Familial hypercholesterolemia

Closing the loop between molecular genetics and personalized medicine

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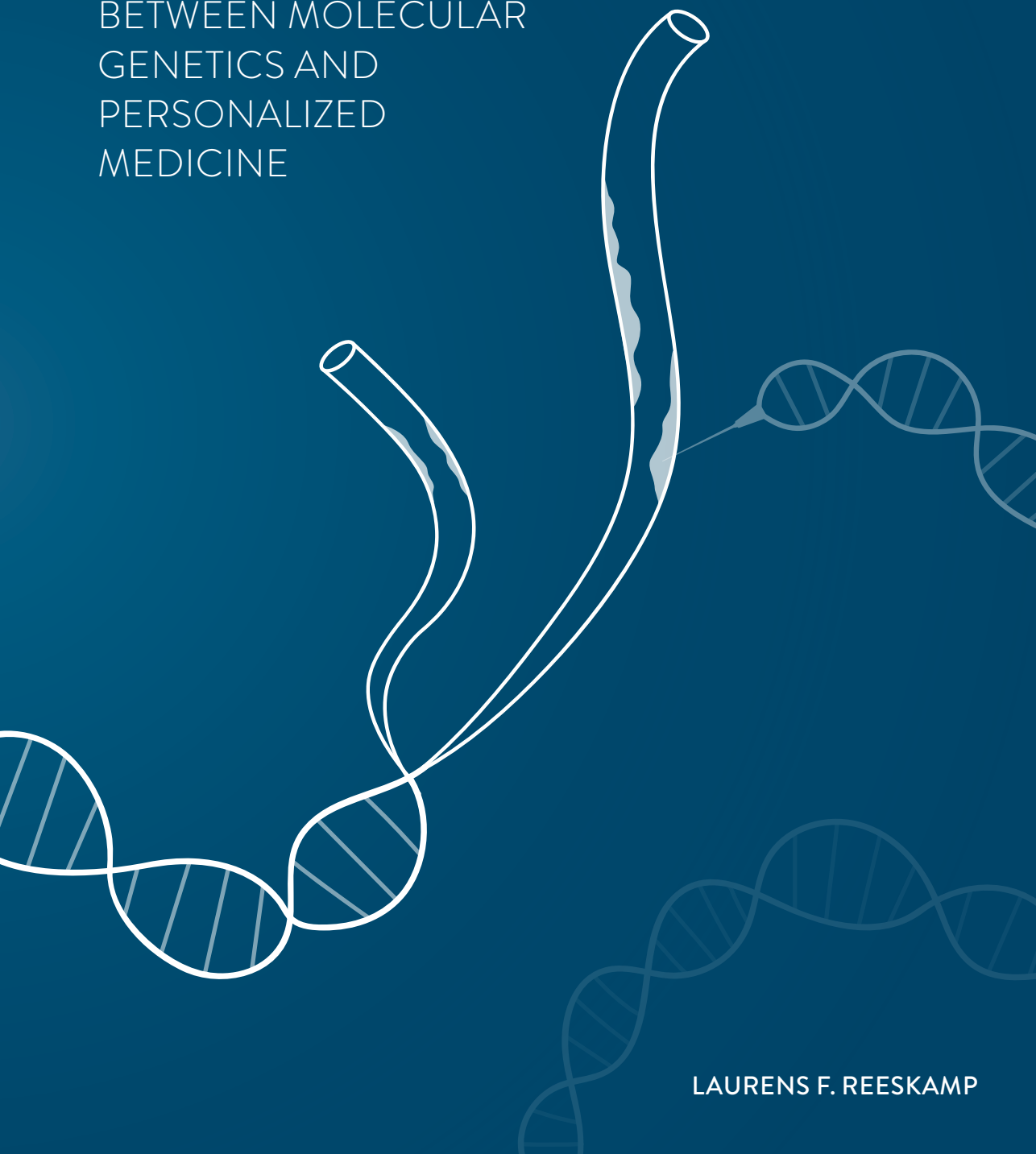

LAURENS F. REESKAMP

Familial Hypercholesterolemia:

closing the loop between molecular genetics
and personalized medicine

Laurens F. Reeskamp

Familial Hypercholesterolemia:
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genetics and personalized medicine

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General introduction, aims, and outline of this thesis

GENERAL INTRODUCTION

Familial Hypercholesterolemia (FH) is a common autosomal disorder characterized by elevated plasma low-density lipoprotein cholesterol (LDL-C) levels. Due to the accumulation of LDL-C in arterial walls over time, patients with FH are at increased risk for premature atherosclerotic cardiovascular disease (ASCVD) compared to people with normal LDL-C levels.[1] Recent meta-analyses have shown that approximately 1:300 persons in the general population have FH[2,3], making it the most common monogenic disorder in humans.[4] Especially in patients with established ASCVD the prevalence of FH is staggering; 1 out of 17 ASCVD patients is carrier of an FH causing variant.[3] However, many patients with FH are not aware of their underlying genetic condition and do not receive adequate lipid lowering therapies to prevent future ASCVD events.[5]

Genetic architecture and molecular diagnosis of FH

FH is diagnosed by the identification of an FH causing genetic variant in one of three genes, namely the genes encoding the low-density lipoprotein receptor (*LDLR*), apolipoprotein B (*APOB*), and proprotein convertase subtilisin/kexin type 9 (*PCSK9*). The proteins encoded by these genes are involved in LDL-C homeostasis, specifically in the adequate removal of LDL particles from the circulation by the liver. Patients with one defective allele, heterozygous FH (heFH) patients, have residual LDL uptake capacity left but usually present with LDL-C plasma levels above 5 mmol/L, which is above the 99th percentile for age and sex.[6] For reference, the mean LDL-C plasma levels for Dutch men and women are 3.4 and 3.0 mmol/L, respectively.[6] Some patients carry two defective alleles in the FH genes, a condition named homozygous FH (hoFH), which is characterized by far higher LDL-C levels (up to 20 mmol/L) and the presence of atherosclerotic plaques at very early age, even before the age of 20 years.[7] The prevalence of hoFH is estimated to be 1:300.000 persons in the general population.[8]

There is, however, significant overlap in phenotype between patients with hoFH, heFH, and patients without pathogenic variants in one of the three FH genes. This latter group is a rather substantial group of hypercholesterolemic patients. In one study 58% of patients with LDL-C levels between 5 and 5.99 mmol/L and

12% of patients with LDL-C levels above 8 mmol/l did not carry an FH causing variant in the three FH genes.[9] This fuels ongoing research into the missing heritability of hypercholesterolemia in these patients, and multiple FH mimicking or hypercholesterolemia traits have been identified, such as high levels of lipoprotein (a)[10], polygenic hypercholesterolemia[11], and variants in other genes involved in LDL-C metabolism (i.e. ATP binding Cassette Subfamily G Member 5 [*ABCG5*] or 8 [*ABCG8*][12], apolipoprotein E [*APOE*][13], and Signal Transducing Adaptor Family Member 1 [*STAP1*][14]).

