from the electronic hospital system of the Academic Medical Center (AMC) in Amsterdam, which is a secondary and tertiary referral center for the Amsterdam region of the Netherlands. The cohort comprised both patients who initially participated in a clinical trial and patients who started using alirocumab or evolocumab in routine care. The decision to start treatment with PCSK9 inhibitors was made by Vascular Medicine specialists. In the Netherlands, PCSK9 inhibitors are reimbursed by standard health insurance (without copayment) for all inhabitants since April 2016 (evolocumab) and June 2016 (alirocumab). The reimbursement criteria include all clinical indications for PCSK9 inhibitors as defined in the European Society of Cardiology (ESC) Guidance (i.e., patients who need additional lipid-lowering despite receiving maximally tolerated statin therapy, including patients with FH or statin intolerance).[12] Prior to market approval, patients were able to receive either of the therapies by participating in clinical trials, or through the open-label Compassionate Use Program.

Patients received either alirocumab (75 mg or 150 mg every two weeks [75Q2W or 150Q2W]) or evolocumab (140 mg every two weeks [140Q2W], or 420 mg every four weeks [420QM]). The choice of the particular PCSK9 inhibitor and dosing regimen was at the discretion of the medical specialist. Alirocumab treatment is typically started at a dose of 75 mg once per two weeks (75Q2W), or 150 mg (150Q2W) if the desired LDL-c reduction is > 60%. For other patients, the dose can be increased from 75Q2W to 150Q2W in case of insufficient response after 4 weeks of therapy. The treatment regimen for evolocumab is typically 140 mg per 2 weeks (140Q2W).

All patients received usage instructions by a nurse practitioner. Follow-up after initiation of the PCSK9 inhibitor typically consisted of bi-annual visits to the outpatient clinic (or more frequently depending on patient preferences, treatment results, tolerability, etc.). Laboratory assessments were performed prior to each outpatient clinic visit. Any changes in treatment, including concomitant (lipid-lowering) therapy, as well as tolerability and therapy adherence were systematically discussed during each visit and documented.

Data were collected during routine visits to the outpatient clinic, or during study visits for patients who initiated PCSK9 inhibitor therapy in clinical studies. Information about demographics, clinical characteristics, relevant medication and outcomes were collected from the hospital electronic health system. For the purpose of this study, CVD was defined as (history of) angina, myocardial infarction, percutaneous coronary intervention, coronary artery bypass graft, cerebrovascular accidents or peripheral vascular disease. Patients

were considered to have FH if they had a documented pathogenic mutation in the genes encoding the LDL receptor, apoB, or PCSK9, or if they had a Dutch Lipid Clinic Network (DLCN) score of ≥ 6. Statin intolerance was defined as inability to tolerate at least 3 statins due to muscle symptoms, in accordance with the European Atherosclerosis Society (EAS) Consensus Panel Statement and Dutch reimbursement criteria for PCSK9 inhibitors.[2]

The primary outcome measures were 1) the %-reduction in LDL-c at the last available measurement from baseline (defined as the lipid measurement prior to starting the PCSK9 inhibitor), and 2) patient-reported side effects. Secondary outcome measures were the effects on total cholesterol, high-density lipoprotein cholesterol (HDL-c), non-HDL-c, and triglycerides, as well as specification of side effects, discontinuation rates and the proportion of patients who attained guideline-recommended treatment goals. The analyses were separately performed for patients with FH or statin intolerance (which are the primary indications for which PCSK9 inhibitors are used) and for the different dosing regimens.

Concomitant use of ezetimibe is a prerequisite for reimbursement of PCSK9 inhibitors in the Netherlands. For patients without available lipid measurements between initiation of ezetimibe and initiation of treatment with PCSK9 inhibitors, the effect of ezetimibe was accounted for by calculating new baseline values using previously reported mean treatment effects of ezetimibe.[13]

All data are reported descriptively and summarized using means and standard deviations (SD) or medians and interquartile ranges (IQR) were appropriate. Categorical data are reported as numbers and percentages.

RESULTS

The cohort consisted of 238 patients; 121 (53.3%) were initially prescribed evolocumab (118 [52.0%] 140Q2W and 3 [1.3%] 420Q4W) and 106 (47.7%) were prescribed alirocumab (42 [18.5%] 75Q2W and 64 [28.2%] 150Q2W). For 11 patients (4.6%) who initiated PCSK9 inhibitor therapy in a blinded clinical study, treatment allocation was not yet available. Patient characteristics are depicted in Table 1. The mean age of patients was 58 years, and 62.6% had a history of CVD. Mean body mass index (BMI) was 27.6 kg/m^2, 40.8% had hypertension and 16.5% were current smokers. In total, 99 patients (41.6%) started using PCSK9 inhibitors in a clinical study (see Table 2).

Table 1. Baseline characteristics

	N = 238
Age, years (SD)	58 (11)
Male, n (%)	139 (58.4)
White, n (%)	232 (97.5)
Previous CVD, n (%)	149 (62.6)
FH, n (%)	160 (67.2)
Statin intolerance*, n (%)	102 (42.9)
Smoking	
Current, n (%)	31 (16.5)
Former, n (%)	66 (35.1)
Never, n (%)	91 (48.4)
Unknown, n (%)	50
BMI, kg/m^2 (SD)	27.6 (4.6)
Hypertension, n (%)	97 (40.8)
Type 2 diabetes, n (%)	40 (16.8)
Concomitant lipid-lowering therapy	
Statins, n (%)	133 (55.9)
Ezetimibe, n (%)	217 (91.2)
Fibrates, n (%)	8 (3.4)
Bile acid sequestrants, n (%)	8 (3.4)

*Unable to tolerate at least three different statins due to muscle symptoms

SD: standard deviation; n: number; BMI: body mass index; CVD: cardiovascular disease

Statin intolerance was the primary indication for initiation of PCSK9 inhibitor therapy for 102 (42.9%) patients and 160 (67.2%) had FH and were unable to achieve lipid goals with maximally tolerated statin therapy (Table 1). Statins were used by 133 patients (55.9%) at the start of PCSK9 inhibitor treatment, 217 (91.2%) used ezetimibe, and 127 (53.4%) both statins and ezetimibe. The 8.8% of patients who did not use ezetimibe at baseline were unable to tolerate this drug due to side effects.

Table 2. PCSK9 inhibitor treatment

	N = 238
Prior study participation, n (%)	99 (41.6)
Initial treatment	
Evolocumab 140 mg / 2 weeks, n (%)	118 (52.0)
Evolocumab 420 mg / months, n (%)	3 (1.3)
Alirocumab 75 mg / 2 weeks, n (%)	42 (18.5)
Alirocumab 150 mg / 2 weeks, n (%)	64 (28.2)
Unknown*	11 (4.6)
Change in PCSK9 inhibitor treatment, n (%)	8 (3.4)
Discontinued PCSK9 inhibitor treatment, n (%)	6 (2.5)