Cumulative LDL-C exposure in FH patients

Despite differences in the exact underlying cause of hypercholesterolemia in FH patients, they share the main determinant for ASCVD risk: high plasma LDL-C levels for a long period of time. Since FH is a genetic disorder, the arteries of these patients are exposed to high LDL-C levels from birth, in contrast to incidental hypercholesterolemia (for example due to environmental factors) in patients who are only exposed to hypercholesterolemia for a short period of time. Hence, the detrimental effects of high LDL-C are more severe/prominent in FH patients. This is well illustrated by a study that investigated the odds for coronary artery disease (CAD) at different LDL-C levels for patients with and without FH causing variants. Patients with LDL-C levels above 5.7 mmol/L with and without FH had an odds ratio for CAD of 25.8 and 7.7, respectively, compared to patients without FH and LDL-C <3.4 mmol/L.[1] This concept of accelerated atherosclerotic plaque formation in FH patients is illustrated in Figure 1, which also shows that early LDL-C lowering in these patients is likely to result in a greater ASCVD risk reduction compared to delayed intervention. This concept was recently shown to hold true in a landmark study that compared the incidence of ASCVD events in FH children (n=214), who started LDL-C lowering treatment with a statin between the ages of 8-18 years, with their parents who had no access to statins in the first decades of their life. Early treatment with statins resulted in only 1 ASCVD event (0.47%) before the age of 40 in a child who had discontinued statin therapy compared to 26% of their parents with FH.[15] Even more striking is the observation that seven percent of the parents with FH had already died before the age of 40 compared to none of the FH children. Unsurprisingly, (inter)national guidelines advice to start LDL-C lowering therapy in FH patients as young as eight years old.[16]

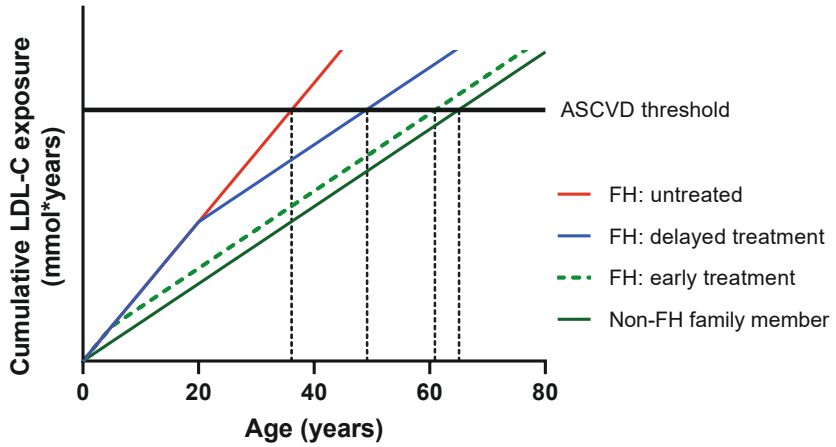

Figure 1: Cumulative LDL-C exposure in FH patients results in early ASCVD
Relationship between age and cumulative LDL-C exposure in FH patients (red line) and unaffected family members (green solid line). Treatment with lipid lowering therapies deflects from accelerated atherosclerotic plaque formation and results in later ASCVD events (blue line). This effect is even greater for those FH patients starting treatment early in life (green dashed line). Adapted from [17]

Current treatment of FH

The corner stone of lipid lowering in FH patients is statin therapy. Statins block HMG-CoA reductase, one of the main enzymes involved in cholesterol synthesis. Inhibition of HMG-CoA reductase results in reduced intracellular cholesterol synthesis and subsequent upregulation of LDLR in hepatocytes, effectively accelerating LDL-C clearance from the circulation.[18] High intensity statins (such as atorvastatin and rosuvastatin) lower LDL-C levels by 50-60%. However, in many FH patients such LDL-C reductions are insufficient to achieve guideline recommended LDL-C goals of 1.4 mmol/L and 1.8 mmol/L in those with and without established ASCVD, respectively.[5] Therefore, many FH patients are also treated with ezetimibe, which inhibits the intestinal uptake of cholesterol via Niemann-Pick Disease Type C like 1 (NPC1L1) protein, as well as PCSK9 by monoclonal antibodies, which increases LDLR recycling and thus LDL-C uptake by the liver. Both ezetimibe and PCSK9 inhibitors have been proven to effectively lower LDL-C in FH patients, with an additional 16.5%[19] and 54%[20] LDL-C lowering on top of statins, respectively. However, despite triple therapy in severe FH cases or due to adverse effects because of these

therapies, a significant proportion of patients will not attain treatment goals[21] and novel drug targets for LDL-C lowering are being investigated.

Novel LDL-C lowering therapies

Two potential new LDL-C lowering therapies are inhibition of apolipoprotein B (APOB) synthesis and inhibition of angiopoietin like 3 (ANGPTL3) protein.

APOB is the main structural protein of LDL and is synthesized in the liver and secreted into the circulation as part of VLDL particles which are LDL precursors. Triglycerides in VLDL are hydrolyzed by lipoprotein lipase (LPL) and used by peripheral tissues such as hearth and muscles for energy consumption or stored in adipocytes for future use. This process results in shrinkage of VLDL particles into smaller, cholesterol-rich particles called intermediate low-density lipoproteins (IDL) and finally LDL. Blocking APOB production, without which VLDL cannot be produced, is thus a logical drug target for LDL-C reduction in FH patients and might be achieved by mipomersen. Mipomersen is an antisense oligonucleotide directed against *APOB* mRNA in the liver, effectively blocking APOB protein synthesis.[22]

Accelerating the lipolysis process by inducing LPL activity and (hence) increasing the uptake of LDL particles from the circulation might be another method to lower LDL-C. ANGPTL3 is a natural inhibitor of LPL and thus inhibition of ANGPTL3 would result in a faster turnover of VLDL to LDL. A pilot study in hoFH patients has shown a 50% LDL-C reduction[23]. However, the exact LDL-C lowering mechanism of ANGPTL3 inhibition remains to be elucidated.