*Study allocation not yet unblinded

SD: standard deviation; n: number

Table 3. Efficacy outcomes

	Baseline	Baseline (ezetimibe corrected)	Last measurement	%-change (95% CI)	%-change (ezetimibe corrected; 95% CI)
Total cholesterol, mmol/L	6.46 (5.26 – 7.96)	6.28 (4.91 – 7.58)	3.82 (3.03 – 4.77)	-38.9 (36.7 – 41.0)	-35.4 (33.1 – 37.7)
LDL cholesterol, mmol/L	4.37 (3.36 – 5.72)	4.07 (3.02 – 5.29)	1.71 (1.09 – 2.52)	-58.3 (55.7 – 60.9)	-55.0 (52.3 – 57.7)
Non-HDL cholesterol, mmol/L	4.99 (4.11 – 6.54)	4.82 (3.56 – 6.18)	2.39 (1.68 – 3.13)	-52.0 (49.4 – 54.6)	-48.1 (45.3 – 51.0)
HDL cholesterol, mmol/L	1.29 (1.06 – 1.54)	1.30 (1.07 – 1.54)	1.42 (1.18 – 1.70)	+13.2 (8.9 – 17.5)	+12.3 (8.1 – 16.6)
Triglycerides, mmol/L	1.50 (0.99 – 2.05)	1.42 (0.98 – 1.98)	1.20 (0.84 – 1.76)	-10.2 (4.0 – 16.3)	-7.0 (0.7 – 13.3)

Absolute values are medians and interquartile ranges, reductions are means and 95% confidence intervals

SD: standard deviation; n: number; LDL: low-density lipoprotein; HDL: high-density lipoprotein; IQR: interquartile range

EFFICACY

The mean LDL-c reduction in the overall cohort was 58.3% (Table 3). In 88 patients (37.0%), ezetimibe was started before initiation of treatment with the PCSK9 inhibitor but after the last available lipid measurement. The mean LDL-c reduction after correction for the effect of ezetimibe was 55.0%. The achieved LDL-c reduction was similar among patients with and without FH (54.6% vs. 55.9%; Figure 1). There were also no substantial differences in the achieved LDL-c reductions across the different dose regimens (Figure 1). The mean reduction in total cholesterol was 35.4%, and the mean reduction in non-HDL-c was 48.1% (Table 3). Treatment with PCSK9 inhibitors resulted in a 12.3% increase of HDL-c, and a 7.0% triglyceride reduction. The overall percentage of patients who achieved guideline recommended LDL-c levels after addition of PCSK9 inhibitors was 62.3%. Among patients with prior CVD, 67.1% achieved the secondary prevention LDL-c goal of < 1.8 mmol/L (70 mg/dL), whereas 54.5% of patients without prior CVD achieved the primary prevention goal of LDL-c < 2.5 mmol/L (100 mg/dL). Among patients with FH, 60.3% reached their LDL-c goal, compared to 66.7% of non-FH patients. Non-HDL-c goals were attained by 70.6% of patients: 72.6% of patients with prior CVD achieved non-HDL cholesterol < 2.6 mmol/L and 67.5% of patients without prior CVD achieved non-HDL cholesterol < 3.4 mmol/L. The percentage of patients with FH achieving their non-HDL-c goal was 68.7%, compared to 74.6% of non-FH patients. Among patients who were still using PCSK9 inhibitors at the time of the last available LDL-c measurement (232 patients [97.5%]), 90.9% had an LDL-c reduction ≥ 25%, 5.2% had an LDL-c reduction smaller than 25% and 1.3% were non-responders. Among the 133 patients (55.9%) using statins at the time of initiation of PCSK9 inhibitor treatment, 61 (45.9%) reported statin associated side effects. In 40 patients (65.5%), statin dose was lowered due to statin-associated side effects after initiating PCSK9 inhibitor therapy, without a meaningful effect on the achieved LDL-c reduction (48.2% and 52.6% in those with and without reduction in statin dose, respectively).

SIDE EFFECTS AND DISCONTINUATION

Side effects attributed to the PCSK9 inhibitor were reported by 37 patients (15.5%) and were the primary reason for discontinuation of therapy in six patients (2.5%; Table 4). Injection-site reactions and muscle complaints were the most commonly reported side-effects (3.4% and 3.8%, respectively). Side effects were reported by 19 of the 102 patients with statin-intolerance (18.6%) and by 18 of the 136 patients without statin-intolerance (13.2%). The proportion of patients reporting muscle symptoms while using PCSK9 inhibitors was similar in patients with and without statin intolerance (3 [2.9%] and 6 [4.4%], respectively). Eleven patients (4.6%) reported missing doses.

Almost all of the patients continued using the same PCSK9 inhibitor in the same dosing

regimen as originally prescribed (96.6%). In three patients, the dose of the PCSK9 inhibitor was reduced due to attributed side effects, whereas one patient switched from evolocumab to alirocumab after reporting side effects on the former. The dose was increased due to insufficient response in 2 patients, and two patients switched from a monthly regimen to a bimonthly regimen after completing a clinical trial.

Figure 1. Percentage reductions and standard errors in LDL-cholesterol for the different dosing regimens and for patients with or without FH, from baseline to the last available lipid measurement

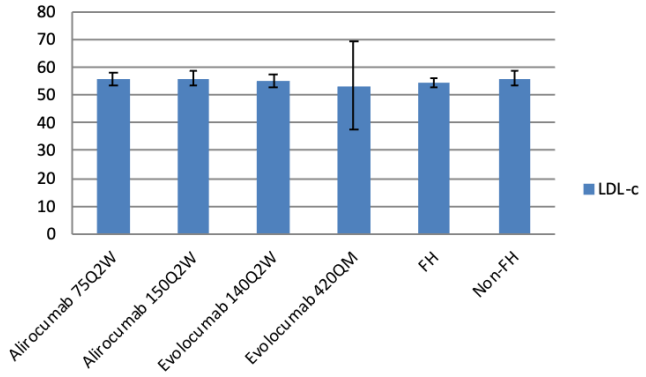

Table 4. Side effects

Discontinuation total, n (%)	6 (2.5)
Discontinuation due to side-effects, n (%)	6 (2.5)
Skipping doses, n (%)	11 (4.6)
Side effects	
Any, n (%)	37 (15.5)
Muscle symptoms, n (%)	9 (3.8)
Injection-site reactions, n (%)	8 (3.4)
Flu-like symptoms / nasopharyngitis, n (%)	6 (2.6)
Joint pain, n (%)	2 (0.8)
Fatigue, n (%)	2 (0.8)
Headache / neurological, n (%)	2 (0.8)
Other, n (%)	8 (3.4)
Non-response, n (%)	3 (1.3)
< 25% LDL-cholesterol reduction, n (%)	15 (6.5)

DISCUSSION

The current report demonstrates that the achieved lipid reductions and side effects profile of PCSK9 inhibitors in a routine care setting are comparable to observations from clinical trials. Notably, treatment with PCSK9 inhibitors resulted in a mean LDL-c reduction of 55%, and side effects were reported by less than one in six patients and rarely led to discontinuation of therapy. In addition, this study shows that there are no meaningful differences in efficacy and tolerability between patient with FH or statin-intolerance, across dosing regimens, or between the two currently approved PCSK9 inhibitors in a real world setting.

Historically, a substantial proportion of patients fail to achieve acceptable lipid control despite optimal use of established lipid-lowering therapies, mostly because of insufficient response or side effects.[14,15] An audit from the UK indicated that only 31% of patients with prior CVD attained the guideline-recommended LDL-c target of < 1.8 mmol/L.[15,16] PCSK9 inhibitors are widely regarded as a welcome addition to the armamentarium of lipid-lowering therapies. In clinical trials and open-label extension studies, PCSK9 inhibitors reduced LDL-c by 50% to 60% when added to statin therapy and enabled approximately 65% to 85% of patients to reach an LDL-c < 1.8 mmol/L (70 mg/dL), and concordantly improved clinical outcomes.[5–8] Discontinuation rates and the proportion of patients reporting side effects were generally similar between patients receiving PCSK9 inhibitors and placebo. PCSK9 inhibitors are increasingly used in clinical practice and are incorporated in updates of clinical treatment guidelines based on these trial results.[1,9–12] Nevertheless, treatment adherence may be better in clinical trials than in routine care, as has been reported for statin therapy, leading to overestimation of treatment effects.[17] To our knowledge, this is the largest study describing the efficacy and tolerability of PCSK9 inhibitors and treatment continuation rates real-life outpatient practice to date.