Lastly, an understudied, but important organ in lipid metabolism is the intestine. Dietary cholesterol is taken up via NP1CL1 from the intestinal lumen after which it is released into the circulation via lymph in chylomicrons. Vice versa, the ABCG5 and ABCG8 proteins function as a heterodimer (ABCG5/G8) responsible for transmembrane transport of cholesterol from hepatocytes into to bile, via which cholesterol ends up in feces. Enterocytes also express the ABCG5/G8 heterodimer which is responsible for export of sterols such as cholesterol from the enterocyte into the intestinal lumen. An interesting and potential target for cholesterol lowering is so called trans intestinal cholesterol excretion (TICE), which is the net resultant

of cholesterol uptake and excretion via NP1CL1 and ABCG5/G8, respectively, by enterocytes. It was shown in mice and healthy volunteers that inhibition of NP1CL1 with ezetimibe in combination with bile acid induced stimulation of cholesterol excretion via ABCG5/G8 results in dramatic cholesterol loss via feces[24]. Targeting this process with ezetimibe/bile acids combination therapy might therefore be a potential lipid lowering pathway in hypercholesterolemic patients.

AIMS AND OUTLINE OF THIS THESIS

The current thesis aims to investigate (novel) genetic causes of hypercholesterolemia in patients with clinical FH and thus to identify those at the highest life time risk of ASCVD. Moreover, it aims to investigate novel therapeutic targets for LDL-C lowering in hypercholesterolemic patients to halt their accelerated cumulative LDL-C exposure.

In **part 1A**, we describes current clinical practice for molecularly diagnosing FH by means of next-generation sequencing (**chapter 2**) and assesses variants in often neglected DNA regions, the introns of *LDLR*, for their role in causing FH (**chapters 3 and 4**). In **part 1B** the research moves towards novel diagnostic and genetic targets in FH patients. Next, genetic variants in the *ABCG5* and *ABCG8* genes in clinical FH patients (**chapter 5**) and the underlying mechanisms by which variants in *STAP1* might by associated with FH are investigated (**chapter 6**). Lastly, we look into epigenetic regulation by means of DNA methylation as a new cause of FH (**chapter 7**).

The **second part** of this thesis is dedicated to the role of ANGPTL3 in dyslipidemia. First, whether targeting ANPGTL3 will reduce ASCVD is reviewed in **chapter 8** and **chapter 9** investigates the efficacy of evinacumab, a monoclonal antibody against ANGPTL3, for LDL-C lowering in hoFH patients. Additionally, we report about the effect of evinacumab on atherosclerotic plaque regression in two hoFH patients (**chapter 10**). Since the exact working mechanism by which ANGPTL3 inhibition lowers LDL-C is unknown, the studies described in **chapter 11** were designed to assess apoB turnover in patients with hoFH before and after ANGPTL3 inhibition.

Lastly, since ANGPTL3 inhibition is likely to be used in conjunction with statin therapy in clinical practice we investigate the effect of statin therapy on ANGPTL3 secretion *in vitro* and on plasma ANGPTL3 levels *in vivo* in **chapter 12**.

The **third and last part** of this thesis focuses on other therapeutic options for LDL-C lowering in hypercholesterolemic patients. In **chapter 13**, we investigate the safety and efficacy of mipomersen for LDL-C lowering in heFH patients and in **chapter 14** we review the evidence for presence of TICE in humans as a targetable mechanism to lower LDL-C.

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PART I A

Diagnosing familial hypercholesterolemia

2

Next-generation sequencing to confirm clinical Familial Hypercholesterolemia

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ABSTRACT

Background: Familial hypercholesterolemia (FH) is characterized by high LDL-C levels and is caused by a pathogenic variant in *LDLR*, *APOB* or *PCSK9*. We investigated which proportion of suspected FH-patients was genetically confirmed, and whether this changed over the past 20 years in The Netherlands.

Methods: Targeted next-generation sequencing of 27 genes involved in lipid metabolism was performed in patients with LDL-C levels >5mmol/L who were referred to our center between May 2016 and July 2018. The proportion of patients carrying likely pathogenic or pathogenic variants in *LDLR*, *APOB*, or *PCSK9,* or the minor FH-genes *LDLRAP1, ABCG5, ABCG8, LIPA,* and *APOE* were investigated. This was compared with the yield of Sanger sequencing between 1999-2016.

Results: 227 out of the 1528 referred patients (14.9%) were heterozygous carriers of a pathogenic variant in *LDLR* (80.2%), *APOB* (14.5%) or *PCSK9* (5.3%). >50% of patients with a Dutch Lipid Clinic Network score of "probable" or "definite" FH, were FH mutation-positive. 4.8% of the FH mutation-negative patients carried a variant in one of the minor FH-genes. The mutation detection rate decreased over the past two decades, especially in younger patients in which it dropped from 45% in 1999 to 30% in 2018.

Conclusions: A rare pathogenic variant in *LDLR*, *APOB*, or *PCSK9* is identified in 14.9% of suspected FH patients and this rate has decreased in the past two decades. Stringent use of clinical criteria algorithms is warranted to increase this yield. Variants in the minor FH-genes provide a possible explanation for the FH phenotype in a minority of patients.

INTRODUCTION

Familial Hypercholesterolemia (FH) is a common autosomal dominantly inherited disease with a prevalence of 1:250[1],hallmarked by elevated low-density lipoprotein cholesterol (LDL-C) levels with a concomitant high risk for premature cardiovascular disease.[2] FH is diagnosed on the basis of clinical criteria (e.g. Dutch Lipid Clinic Network [DLCN] criteria), ideally followed by DNA sequencing to identify variants in the *LDLR*, *APOB*, and *PCSK9* genes that cause FH. A genetic diagnosis of FH allows better CVD risk stratification[2], increases therapy adherence of the diagnosed patient[3], and allows cascade testing of first-degree relatives[4].

Apart from the three canonical FH-genes, pathogenic variants in other so called "minor FH" genes may also contribute to or mimic the FH phenotype.[5] Patients with variants in *APOE*[6], *ABCG5* and/or *ABCG8*[7], or homozygous variants in *LIPA*[8] or *LDLRAP1*[9], often present with hypercholesterolemia. Advances in targeted next-generation sequencing (NGS) methods enabled sequencing of multiple genes at once, providing genetic information in the classical FH-genes, the minor FH-genes, as well as other lipid metabolism related genes in a clinical diagnostic setting[7,10]. However, it is yet unknown whether addition of these extra genes to NGS panels increases the proportion of molecularly diagnosed FH patients.