Our findings demonstrate that the results of PCSK9 in intervention studies are maintained when these drugs are applied in a routine care setting. Specifically, both the mean LDL-c reduction of 55% and the proportion of patients achieving guideline-recommended LDL-c goals (62%) in our study are in line with observations from clinical trials. There were no meaningful differences between alirocumab and evolocumab. A recent network meta-analyses of clinical trials showed that evolocumab produced greater LDL-c reductions than alirocumab in patients receiving medium- or high-intensity statins.[18] Our cohort might have been too small to detect any such differences between the therapies. In line with trial evidence, there were no major differences in the achieved lipid reductions between patients with FH or statin-intolerance in our cohort (which represent the major categories of patients who will use PCSK9 inhibitors).[5,6]

Approximately 16% of patients in our cohort reported side effects, which led to discontinuation in 2.5% of the total cohort. Although it is not meaningful to numerically compare side effect rates in our study with those from intervention studies due to differences in reporting, blinded studies generally showed favorable tolerability with similar rates of side effect in patients receiving placebo or PCSK9 inhibitors.[5,6] The proportion of patients reporting side effects attributed to PCSK9 inhibitors in our study is comparable to estimates of the occurrence of side effects attributed to statins from patient registries.[2]

While the efficacy and tolerability of statins have been firmly established, approximately 10% to 20% of statin users reported side effects in cohort studies, mostly in the form of muscle complaints.[2,19] Statin-associated muscle symptoms underlie the majority of cases of discontinuation of statin therapy.[19] Meta-analyses of observational studies revealed adherence and discontinuation rates of approximately 50% one year after initiating therapy.[17] Even among patients in the highest risk categories, such as those with prior acute coronary syndromes, < 50% of patients were still using statin therapy 2 years after the initial prescription.[20] It has been confirmed that poor adherence to statin therapy translates into an increased risk of CVD.[21] In the ODYSSEY ALTERNATIVE and GAUSS trials, more than 80% of statin-intolerant patients were able to tolerate PCSK9 inhibitors, suggesting that these drugs are a valuable treatment option for high-risk patients who are unable to tolerate statins at adequate doses.[22–24] Only three out of the 102 statin-intolerant patients in our cohort reported muscle complaints while using PCSK9 inhibitors, and these complaints did not lead to discontinuation. Our study therefore suggests that PCSK9 inhibitors are a valuable alternative for statin-intolerant patients in the routine outpatient setting.

To date, the total body of evidence about the results with PCSK9 inhibitors in routine care is scarce. One audit from the UK reported the results of PCSK9 inhibitor therapy in 105 patients – 75% with FH and 67% with statin intolerance.[25] The mean LDL-c reduction in their cohort was 49%, 30% attained LDL-c < 2.5 mmol/L, and 15% discontinued therapy. Despite a similar mean LDL-c reduction, both the proportion of patients achieving LDL-c goals and continuation rates were lower in the UK audit than those in our cohort as well as a second report from the Netherlands.[26] In the other report from the Netherlands, which comprised 83 FH patients (29% with statin intolerance), the mean LDL-c reduction was 55%, 58% achieved guideline-recommended treatment goals, and 5 patients (6%) discontinued treatment due to side effects. The higher rates of LDL-c goal attainment in our study and in the other Dutch study can be partially explained by differences in baseline LDL-c levels (5.3 mmol/L in the UK audit versus 4.4 mmol/L and 5.0 mmol/L in our study and the other Dutch study, respectively). The higher discontinuation rates in the UK report compared to both Dutch studies can possibly be explained partially by differences in drug dispensing.

In the Netherlands PCSK9 inhibitors are largely dispensed through home delivery or local pharmacies, while in the UK audit repeat prescriptions were only available through hospital pharmacies.

LIMITATIONS

Despite being the largest real life cohort study of PCSK9 inhibitors to date, the generalizability of our study to other centers and clinical settings may be hampered by the fact that our study comprises a single-center experience. Our sample may not resemble the entire population of patients who are eligible for treatment with PCSK9 inhibitors. We only included patients who were actually prescribed PCSK9 inhibitors, which may represent a selection of especially motivated patients. Moreover, discontinuation rates are likely to be influenced by factors such as counseling practices and the frequency of follow-up visits. Also, the Dutch reimbursement system and the absence of copayment could positively influence adherence in the Netherlands as compared to other countries. In addition, while therapy adherence and side effects are systematically discussed and documented during visits to the outpatient clinics for patients in our study, it is recognized that the majority of the data were collected in routine care and we did not use standardized data collection methods. In addition, patients in our cohort received PCSK9 inhibitor in routine care for a maximum of 17 months because PCSK9 inhibitors were only introduced in early 2016. Longer-term studies did not indicate attenuation of the effect over time or increased rates of side effects with prolonged dosing.[27,28]

In conclusion, our study demonstrates that the results of treatment with PCSK9 inhibitors in terms of efficacy and tolerability in routine care are similar to those in intervention studies, and that there were no substantial differences between patients with FH or statin intolerance or between the different dosing regimens. These findings highlight the value of treatment with PCSK9 inhibitors for patients who require additional lipid lowering and support their incorporation in clinical treatment guidelines.

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SUMMARY AND PERSPECTIVES

SUMMARY

The studies described in this thesis were conducted to a) address the relationship between the genotype and phenotype of (novel) causative genetic variants in familial hypercholesterolemia (FH) and b) explore the efficacy and safety of the therapeutic management of FH patients, with a strong emphasis on the role of PCSK9 inhibitors. **Chapter 1 and 2** provide a rationale for this thesis, as well as a general overview about FH.

Part I focuses on the genetic and clinical diversity in FH. **Chapter 3** illustrates how the genotype can be linked to the phenotype in genetic variant that are already established. However, in a large proportion of clinical FH patients no molecular defect can be detected. **Chapters 4, 5 and 6** therefore focus on novel genetic variants that cause FH.

Chapter 3 describes the challenge when it comes to linking the genotype to the phenotype. Over 1,700 variants have been described to cause FH. The described variants in *LDLR* result in a wide variety of mutated LDLRs with variable residual activity and therefore different abilities to remove LDL-c from the circulation. Hence, the clinical variability in terms of LDL-c levels and coronary artery disease (CAD) observed in patients with FH can largely be explained by the severity of the underlying genetic variant. Guidelines advise to classify *LDLR* mutations as 'deficient' or 'defective' based on *in silico* prediction tools and fibroblast studies. However, this classification is likely an oversimplification since it does not reflect the wide range of effects on LDLR function and this likely reflects in a relative crude CAD risk prediction. Therefore, we aimed to classify *LDLR* variants based on severity and perform a CAD risk assessment based on lipid phenotype. We therefore calculated the mean percentile LDL-c per *LDLR* variant for a total of 456 different *LDLR* variants in over 12,000 FH patients identified by the Dutch national screening program. We subsequently stratified these in six groups depending on the LDL-c percentile levels and calculated the CAD risk for each group. We found that compared to healthy non-FH family controls, CAD risk gradually increased from 2.2 in carriers of *LDLR* mutations resulting in LDL-c levels below the 75th percentile for age and gender, up to a 12-fold higher risk for variants causing LDL-c levels above the 98th percentile. These data imply that carrying a pathogenic *LDLR* variant per se results in a higher CAD risk, even in patients with variants of modest severity. Moreover, this study provides a new approach of classifying *LDLR* variants based on percentile LDL-c resulting in a more precise CAD risk prediction in FH than the dichotomous classification based on receptor deficient or defective *LDLR* variants. This adds value to the counselling relatives of FH patients as it allows for a more accurate CAD risk estimate based on the specific *LDLR* variant found in their family.

In the study described in **chapter 4,** we evaluated several novel *PCSK9* variants identified in a clinical cohort of FH patients from Cape Town, South Africa. In contrast to the aforementioned large proportion of variants found in *LDLR* (around 90% of the known genetic variants causing FH are found in the *LDLR*), variants in *PCSK9* that result in an FH phenotype are very rare (< 1%). The identification of novel *PCSK9* gain of function variants is not only of interest for providing a molecular diagnosis in FH patients, but also for the improvement of our understanding and knowledge of *PCSK9* biology. We aimed to determine the pathogenicity of seven novel *PCSK9* variants by segregation analysis and subsequently by *in vitro* studies. One of these novel PCSK9 variants, G516V variant indeed cosegregated with the FH phenotype. This variant was identified in five index patients and cascade screening resulted in the identification of 15 additional carriers. LDL-c levels were significantly higher in carriers in comparison to non-carriers and *in vitro* studies confirmed pathogenicity of the G516V variant. This variant encodes for a PCSK9 protein with increased affinity for the LDLR which leads to reduced dissociation of the LDLR from LDL particles and inability to subsequently recycle the LDLR. As a consequence, the expression of this PCSK9 protein results in lesser LDLR capacity on the surface of the hepatocyte and high LDL-c levels. In addition, this study shows that 1.14% of patients with a clinical FH phenotype presenting to an outpatient clinic in the Western Cape area of South Africa had a pathogenic *PCSK9* variant.

The search for novel genetic variants has been predominantly focused on the coding regions of genes. However, with recent advances in sequencing methods it is now possible and relatively affordable to sequence not only the exome, but also non-coding regions of the genome. **Chapter 5** describes the discovery of a novel deep intronic variant in *LDLR***.** For this, whole genome sequencing was performed in members of a large family with severe clinical FH with no causative variants in the exons of *LDLR*, *APOB* and *PCSK9*. This led to the identification of a deep intronic variant in *LDLR* (c.2140+103G>T) that cosegregated with the FH phenotype. cDNA sequencing showed that this variant resulted in a frameshift and premature stop codon in the *LDLR*. These findings imply that it is important to not only sequence the coding regions, but to also analyze the intronic regions of *LDLR* in FH patients, in particular in patients with clinical FH in whom no causative genetic variants was identified by means of conventional genetic (i.e. Sanger) analysis.

While in **chapters 4 and 5** we concentrate on studies to assess the impact of variants in established FH causing genes, the focus in **chapter 6** is on a previously described putative FH gene: *STAP1*. This gene was identified as a potential FH gene in 2014 and has thereafter been studied by several groups with ambiguous results when it comes to the lipid levels in carriers of variants in *STAP1*. STAP1 is mainly expressed in immune cells, but unlike the

three established FH genes, STAP1 is not expressed in the liver, the organ that plays a crucial role in cholesterol homeostasis. In depth studies on the involvement of STAP1 in cholesterol metabolism were lacking. Therefore, the studies in chapter 6 evaluated the functional impact of STAP1 in lipid metabolism *in vivo* and *in vitro*. A whole-body *Stap1* knockout mouse model (*Stap1*$^{-/-}$) showed that no effect on lipid levels or atherosclerotic lesions compared to control mice. Moreover, transplantation of bone marrow of *Stap1*$^{-/-}$ mice into *Ldlr*$^{-/-}$ mice did not affect lipids and atherosclerosis. Coculture of the human hepatoma cell line HepG2 with peripheral blood mononuclear cells (BMCs) from *STAP1* variant carriers, i.e. the putatively pathogenic variants p.Leu69Ser and p.Glu97Asp, did not affect the *LDLR* mRNA and protein levels as well as the LDL uptake by HepG2 cells. This, combined with the lack of difference in plasma LDL-c levels between *STAP1* variant carriers and their family controls, emphasize that *STAP1* should not be considered an FH gene and that it should be excluded of targeted sequencing panels for FH.

Part II focuses on the treatment of FH patients. **Chapter 7** reviews therapies in FH and discusses the role of PCSK9 inhibitors in the prevention of cardiovascular disease in FH patients.

In a more detailed study, **chapter 8** describes the effect of the PCSK9 inhibitor alirocumab in patients with double heterozygous, compound heterozygous or homozygous FH. To set out for this, almost 1,200 patients from 6 trials were sequenced leading to the identification of 7 double heterozygous (n = 3 *APOB* defective/*LDLR* negative, n = 3 *APOB* defective/*LDLR* defective, n = 1 *LDLR* negative/*PCSK9* gain of function); 10 compound heterozygous (n = 3 *LDLR* defective/*LDLR* negative n = 7 *LDLR* defective/*LDLR* defective) and 3 true homozygous patients (n = 1 *LDLR* defective, n = 2 *LDLRAP1* negative). It was shown that double heterozygous mutations in *APOB* and *LDLR* did not affect the efficacy of alirocumab since the maximal LDL-c reduction was 55.1% to 62%, which is comparable to the effect in patients with heterozygous FH. For the compound heterozygous patients, these percentages were 21.7% to 65.1%. The true homozygous FH patients showed maximal reductions of 22.9% to 34.3%. This study shows that the magnitude of the effect of alirocumab is attributable to maintaining the function of the LDLR protein expressed by the allele with at least some residual LDLR functional activity.

A cross-sectional analysis in a large cohort of over 10,000 FH patients was performed in **chapter 9.** Statins are the cornerstone when it comes to the treatment of patients with FH but in a large proportion of patients the recommended LDL-c levels by international guidelines cannot be reached. Therapies such as CETP inhibitors and PCSK9 inhibitors that (potentially) will help to reduce to LDL-c levels to recommended levels have been

extensively studied in trials. However, patients enrolled in such trials are highly subjective to selection bias and in general have higher LDL-c levels than patients identified by a nationwide cascade screening program. To determine the efficacy of those lipid lowering therapies in real life, this study describes a model to calculate the proportion of patients that would achieve their treatment target when using these compounds. To set out for this, the applied model discriminates four different treatment regimens: 1. high intensity statin therapy; 2. high intensity statin therapy in combination with ezetimibe; 3. high intensity statin therapy and ezetimibe in combination with a CETP inhibitor; 4. high intensity statin therapy and ezetimibe in combination with a PCSK9 inhibitor. Adherence rates of 100% and adherence rates of 62% to 80% (described to reflect the situation in real life) were applied. The results show that less than 10% and 50% of FH patients with and without CVD, respectively, would reach recommended LDL-c levels with high intensity therapy alone. The proportion of patients attaining LDL-c targets increased considerably when ezetimibe was added to the statin therapy or if a CETP- or a PCSK9 inhibitor was added to statin therapy plus ezetimibe. For example, 99.8% of the FH patients with and 100% of the FH patients without CHD would reach LDL-c targets when PCSK9 inhibitors are added to potent statin and ezetimibe therapy. However, when taken into account adherence to therapy, as suggested from literature, these reductions are likely to be lower in real life.