Since diagnostic resources in many countries are limited and FH-causing variants in one of the three culprit genes are not always identified in patients with severe hypercholesterolemia[11–13], selection of FH patients who are deemed eligible for molecular testing is mostly based on clinical FH criteria such as the DLCN criteria.[14] Moreover, the yield of FH sequencing is proportionally related to the severity of the FH phenotype (i.e., LDL-C levels). For example, in a general-population study with patients with LDL-C levels above 5 mmol/L only 2% of patients carried an FH-causing variant.[2] This percentage is proportionally associated to the measured LDL-C level and increases when patients are selected based on additional clinical FH criteria, such as xanthomas, which are incorporated in algorithms such as the DLCN score. Also, Wang and colleagues showed that variants were found in approximately 40% in clinical FH patients with LDL-C levels between 5 and 6 mmol/L

while approximately 90% of clinical FH patients with LDL-C levels above 8 mmol/L carried a pathogenic variant in *LDLR*, *APOB*, or *PCSK9*.[13]

In the present study, we set out to quantify the percentage of patients in whom a pathogenic variant in established and minor FH-genes is found, with a targeted NGS approach in a large cohort of patients that was referred to our molecular diagnostics laboratory. Furthermore, we investigated whether, and to which extent, the use of two clinical FH criteria algorithms (DLCN and MedPed) impacted the diagnostic yield and we evaluated the diagnostic yield during the last two decades.

METHODS

Referral procedures

The Amsterdam University Medical Center is the national referral center for DNA diagnostics for patients with suspected genetic dyslipidemias. Physicians who consider a genetic cause to underlie the encountered dyslipidemia in their patient, ship a blood sample to the central facility along with questionnaire containing clinical data about lipid levels, the results of the physical examination, use of medication, and the medical and family history of the patient. For this study we analyzed data from patients whose referring physician requested molecular FH analysis based on their clinical judgement.

DNA sequencing

Since May 2016, all referrals for genetic dyslipidemia are analyzed using an in house next-generation sequencing (NGS) capture covering 27 lipid genes, including *LDLR*, *APOB*, and *PCSK9* (SeqCap easy choice, Roche NimbleGen Inc., Pleasanton, USA). A full list of this gene panel is provided in Supplementary Table 1. Two trained molecular geneticists assessed all variants in the exons and at least 20 base pairs in the adjacent introns of these sequenced genes for their pathogenicity according to standard guidelines for variant classification of the American College of Medical Genetics and Genomics.[15] Detection of copy number variants (CNVs) was based on normalized depth of coverage analysis as its principal method. Using this scoring system, class 4 (likely pathogenic) and class 5 (pathogenic) variants in

LDLR, *APOB*, and *PCSK9* were considered to be causal. Additionally, we investigated class 4 and 5 variants in "minor FH-genes", comprising *LDLRAP1*, *APOE, LIPA, ABCG5*, and *ABCG8*.[5] We excluded *STAP1* variants from this analysis, since this is no longer considered an FH gene.[16,17] Next, the allele frequency of variants in the minor FH genes in FH mutation-negative patients was numerically compared with the allele frequency reported in the European non-Finnish population in gnomAD V2.1.1. (https://gnomad.broadinstitute.org/[18]). The E2/E2 genotype was excluded from this analysis, because this causes dysbetalipoproteinemia which is a distinct phenotype from FH. All variants classified as class 4 and class 5 are hereafter referred to as pathogenic variants.

Before May 2016 (n = 20912), a molecular diagnosis of FH was made by means of Sanger sequencing of *LDLR*, *APOB*, and *PCSK9* and, when negative with LDL-C levels above age-specific thresholds[19], subsequent analysis for CNVs in *LDLR* by Multiplex Ligation-dependent Probe Amplification.

Patient selection

All consecutive patients who were referred for genetic FH testing, who gave written informed consent and were analysed for variants between May 2016 and July 2018, were eligible for this analysis. Patients were excluded when LDL-C level was below 5 mmol/L (cutoff for a DLCN score of at least "possible FH"), when data on LDL-C was missing or reported LDL-C was obtained during active lipid-lowering treatment. Patients with triglyceride levels >4.5 mmol/L were excluded to prevent enrolling patients with unreliable LDL-C levels. For the comparison with historical data on Sanger sequencing, we selected all referred patients that were analysed for FH variants between 1999 and May 2016 and compared the data to the NGS yield for all patients with an indication for genetic FH diagnostics, also those with LDL-C levels below 5 mmol/L. All included patients referred for genetic analysis are index cases. Genetic analysis for cascade screening of members of families with a proven FH variant is performed in a separate program in which targeted variant identification is performed in these individuals. FH patients with homozygous or compound heterozygous pathogenic variants in *LDLR*, *APOB*, or *PCSK9* were excluded from all analyses, except from the overall frequency tables (Table 1, Supplementary Table 2).

Clinical FH scoring

We applied two clinical scoring systems for FH. First, as collected data were limited, the Dutch lipid clinical network criteria[14] were modified to fit the collected clinical data (Supplementary Table 5) and applied to the FH cohort. Second, the MedPed criteria for LDL-C levels in the general population were applied (Supplementary Table 6).[20]

Statistical analysis

Normally and non-normally distributed data were compared between groups using independent t-test and Mann-Whitney U test, respectively. Categorical data were compared using the Fisher-exact test. For analysis of the risk for carrying an FH-causing variant over time, the year of sequencing and the age of the patient were used as covariates in a logistic regression model with FH-causing variant status as outcome (yes/no). The variable year of sequencing was added to the model with use of a natural spline and age as a categorical variable consisting of age tertiles. A p-value <0.05 was considered statistically significant. All statistical analyses were performed in R, version 3.6.1 (R Foundation, Vienna, Austria).

RESULTS

Yield of NGS sequencing

Between May 2016 and July 2018, 2320 index patients with clinical FH were referred to our laboratory for molecular analysis. After excluding patients who were using lipid-lowering therapy, had incomplete lipid data or TG levels above 4.5 mmol/L, a total of 1858 patients remained. 1528 of those patients had LDL-C levels above 5 mmol/L, corresponding to a DLCN score of "possible FH". (Figure 1). Genetic analysis of the three culprit FH-genes (*LDLR*, *APOB*, and *PCSK9*) showed that 14.9% of these clinical FH patients were heterozygous carriers of pathogenic variants in these genes (Table 1, Figure 2A). Three homozygous FH patients were identified (0.2%, Table 1).

In total, 227 (14.9%) patients were identified who were heterozygous carrier of a pathogenic variant in *LDLR*, *APOB* or *PCSK9*. The proportion of clinical FH patients with such a variant increased with LDL-C levels and ranged from 7.9% in patients with

untreated LDL-C levels between 5-5.99 mmol/L to 54.3% in patients with LDL-C levels above 8 mmol/L (Figure 2A). In the vast majority of FH mutation-positive patients, *LDLR* variants were identified (80.2%, Table 1, Figure 2B), followed by *APOB* variants and *PCSK9* variants in 14.5% and 5.3% of FH mutation-positive patients, respectively. With increasing LDL-C levels, the proportion of FH mutation-positive patients with an *LDLR* variant increased from 74.5% to 94.1% (Figure 2B). The distribution of LDL-C levels for carriers of pathogenic *LDLR*, *PCKS9* and *APOB* variants is shown in Supplementary Figure 1, and the mutation type frequencies and associated LDL-C levels are reported in Supplementary Table 4 and Supplementary Figure 2.

Table 1: FH genotype frequencies

Genotype	n	% in all clinical FH patients	% of heterozygous FH mutation-positive patients
FH mutation-negative	1298	84.9%	-
LDLR heterozygote	182	11.9%	80.2%
APOB heterozygote	33	2.2%	14.5%
PCSK9 heterozygote	12	0.8%	5.3%
LDLR-PCSK9 double heterozygote	1	0.1%	-
LDLR compound heterozygote	1	0.1%	-
LDLR homozygote	1	0.1%	-
Total	1528	100	100

Clinical characteristics of FH mutation-positive and FH mutation-negative patients

Genetically confirmed FH patients were younger (mean age 43.6 ± 16.4 vs. 54.5 ± 11.7 years, p <0.001) and had higher total cholesterol (9.2 ± 1.6 vs 8.4 ± 1.0 mmol/L, p <0.001) and LDL-C levels (7.1 ± 1.4 vs. 6.2 ± 0.9 mmol/L, p <0.001) compared to those without a pathogenic variant in one of the three FH-genes (Table 2). The prevalence of CVD of the patient and their parents was similar between the two groups, except for peripheral artery disease (PAD) and stroke, which were observed more frequently in FH mutation-negative patients (4.5 vs 1.0%, p = 0.026 and 5.0 vs 1.4%, p = 0.031, respectively). Furthermore, a history of liver enzyme abnormalities and the use of hormone replacement therapy were significantly more prevalent in FH mutation-negative compared with FH mutation-positive patients (6.6 vs 2.1%, p = 0.027 and 8.2 vs 4.7%, p = 0.049 respectively, see Table 2).

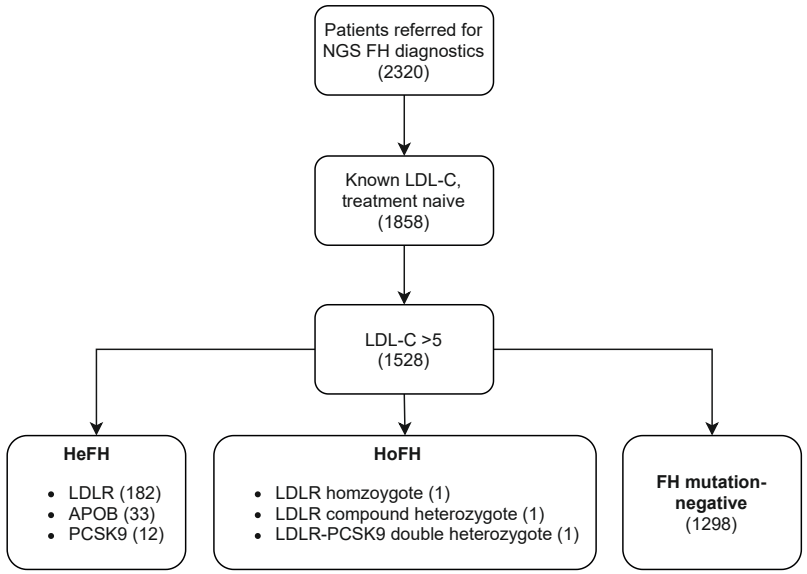

Figure 1: Study design flowchart
Flowchart showing selection of patients included in the analyses. NGS, next-generation sequencing; HeFH, heterozygous Familial hypercholesterolemia; HoFH, homozygous Familial Hypercholesterolemia.

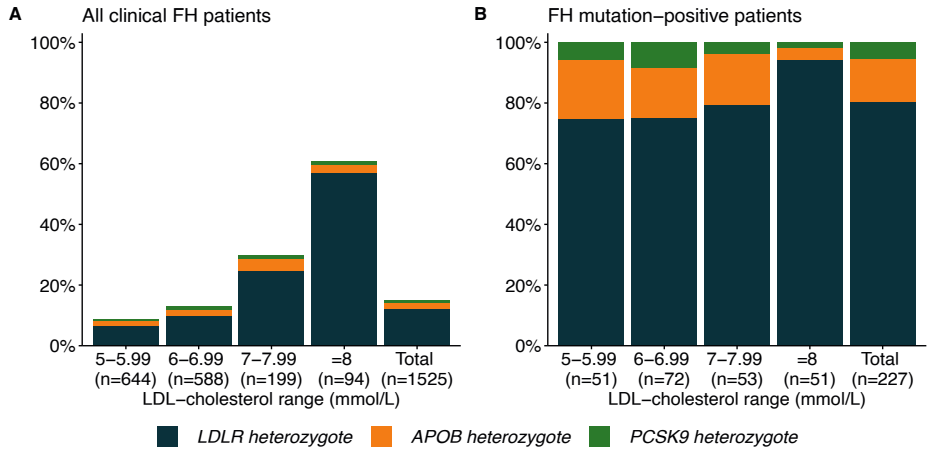

Figure 2: Distribution of FH-causing variants across LDL levels
Proportion of patients with pathogenic variants in *LDLR*, *APOB*, or *PCSK9* stratified according to LDL-C levels. (A) All clinical FH patients and (B) Only patients with genetically confirmed FH. Patients who were found to be homozygote or compound heterozygote for pathogenic variants in *LDLR*, *APOB*, *PCSK9* were excluded from all graphs.