The issue about real life effects of PCSK9 inhibitors is also addressed in **chapter 10** in which the effect of PCSK9 inhibitors in real life is evaluated. Since trial results may not reflect the effectiveness in the real world, this chapter explores the efficacy and tolerability of PCSK9 inhibitors in routine care. For this, a cohort of FH patients and statin intolerant patients from the outpatient clinic of the Academic Medical Center was evaluated. Patients were eligible to receive evolocumab or alirocumab if they required additional lipid lowering therapy because they did not reach LDL-c target despite maximal tolerated statin therapy and ezetimibe, or because they were statin intolerant. This study showed an overall mean LDL-c reduction of 55.0% upon PCSK9 inhibitor therapy in real life. This reduction did not differ between patients with and without FH (54.6% and 55.9%, respectively) and is comparable to the results described in clinical trials. Altogether, this study underlines the potential of PCSK9 inhibitors in real life for FH patients with generally high untreated LDL-c levels with an apparent need of aggressive LDL-c lowering therapies on top of statins.

PERSPECTIVES

As outlined in **chapter 1 and 2**, FH is a common disease that can lead to severe morbidity and mortality, which can be prevented by early diagnosis and treatment. It is therefore crucial to identify patients who are at highest risk. Although it is accepted that CVD risk in FH patients is driven by LDL-c levels, consensus on risk stratification in FH patients is lacking. **Chapter 3** therefore contributes to risk stratification by providing a genotype specific CAD risk estimation, which can be used by physicians to counsel patients and relatives about their CAD risk, based on the specific variant in their family. Moreover, it can be used in evaluation of the cost-effectiveness of genetic cascade screening for specific pathogenic variants that are prevalent in some regions.

However, in a substantial proportion of patients, the genetic basis of the FH phenotype cannot be established.[1] Once a novel variant is identified that might cause FH, is it crucial to evaluate whether this variant is indeed pathogenic. This was done in **chapter 4** for several *PCSK9* variants, in **chapter 5** for an intronic variant in the *LDLR*, and in **chapter 6** for three variants in the *STAP1* gene. The findings from these studies contribute to our knowledge on the molecular basis of FH. The identification of a novel *PCSK9* variant in **chapter 4** does not only have implications for diagnosing FH patients in whom no mutations in *LDLR* or *APOB* are found, but it might also provide new insights on the functionality of the PCSK9 protein. GOF mutations, such as found in our study, can influence LDL-metabolism by encoding for PCSK9 proteins that have an increased infinity for the LDLR. It was also shown recently that some *PCSK9* GOF variants might result in a PCSK9 protein with a diminished ability to bind LDL, and PCSK9 bound to LDL is known to inhibit PCSK9 activity.[2] The PCSK9 field is changing rapidly, however, still many aspects of PCSK9 biology have to be unraveled. This is of interest since PCSK9 is an established target for therapy and additional therapies are being developed. For example, inclisiran, a small interfering RNA that prevents the production of PCSK9, shows a similar efficacy and only has to be administered twice a year.[3]

The discovery of a novel pathogenic intronic variant in **chapter 5** underlines the need for more extensive genetic analysis of the established FH genes *LDLR*, *APOB* and *PCSK9*: not only the exonic but also the (deep) intronic regions should be investigated. Advances in genetics such as whole genome sequencing allow us to explore the impact of variants in genomic regions that have not been extensively studied. Eventually, whole genome sequencing can be applied for the screening of FH patients, especially in those in whom no causative genetic variants can be identified.

In contrast, **chapter 6** shows that *STAP1* should be excluded from sequencing panels in

the future, since in-depth validation studies have shown that *STAP1* is not an FH gene. In the studied families, other causes might be responsible for the suspicion on FH. LDL-c levels from the initial publication were only 11% higher in comparison to controls,[4] which is a smaller effect than seen in carriers of a pathogenic *LDLR*, *APOB* or *PCSK9* variant. It is known that polygenic FH results in lower levels of LDL-c in comparison to monogenic FH.[5] Family members with polygenic FH might have been included, since the FH definition was not strict (TC or LDL-c levels above the 95th percentile). In addition, Lp(a) might be a causal factor for clinical FH. It has been shown that 25% of all clinically diagnosed FH is due to elevated Lp(a) levels.[6] Indeed, in **chapter 6** we observed overall higher mean Lp(a) levels in carriers. However, this was largely due to increased Lp(a) levels in one family carrying the p.Ile71Thr variant. When Lp(a) levels were compared within the family, there was no statistical significance, rather indicating a genetic susceptibility for high Lp(a) in this family. Last, variants in known genes (such as *ABCG5* and *ABCG8*)[7] or unknown genes might be responsible for a clinical diagnosis of FH.

As outlined in **chapter 7**, inhibition of PCSK9 is of great interest in the treatment of FH. The data on the PCSK9 inhibitor alirocumab in **chapter 8** showed that this might not only be of interest for patients with one mutation (such as in heterozygous FH), but also for patients who are double or compound heterozygous or homozygous for pathogenic genetic variants in the *LDLR*, *APOB* or *PCSK9*. In these patients that were treated with alirocumab, clinically relevant LDL-c reductions were observed. However, the LDL-c reduction is likely dependent on the presence of a partial functional allele. This implies that PCSK9 inhibition might be suitable for patients with more than one FH causing genetic variant.

How such trial results can be extrapolated to the real world situation, was explored in **chapter 9** where a model was used to estimate the proportion of FH patients that would reach treatment targets in different treatment regimens. Our results imply that 'the general FH patient' will reach treatment targets if treated adequately and if the adherence is high. In addition, this model might provide for an approach for quantification and estimation of the efficacy of novel therapies, before these are implemented in routine care. And since clinical trial results might be not be representative in the real world situation, the study described in **chapter 10** was the first to investigate the efficacy of PCSK9 inhibition in real life in the Netherlands. The findings from this study showed comparable LDL-c reductions in comparison to clinical trial results and therefore support the potential of PCSK9 inhibition in routine care.

Since a large proportion of patients does not reach recommended LDL-c levels, PCSK9 inhibition is a valuable addition to the armamentarium that can be applied to strive for LDL-c

targets in high-risk FH patients, as proposed by several guidelines.[8,9] However, lifelong treatment with monoclonal antibodies is costly and frequent injections can lead to side effects such as injection side reactions. The siRNA inclisiran is less costly and it provides effective LDL-c lowering with only two injections per year, which is favorable for adherence to therapy.[9] In the future this might be a viable alternative to monoclonal antibodies. Eventually, a bright future is foreseen for FH patients where almost all will achieve unprecedented low LDL-c levels resulting in significant CAD risk reduction.

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A

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NEDERLANDSE SAMENVATTING

De in dit proefschrift beschreven studies zijn uitgevoerd om: a) de relatie tussen het genotype en het fenotype van (nieuwe) genetische varianten bij familiaire hypercholesterolemie (FH) te beschrijven en b) de effectiviteit en veiligheid van medicamenteuze therapie bij patiënten met FH te onderzoeken, met name PCSK9 remmers. In **hoofdstuk 1 en 2** wordt de rationale voor dit proefschrift beschreven, evenals een algemeen overzicht over FH.

Deel I richt zich op de genetische en klinische diversiteit bij FH. **Hoofdstuk 3** illustreert hoe het genotype kan worden gekoppeld aan het fenotype, bij genetische varianten in een reeds bekend FH gen**.** Bij een groot deel van de patiënten met klinische FH kan echter geen genetische oorzaak worden gevonden. Daarom richten **hoofdstukken 4, 5 en 6** zich op nieuwe genetische varianten die FH veroorzaken.