Minor FH-genes

Apart from the three major genes, variants in a number of other genes have been described to result in a phenotype that mimics FH (i.e. *APOE*, *ABCG5, ABCG8, LIPA, LDLRAP1* are implied to cause FH). We therefore investigated how many patients were carriers of pathogenic variants in these genes. Of the FH-mutation-negative patients, 4.8% (n=63), which equals 4.1% of all patients, (excluding those with a combination of a pathogenic variant in both a minor FH gene and in *LDLR*, *APOB,* or *PCSK9*) were heterozygous carrier of a pathogenic variant in one of these genes. Eight were true dysbetalipoproteinemia patients with the E2/E2 genotype and two had hypercholesterolemia associated with the recessive disease sitosterolemia (one *ABCG8* true homozygote and one *ABCG8* compound heterozygote; Supplementary Table 2). 16 patients had a combination of an FH-causing variant in *LDLR*, *APOB*, or *PCSK9* with one or more homozygous or compound heterozygous variants in the minor FH-genes (Supplementary Table 2). The allele frequency of variants in *APOE*, *ABCG5*, and *ABCG8* was numerically higher in FH mutation-negative patients compared to an European, non-Finnish, reference population in gnomAD (supplementary Table 3). An additional 8 variants, not reported in this gnomAD population, were identified. None of the patients were found to be homozygous or compound heterozygous for pathogenic variants in *LDLRAP1* or *LIPA*.

Clinical FH criteria

The DLCN and MedPed criteria are often used in clinical practice to diagnose FH. We investigated whether the proportion of patients with an FH-causing variant increased with the use of clinical FH criteria (Supplementary Table 5 & 6). While we found that 14.9% of the subjects in our cohort carried a variant in the culprit genes, this percentage was 9.9%, 23.6%, and 40.2% when patients were stratified for possible, probable, and definite FH according to the modified DLCN criteria, respectively (Figure 3A, Supplementary Table 7). These percentages increased to 25.0%, 50.9%, and 50.0% when the modified DLCN criteria were only applied to patients with complete data for DLCN criteria (n = 219, Figure 3B, Supplementary Table 7). 32.8% of the patients classified as having FH using the MedPed criteria had genetically confirmed FH (Figure 3C, Supplementary Table 8).

Table 2: Clinical characteristics of the study population

	FH mutation-negative (n = 1298)	FH mutation-positive (n = 227)	p-value
Female – No (%)	781 (60.2)	129 (56.8)	0.382
Age (years) – mean (SD)	54.5 (11.7)	43.55 (16.4)	<0.001
BMI (kg/m²) – mean (SD)	26.7 (4.0)	26.29 (4.7)	0.17
Total cholesterol (mmol/L) – mean (SD)	8.4 (1.0)	9.18 (1.6)	<0.001
LDL-C (mmol/L) – mean (SD)	6.2 (0.9)	7.09 (1.4)	<0.001
HDL-C (mmol/L) – mean (SD)	1.4 (0.4)	1.36 (0.5)	0.037
Triglycerides (mmol/L) – mean (SD)	1.9 [1.4, 2.6]	1.50 [1.0, 2.1]	<0.001
CVD history			
Myocardial infarction – No (%)	116 (9.1)	17 (7.8)	0.592
Angina – No (%)	125 (10.0)	17 (7.8)	0.363
Percutaneous Coronary Intervention – No (%)	111 (8.8)	13 (6.2)	0.258
Coronary Artery Bypass Graft – No (%)	43 (3.4)	9 (4.3)	0.68
Peripheral Artery Disease – No (%)	56 (4.5)	2 (1.0)	0.026
Stroke – No (%)	63 (5.0)	3 (1.4)	0.031
Parents with CVD– No (%)			
- Both parents	252 (19.4)	40 (17.6)	
- One parent	619 (47.7)	101 (44.5)	0.388
- No parent or unknown	427 (32.9)	86 (37.9)	
Other potential causes of hypercholesterolemia			
Hypothyroidism – No (%)	64 (5.6)	5 (2.7)	0.138
Proteinuria – No (%)	28 (2.6)	5 (3.1)	0.953
Liver enzyme abnormalities – No (%)	74 (6.6)	4 (2.1)	0.027
Hormone use – No (%)	58 (4.7)	17 (8.2)	0.049
Diabetes Mellitus – No (%)	65 (5.2)	5 (2.4)	0.118
Alcoholism – No (%)	48 (3.9)	10 (4.8)	0.698
DLCN score – No (%)			
Possible	967 (74.5)	106 (46.7)	
Probable	279 (21.5)	86 (37.9)	<0.001
Definite	52 (4.0)	35 (15.4)	

Characteristics of all included subjects stratified according to molecular diagnosis (FH mutation-positive vs. FH mutation-negative patients). Age, BMI, Total cholesterol, LDL-C, HDL-C are reported as mean (±SD), triglycerides as median [inter quartile range]. BMI, body mass-index; CVD, cardiovascular disease; DLCN, Dutch Lipid Clinic Network.

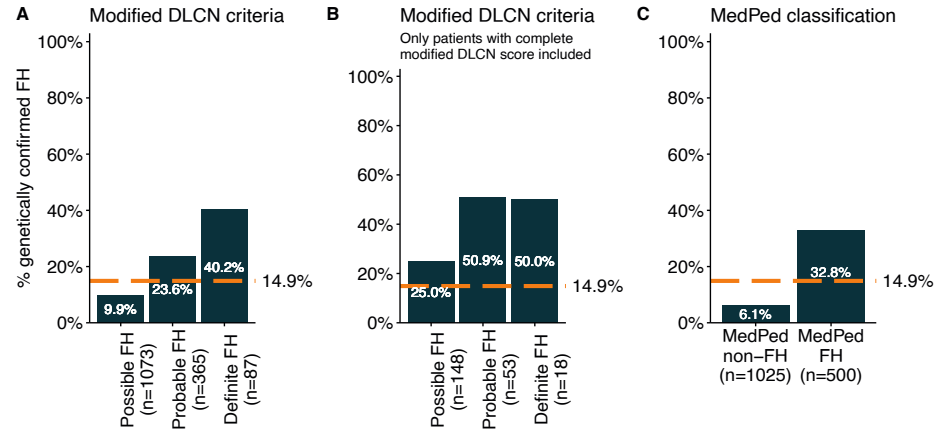

Figure 3: Percentage of patients with genetically confirmed FH per clinical criteria categories
Proportion of patients with genetically confirmed FH stratified according to clinical FH diagnosis: (A) Modified DLCN criteria, (B) Modified DLCN criteria, only included patients with complete data, (C) MedPed classification. Patients who were found to be homozygote or compound heterozygote for pathogenic variants in *LDLR*, *APOB*, *PCSK9* were excluded from all graphs. DLCN, Dutch Lipid Clinic Network.