Hoofdstuk 3 beschrijft de uitdaging als het gaat om het linken van het genotype aan het fenotype bij FH. Er zijn meer dan 1,700 genetische varianten beschreven die FH veroorzaken. De beschreven varianten in het gen coderend voor de low-density lipoprotein receptor (*LDLR*) resulteren in een grote verscheidenheid aan LDLR's met variabele restactiviteit en als gevolg een verschillend vermogen om LDL-cholesterol uit de circulatie te verwijderen. Om deze reden kan de klinische variabiliteit die wordt waargenomen bij patiënten met FH, grotendeels worden verklaard door de ernst van de onderliggende genetische variant. Richtlijnen adviseren om *LDLR* varianten te classificeren als 'deficiënt' of 'defect', op basis van *in silico* predictiemodellen en studies in fibroblasten. Deze classificatie is echter waarschijnlijk een oversimplificatie van de werkelijkheid, omdat hierin het brede scala van effecten op de LDLR functie niet wordt weerspiegeld. Dit leidt waarschijnlijk tot een relatief ruwe risico inschatting. Om deze reden besloten we *LDLR* varianten te classificeren op basis van het fenotype (LDL-cholesterolspiegels) en het daarbij behorende hart- en vaatziekte risico. We berekenden daarom het gemiddelde percentiel LDL-cholesterol per *LDLR* variant voor 456 verschillende *LDLR* varianten bij meer dan 12,000 FH patiënten, die geïdentificeerd waren door het Nederlandse nationale screeningsprogramma. We hebben deze vervolgens in zes groepen verdeeld op basis van percentiel LDL-cholesterol, en vervolgens het bijbehorende risico op coronaire hartziekte berekend voor elke groep. We vonden dat in vergelijking met gezonde familiecontroles, het risico op coronaire hartziekte geleidelijk steeg van 2.2 bij dragers van *LDLR* varianten die resulteerden in LDL-cholesterol plasmaspiegels onder het 75e percentiel voor leeftijd en geslacht, tot een 12-maal hoger risico voor varianten die leiden tot een LDL-cholesterol plasmaconcentratie ver boven het 98e percentiel. Deze gegevens impliceren dat het hebben van een pathogene *LDLR* variant op zichzelf resulteert in een hoger risico op coronaire hartziekte, zelfs bij

patiënten met een milde variant. Bovendien biedt deze studie een nieuwe benadering voor het classificeren van *LDLR* varianten op basis van percentiel LDL-cholesterol, hetgeen resulteert in een meer precieze risico inschatting van coronaire hartziekte in FH dan de dichotome classificatie op basis van receptor 'deficiënte' of 'defecte' *LDLR* varianten. Dit is waardevolle informatie voor familieleden van patiënten met FH, omdat het een meer accurate risicoschatting mogelijk maakt op basis van de specifieke *LDLR* variant die in de familie wordt gevonden.

In de studies beschreven in **hoofdstuk 4,** hebben we verschillende nieuwe *PCSK9* varianten geëvalueerd die waren geïdentificeerd in een klinisch cohort van patiënten met FH in Kaapstad, Zuid-Afrika. In tegenstelling tot het grote aantal varianten dat wordt gevonden in *LDLR* (ongeveer 90% van de bekende genetische varianten die FH veroorzaken, wordt gevonden in *LDLR*), zijn varianten in *PCSK9* die resulteren in een FH fenotype zeer zeldzaam (< 1%). De identificatie van nieuwe PCSK9 gain of function-varianten is niet alleen van belang voor een moleculaire diagnose bij patiënten met FH, maar ook voor de verbetering van ons begrip en onze kennis van de biologische aspecten van PCSK9. Het doel van de beschreven studies was om de pathogeniciteit van zeven nieuwe *PCSK9* varianten te bepalen middels segregatie analyse en deze varianten verder te onderzoeken middels *in vitro* studies. Een van deze nieuwe *PCSK9* varianten, de G516V-variant, bleek inderdaad te cosegregeren met het FH fenotype. Deze variant werd geïdentificeerd bij 5 indexpatiënten en cascade screening resulteerde in de identificatie van 15 extra dragers. LDL-cholesterolspiegels waren significant hoger in dragers vergeleken met niet-dragers en *in vitro* studies bevestigden de pathogeniciteit van de G516V variant. Deze variant codeert voor een PCSK9 eiwit met verhoogde affiniteit voor de LDLR, wat leidt tot verminderde dissociatie van de LDLR en LDL-deeltjes, en hierdoor een verminderd vermogen om de LDLR te recyclen. Als gevolg hiervan resulteert de expressie van dit PCSK9 eiwit in een lagere LDLR capaciteit op het oppervlak van de hepatocyten en daarmee hoge LDL-cholesterolspiegels. Bovendien laat deze studie zien dat 1.14% van de patiënten met een klinisch FH fenotype die een polikliniek in het West-Kaapse gebied van Zuid-Afrika bezocht, een pathogene *PCSK9* variant had.

De zoektocht naar nieuwe genetische varianten is voornamelijk gericht op de coderende regio's van genen. Recente ontwikkelingen op het gebied van DNA analyse maken het nu echter mogelijk en relatief betaalbaar om niet alleen de coderende, maar ook niet-coderende gebieden van het genoom te sequencen. **Hoofdstuk 5** beschrijft de ontdekking van een nieuwe diepe intronische variant in het *LDLR* gen. Hiervoor werd whole genome sequencing uitgevoerd bij leden van een grote familie met ernstige klinische FH waarbij er geen pathogene varianten werden gevonden in de exonen van *LDLR*, *APOB* en *PCSK9*.

Dit leidde tot de identificatie van een diepe intronische variant in de *LDLR* (c.2140+103G>T) die cosegregeerde met het FH fenotype. cDNA-sequentiebepaling toonde aan dat deze variant resulteerde in een frameshift en voortijdig stopcodon in de *LDLR*. Deze bevindingen impliceren dat het belangrijk is om niet alleen de coderende regio's te sequencen, maar ook om de intronische regio's van *LDLR* bij patiënten met FH te analyseren, in het bijzonder bij patiënten met klinische FH bij wie geen genetische varianten werden geïdentificeerd door middel van conventionele genetische (Sanger) analyse.

Terwijl we ons in **hoofdstuk 4 en 5** concentreren op studies die de impact beoordeelt van varianten in reeds bekende FH genen, ligt de nadruk in **hoofdstuk 6** op een verondersteld FH gen: *STAP1.* Dit gen werd geïdentificeerd als een potentieel FH gen in 2014 en is daarna onderzocht door verschillende groepen met wisselende resultaten. STAP1 komt voornamelijk tot expressie in immuuncellen, maar in tegenstelling tot de drie bekende FH genen, komt STAP1 niet tot expressie in de lever, het orgaan dat een cruciale rol speelt bij cholesterolhomeostase. Studies over de betrokkenheid van STAP1 bij het cholesterolmetabolisme ontbreken. Om deze reden wordt in hoofdstuk 6 de functionele impact van STAP1 op het lipidenmetabolisme *in vivo* en *in vitro* geëvalueerd. Een *Stap1* knock-out muismodel toonde aan dat er geen effect was op cholesterolconcentraties of atherosclerotische laesies in vergelijking met controlemuizen. Bovendien had een beenmergtransplantatie van *Stap1*-/- muizen in *Ldlr*-/- muizen geen invloed op lipiden en atherosclerose. Coculture experimenten van de menselijke hepatoomcellijn HepG2 met mononucleaire cellen uit perifeer bloed van *STAP1* variant dragers (van de verondersteld pathogene varianten p.Leu69Ser en p.Glu97Asp) hadden geen invloed op het *LDLR*-mRNA en eiwitniveaus, evenals de LDL opname door HepG2-cellen. Dit, gecombineerd met het gebrek aan verschil in LDL-cholesterol plasmaspiegels tussen *STAP1* variant dragers en hun familiecontroles, laat zien dat *STAP1* niet als een FH gen moet worden beschouwd en dat deze moet worden uitgesloten van panels voor genetische diagnostiek bij FH.

Deel II richt zich op de behandeling van FH patiënten. **Hoofdstuk 7** bespreekt therapieën bij FH en de rol van PCSK9 remmers bij de preventie van hart- en vaatziekten. In een meer gedetailleerd onderzoek beschrijft **hoofdstuk 8** het effect van de PCSK9 remmer alirocumab bij patiënten met dubbele heterozygote, compound heterozygote of homozygote FH. Hiertoe werden bijna 1,200 patiënten uit 6 onderzoeken gesequenced, wat leidde tot de identificatie van 7 dubbele heterozygote, 10 compound heterozygote en 3 homozygote patiënten. Er werd aangetoond dat dubbele heterozygote mutaties in *APOB* en *LDLR* geen invloed hadden op de werkzaamheid van alirocumab, omdat er een maximale LDL-cholesterolreductie 55,1% tot 62% werd geobserveerd, wat vergelijkbaar is met het effect bij patiënten met heterozygote FH. Voor de samengestelde heterozygote patiënten waren

deze percentages 21,7% tot 65,1%. Patiënten met homozygote FH vertoonden maximale LDL-cholesterolverlagingen van 22,9% tot 34,3%. Deze studie toont aan dat de grootte van het effect van alirocumab, afhangt van de functie van het LDLR eiwit dat tot expressie wordt gebracht door het allel met ten minste enige residuale LDLR activiteit.

In **hoofdstuk 9** werd er een crosssectionele analyse uitgevoerd in een groot cohort van meer dan 10,000 FH patiënten. Statines vormen de hoeksteen voor de behandeling van patiënten met FH. Echter, bij een groot deel van de patiënten wordt de LDL-cholesterol streefwaarden, aanbevolen door internationale richtlijnen, niet bereikt. Therapieën om LDL-cholesterolspiegels tot aanbevolen niveaus te verlagen zoals CETP- en PCSK9-remmers, zijn uitgebreid onderzocht. Patiënten die deelnemen aan dergelijke onderzoeken zijn echter onderhevig aan selectiebias en hebben over het algemeen hogere LDL-cholesterolspiegels dan patiënten die worden opgespoord door een landelijk cascade screeningprogramma. Om de effectiviteit van cholesterolverlagende therapieën in de dagelijkse klinische praktijk te bepalen, beschrijft deze studie een model om het percentage patiënten te berekenen dat hun behandeldoel zou bereiken bij gebruik van deze therapeutische middelen. Hiertoe onderscheidt het toegepaste model vier verschillende behandelingsscenario's: 1. hoge intensiteit statinetherapie; 2. hoge intensiteit statinetherapie in combinatie met ezetimib; 3. statines met hoge intensiteit en ezetimib in combinatie met een CETP remmer; 4. statines met hoge intensiteit en ezetimib in combinatie met een PCSK9 remmer. Voor therapietrouw werden percentages van 100% en 62% tot 80% toegepast, om de werkelijke situatie weer te geven. De resultaten tonen aan dat minder dan 10% en 50% van de FH patiënten met en zonder coronaire hartziekte respectievelijk, de aanbevolen LDL-cholesterol streefwaarden zullen bereiken met alleen hoge intensiteit statinetherapie. Het deel van de patiënten dat LDL-cholesterol streefwaarden bereikte nam aanzienlijk toe wanneer ezetimib werd toegevoegd aan de statinetherapie of wanneer een CETP- of een PCSK9 remmer werd toegevoegd aan hoge intensiteit statinetherapie in combinatie met ezetimib. Bijvoorbeeld, 99.8% van de FH-patiënten met en 100% van de FH patiënten zonder coronaire hartziekte zal de LDL-cholesterol streefwaarde bereiken wanneer PCSK9 remmers worden toegevoegd aan krachtige statine therapie in combinatie met ezetimib. Wanneer echter rekening wordt gehouden met therapietrouw zoals die in de literatuur wordt gesuggereerd, zullen deze verminderingen in de klinische praktijk waarschijnlijk lager zijn.

De kwestie van de 'real life' effecten van PCSK9 remmers wordt ook behandeld in **hoofdstuk 10**, waarin het effect van PCSK9 remmers in de klinische praktijk wordt geëvalueerd. Omdat onderzoeksresultaten mogelijk niet de effectiviteit in de klinische praktijk weerspiegelen, onderzoekt dit hoofdstuk de effectiviteit en verdraagbaarheid van PCSK9 remmers in de reguliere zorg. Hiertoe werd een cohort van FH patiënten en statine-intolerante patiënten

van de polikliniek van het Academisch Medisch Centrum geëvalueerd. Patiënten kwamen in aanmerking voor evolocumab of alirocumab als ze aanvullende cholesterolverlagende therapie nodig hadden omdat ze ondanks de maximaal getolereerde statinetherapie en ezetimib hun LDL-cholesterol streefwaarde niet konden bereiken, of omdat ze statine-intolerant waren. Deze studie toonde een algehele gemiddelde LDL-cholesterolreductie van 55.0% bij behandeling met PCSK9 remmers bij gebruik in de klinische praktijk. Deze reductie verschilde niet tussen patiënten met en zonder FH (respectievelijk 54,6% en 55,9%) en is vergelijkbaar met de resultaten die zijn beschreven in klinische onderzoeken. Al met al onderstreept deze studie het potentieel van PCSK9 remmers in de klinische praktijk voor FH patiënten met over het algemeen hoge onbehandelde LDL-cholesterolwaarden met een duidelijke behoefte aan agressieve cholesterolverlagende therapieën bovenop statines.

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PORTFOLIO

Name PhD student: Merel Louisa Hartgers
PhD period: February 2015 – February 2019
Name PhD supervisor: Prof. dr. G.K. Hovingh

1. PhD training

	Year	ECTs
Courses, seminars and masterclasses		
Good Clinical Practice (GCP) *Amsterdam UMC-AMC, Amsterdam, The Netherlands*	2015	0.1
Basiscursus Regelgeving en Organisatie van Klinisch Onderzoek (BROK) *Amsterdam UMC – Vumc, Amsterdam, The Netherlands*	2015	0.9
Practical Biostatistics *Graduate School, Amsterdam UMC-AMC, Amsterdam, The Netherlands*	2015	1.1
Scientific Writing in English *Graduate School, Amsterdam UMC-AMC, Amsterdam, The Netherlands*	2015	1.5
Project Management *Graduate School, Amsterdam UMC-AMC, Amsterdam, The Netherlands*	2017	0.6
Clinical Epidemiology: randomized controlled trials *Graduate School, Amsterdam UMC-AMC, Amsterdam, The Netherlands*	2015	0.6
Clinical Epidemiology: evaluation of medical tests *Graduate School, Amsterdam UMC-AMC, Amsterdam, The Netherlands*	2015	0.6
Advanced Lipid Course *University of Cape Town, Cape Town, South-Africa*	2018	2.1
Weekly Journal Club, department of Vascular Medicine *Amsterdam UMC-AMC, Amsterdam, The Netherlands*	2015-2019	4.0
Weekly Clinical Education, department of Vascular Medicine *Amsterdam UMC-AMC, Amsterdam, The Netherlands*	2015-2019	4.0
Oral presentations		
Attainment of low-density lipoprotein cholesterol treatment target in familial hypercholesterolemia patients: a model exploring efficacy of current and novel lipid lowering therapies *American Heart Association Conference, New Orleans, USA*	2016	0.5
Familial hypercholesterolemia: Classification of Mutation Severity According to Percentile Low-Density Lipoprotein Cholesterol Useful for Predicting Coronary Artery Disease Risk *American Heart Association Conference, New Orleans, USA*	2016	0.5
HICC, The Homozygous FH Clinical Collaborators Registry *CareHigh Symposium, Mannheim, Germany*	2016	0.5
STAP1: what should be the next step? *TRANSCARD meeting, Zandvoort, The Netherlands*	2017	0.5