Yield of genetic sequencing over time

Next, we evaluated the yield of genetic FH testing in The Netherlands during the last 2 decades. Figure 4 and supplementary figures 3 show that the percentage of genetically confirmed FH patients per year decreased since 1999. We observed that this decrease over time differed significantly between age tertiles (Figure 5, Supplementary table 9); patients in the lowest tertile (younger than 44 years old) were most likely to carry an FH-causing variant, but also showed the largest decrease in yield over time from 40.4% in 1999 to 19.5% in 2018. Overall, the average age of the sequenced clinical FH patients increased from 44.1±14.5 to 51.3±14 years during this period (Supplementary Figures 4 and 5).

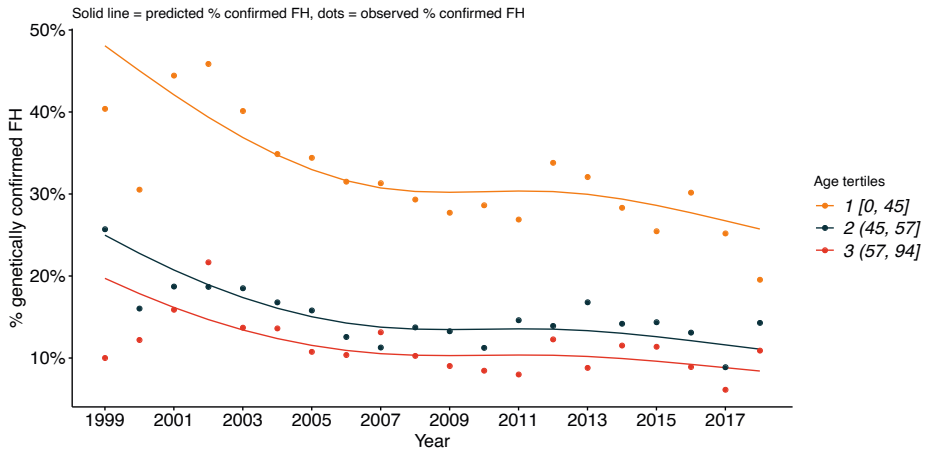

Figure 4: Predicted and observed risk for genetically confirmed FH as a function of year and age
Predicted and observed risk for genetically confirmed FH as a function of year and age. Analysis was performed with a logistic regression analysis with the natural spline of year and age tertiles as covariates. The solid line represents the predicted risk (or proportion) of genetically confirmed FH patients over time. Dots represent the observed proportion of genetically confirmed FH in a given year.

DISCUSSION

We investigated the diagnostic yield of next-generation sequencing in a large number of patients referred for molecular testing for FH variants. An FH-causing genetic variant was identified in only 14.9% of FH patients with LDL-C levels ≥5 mmol/L. This percentage increased to more than 50% when patients were stratified according to either higher LDL-C levels or more stringent diagnostic FH criteria ascertained by the DLCN criteria. 4.8% of FH mutation-negative patients were heterozygous carriers of pathogenic variant in a minor FH gene. When comparing the yield of next-generation sequencing for FH with historical Sanger sequencing data, we found that the likelihood of finding a pathogenic FH variant has decreased from 30% in the early 2000s to approximately 15% in the last few years. This decrease was largely due to a decline of the prevalence of variants in younger patients.

An average genetic yield of approximately 15% in our cohort is relatively low compared to previous studies. A Canadian study with 313 patients reported

that 42% and 88% of patients with LDL-C between 5 and 6 mmol/L and above 8 mmol/L, respectively, carried an FH-causing variant.[13] A more recent study from the same research group found that 38% of 924 patients with an "FH phenotype" (typically LDL ≥ 5 mmol/L) who were referred for NGS carried a pathogenic FH-causing variant.[12] In an unrelated Canadian FH cohort, a pathogenic FH variant was identified in 275 of 626 patients (44%) with clinically diagnosed FH[21] while 30% of the patients were found to be mutation-positive in an Australian cohort of adult patients with at least possible FH.[22] In the Italian LIPIGEN study, 1592 patients with probable or definite FH according to the DLCN were sequenced using NGS and 1076 (68%) were found to carry a pathogenic FH mutation.[23]

Our lower yield likely reflects the mode of referral that led to our cohort and might be caused by the fact that NGS diagnostics is accessible to a heterogeneous group of referring physicians in the Netherlands (e.g., general practitioners, cardiologists, internists) as opposed to a request for NGS analysis in specified lipid clinics in other countries.[12,13,22] Additionally, NGS analysis costs are covered by the basic health insurance of all Dutch inhabitants, and this unrestricted access may lower the threshold for requesting molecular testing compared to regions where more restrictions apply. In such a situation it is for a physician also easier to use genetic testing as a method to exclude FH.

In our analyses, we included all patients who were referred for sequencing and had an LDL-C above or equal to 5 mmol/L, regardless of other phenotypic characteristics. However, when we retrospectively applied clinical FH criteria to our dataset, the yield increased, and was larger than the overall observed yield in our study. Thus, more than 50% of patients with a DLCN score indicating "probable" or "definite" FH, of whom we obtained complete data, were carriers of an FH-causing variant. This, however, has to be interpreted with caution since only 219 patients (14.4%) had complete data for DLCN calculation, potentially introducing a bias as forms may have been filled out more completely for patients where the referring physician was more confident that a variant would be identified. However, a positive MedPed score, which only requires age and an untreated LDL-C level for its calculation, also increased the likelihood of identification; 32.8% of the patients with FH based on the MedPed score carried an FH-causing variant. These findings

underscore that a strict application of clinical criteria for FH will increase the proportion of patients in whom a variant is identified.

The inclusion of phenotypically less severe hypercholesterolemic patients nowadays may also explain the decline of the yield we observed over time to the current 14.9%, and might be a result of the successful cascade-screening program for FH conducted in the Netherlands between 1993 and 2014.[24] We hypothesize that subjects with frequently occurring FH-causing variants had already been identified in the early years of the program, leaving the remainder of the population enriched for hypercholesterolemia of other origins, especially in subjects with lower LDL-C levels compared to those with extremely elevated LDL-C levels. Unfortunately, we cannot confirm this screening success hypothesis with the current data.