	Year	ECTs
Poster presentations		
Attainment of low-density lipoprotein cholesterol treatment target in familial hypercholesterolemia patients: a model exploring efficacy of current and novel lipid lowering therapies *Rembrandt Symposium, Noordwijkerhout, The Netherlands*	2015	0.5
Attainment of low-density lipoprotein cholesterol treatment target in familial hypercholesterolemia patients: a model exploring efficacy of current and novel lipid lowering therapies *Cardio Vasculaire Conferentie, Amersfoort, The Netherlands*	2015	0.5
Clinical characteristics and prevalence of cardiovascular disease risk factors in heterozygous FH patients: a cross-sectional study from the Netherlands *European Atherosclerosis Society, Innsbruck, Austria (moderated poster)*	2016	0.5
Attainment of low-density lipoprotein cholesterol treatment target in familial hypercholesterolemia patients: a model exploring efficacy of current and novel lipid lowering therapies *European Atherosclerosis Society, Innsbruck, Austria (moderated poster)*	2016	0.5
Treatment effect of alirocumab in patients with heterozygous familial hypercholesterolemia with baseline low-density lipoprotein cholesterol levels > 130 mg/dL receiving high intensity statin *American Heart Association Conference, New Orleans, USA*	2016	0.5
Familial hypercholesterolemia: classification of mutation severity according to percentile LDL-cholesterol useful for predicting coronary artery disease risk *Rembrandt Symposium, Noordwijkerhout, The Netherlands*	2016	0.5
Familial hypercholesterolemia: classification of mutation severity according to percentile LDL-cholesterol useful for predicting coronary artery disease risk *Cardio Vasculaire Conferentie, Amersfoort, The Netherlands*	2016	0.5
Clinical, demographic and genetic characteristics of homozygous familial hypercholesterolemia patients worldwide: interim results from the Homozygous FH (HoFH) InternationI Clinical Collaborators (HICC) registry *European Atherosclerosis Society, Lisbon, Portugal (moderated poster)*	2018	0.5
Congresses and symposia		
International Symposium on Atherosclerosis (ISA), *Amsterdam, The Netherlands*	2015	1.0
Rembrandt Symposium, *Noordwijkerhout, The Netherlands*	2015	0.25
Cardio Vasculaire Conferentie, *Amersfoort, The Netherlands*	2015	0.25
Rembrandt Symposium, *Noordwijkerhout, The Netherlands*	2016	0.25
Cardio Vasculaire Conferentie, *Amersfoort, The Netherlands*	2016	0.25
European Atherosclerosis Society, *Innsbruck, Austria*	2016	1.0
FH Global Summit, *Dallas, USA*	2016	0.5
American Heart Association Conference, *New Orleans, USA*	2016	1.0
European Atherosclerosis Society, *Prague, Czech Republic*	2017	0.75
Familial Hypercholesterolemia Studies Collaboration (FHSC) Lead Investigator Meeting, *Prague, Czech Republic*	2017	0.25
European Atherosclerosis Society, *Lisbon, Portugal*	2018	1.0

	Year	ECTs
Other		
Research fellowship Groote Schuur Ziekenhuis, University of Cape Town Supervisors: A.D. Marais, D.J. Blom, R. Huijgen, G.K. Hovingh *Cape Town, South-Africa*	2018	
Personal research grant AMC Young Talent Fund, AMC Foundation	2018	
Personal research grant Atheros Foundation	2018	
Funding for research fellowship South-Africa by Sanofi	2018	

2. Teaching

Lecturing		
Epidemiology and diagnostics of familial hypercholesterolemia lecture for clinical trial unit *Amsterdam UMC-AMC, Amsterdam, The Netherlands*	2016	0.5
Clinical characteristics of (familial) hypercholesterolemia lecture for clinical chemists in training *Amsterdam UMC-AMC, Amsterdam, The Netherlands*	2017	0.5
Epidemiology and diagnostics of familial hypercholesterolemia lecture for general practitioners *Academisch Netwerk Huisartsen (ANH), Amsterdam, The Netherlands*	2018	0.5
Guest speaker Advanced Lipid Course *University of Cape Town, South-Africa*	2018	0.5
Supervising		
S. Moussane, bachelor thesis *'Screening for familial hypercholesterolemia, what is the best way forward?'* *Amsterdam UMC-AMC, Amsterdam, The Netherlands*	2018	1.0
Supervision of D. Bos, D. Schweitzer, J. Moekotte during their student jobs as junior researcher for the BEAVER study *Amsterdam UMC-AMC, Amsterdam, The Netherlands*	2018	3.0
Supervision of A. Cupido, J. Hooglugt and D. Schalkers during their student jobs as junior researcher for the HICC registry *Amsterdam UMC-AMC, Amsterdam, The Netherlands*	2016 - 2019	3.0

CURRICULUM VITAE

Merel Louisa Hartgers werd geboren in Amsterdam, waar zij opgroeide met haar ouders Wim en Loes, haar zus Myrthe en broer Menso. Zij behaalde haar gymnasiumdiploma met profiel Economie en Maatschappij in 2003 aan het Barleaus Gymnasium te Amsterdam. Hierna volgde zij een studie Psychologie aan de Universiteit van Amsterdam. Na een jaar startte zij met een een versneld traject VWO voor de exacte vakken, waarna zij in 2006 werd toegelaten voor de studie Geneeskunde aan de Universiteit van Amsterdam. In deze periode deed zij een onderzoeksstage aan het Tytgat Instituut in Amsterdam, waar zij de effecten van o.a. TNF-α remmers op de darm van patiënten met inflammatoire darmziekten onderzocht. Haar artsexamen behaalde zij cum laude in Januari 2014. Aansluitend werkte zij een jaar als arts (ANIOS) interne geneeskunde in het Flevoziekenhuis in Almere, waarna zij in 2015 startte met een promotietraject aan de afdeling Vasculaire Geneeskunde van het Amsterdam UMC, locatie AMC. Haar onderzoek naar familiaire hypercholesterolemie werd gesuperviseerd door prof. dr. Kees Hovingh en mede gesuperviseerd door dr. Roeland Huijgen en dr. Aldo Grefhorst. De uitkomsten van dit onderzoek resulteerden uiteindelijk in dit proefschrift. In 2018 vertrok zij voor drie maanden naar Kaapstad, Zuid-Afrika, voor een onderzoeksstage aan de Universiteit van Kaapstad in het Groote Schuur Ziekenhuis. Hier bestudeerde zij nieuwe varianten in het *PCSK9* gen als mogelijke oorzaak voor familiaire hypercholesterolemie. Merel woont momenteel in Singapore met haar man Dick en hun zoon Siem, geboren in 2019.