Despite having a clinical FH diagnosis based on clinical criteria, a relatively large proportion of FH patients remains undiagnosed at a molecular level after NGS sequencing. The FH phenotype in these subjects could be explained by (a combination of) other factors, including: pathogenic (intronic) variants in regions that are not covered by NGS[25], polygenic hypercholesterolemia[26], high Lp(a) levels[27,28], mixed dyslipidemia (familial combined hyperlipidemia)[29] or incidental and secondary hypercholesterolemia.[30] Our data did not permit to rule out these factors in our current cohort of FH patients. Unfortunately, our sequencing capture did not include the single nucleotide polymorphisms to calculate an FH polygenic risk score. It is likely that a significant proportion of FH mutation-negative patients suffers from polygenic hypercholesterolemia as described by others, and that the proportion of polygenic hypercholesterolemia patients has relatively increased compared to monogenic FH over the years.[13,26] Furthermore, patients with high Lp(a) levels and incidental hypercholesterolemia have recently been reported to be prevalent in this group as these patients present with moderately elevated LDL-C levels.[28,30] This was illustrated by 2219 healthy blood donors who had FH according to the MedPed criteria, and of which 71% no longer met the MedPed criteria for FH at a second LDL-C measurement at a later time point.[30] This clearly illustrates the variability in LDL-C levels and may cause overestimation of the FH prevalence in cohorts selected based on only one LDL-C measurement, such as ours. Therefore,

it might be beneficial to only request molecular testing for FH when elevated LDL-C has been observed with multiple measurements.

Apart from the three major FH-genes (i.e., *LDLR*, *APOB*, *PCSK9*), additional "minor FH-genes" (i.e., *APOE, ABCG5, ABCG8* and *LIPA*) are implied to be associated with the FH phenotype.[5] However, more research on the association between variants in these genes and the occurrence of the FH phenotype is needed. For example, it is still unclear whether variants in *ABCG5* or *ABCG8* are causative for autosomal dominant FH, or are associated with an overlapping FH phenotype.[7,31] Equally challenging are variants in *APOE*. The E2/E2 *APOE* genotype is best known to cause the distinct dysbetalipoproteinemia phenotype, which is observed in 8 individuals in our cohort and might thus have been clinically misdiagnosed. Furthermore, other *APOE* variants are implied to be causative for FH instead of dysbetalipoproteinemia.[6] Taken together, pathogenic variants in minor FH-genes were found in 4.8% of FH mutation-negative patients and 4.1% of all included patients and the allele frequency of these variants was higher in FH mutation-negative patients compared to a reference population. However, the degree to which these variants explain theFH phenotype in FH mutation-negative patientsremains uncertain. This implies that only a minority of patients might benefit from a genetic diagnosis in one of these genes.

Our study has several strengths. Firstly, our NGS platform offers comprehensive coverage of a large number of genes associated with dyslipidemia. Secondly, our center is the national referral center for genetic dyslipidemia and we were therefore able to assemble a large cohort of treatment-naïve patients that underwent NGS for clinical suspicion of FH. However, this study also has several limitations. Although data was obtained through a standardized referral form, correct and complete data entry was entirely dependent on the referring physician. As a consequence, FH stigmata and clinical data may have either been over- or underreported, depending on the level of expertise of the referring physician. Additionally, we excluded all patients who were taking lipid-lowering therapy upon referral and/or had no known LDL-C levels, greatly reducing the number of patients for analysis.

In conclusion, we have shown that comprehensive next-generation sequencing of a large cohort (n=1528) of patients with clinical suspicion of FH and LDL-C levels ≥ 5mmol/L yielded a molecular diagnosis in almost 15% of the cases. Patients with a pathogenic FH variant were younger, had higher plasma LDL-C levels and lower triglycerides. Diagnostic yield increased when more stringent selection according to DLCN score, MedPed criteria, or plasma LDL-C level was applied. Furthermore, the diagnostic yield decreased over time from ~30% in the early 2000s to ~15% nowadays, and this decline was most salient in younger patients with suspected FH. The relatively low and declining diagnostic yield advocates more stringent preselection of clinical FH patients before diagnostic next-generation sequencing is performed.

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DISCLOSURES

G.K.H. has served as consultant and speaker for biotech and pharmaceutical companies that develop molecules that influence lipoprotein metabolism, including Regeneron, Pfizer, MSD, Sanofi and Amgen. Until April 2019 Hovingh served as PI for clinical trials conducted with Amgen, Sanofi, Eli Lilly, Novartis, Kowa, Genzyme, Cerenis, Pfizer, Dezima and Astra Zeneca; and with current and past research grants from ZonMW (ViDi 016.156.445), EU, AMGEN, Sanofi, AstraZeneca, Aegerion, and Synageva. The Department of Vascular Medicine, Amsterdam University Medical Center, receives honoraria and investigator fees for sponsor driven studies/lectures for companies with approved lipid lowering therapies in the Netherlands. Since April 2019, G.K.H. is partly employed by Novo Nordisk (0.7FTE) and the AMC (0.3FTE). E.S.G.S. reports personal fees from Novartis, personal fees from Amgen, personal fees from Sanofi-Regeneron, personal fees from Mylan, personal fees from Esperion, unrelated to the submitted work. All fees were paid to the institution.

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SUPPLEMENTARY DATA

Supplementary Table 1: 27 gene panel NGS

GENES	
LDLR	*SCARB1*
APOB	*CETP*
PCSK9	*LIPG*
LIPA	*LIPC*
LDLRAP1	*APOC3*
ABCG5	*LPL*
ABCG8	*APOC2*
STAP1	*APOA5*
ANGPTL3	*GPIHBP1*
MTTP	*LMF1*
MYLIP	*APOE*
ABCA1	*SAR1B*
LCAT	*CYP27A1*
APOA1	

Supplementary Table 2: FH genotypes including minor FH genes

Genotype	n	% in all clinical FH patients	% of FH mutation-negative patients	% of FH mutation-negative patients with variant in minor FH gene
FH mutation-negative	1236	80.9	95.2	-
LDLR heterozygote	171	11.2	-	-
APOB heterozygote	32	2.1	-	-
PCSK9 heterozygote	11	0.7	-	-
APOE heterozygote	22	1.4	1.7	35.5
ABCG8 heterozygote	17	1.1	1.3	27.4
ABCG5 heterozygote	12	0.8	0.9	19.4
APOE homozygote (E2/E2) (dysbetalipoproteinemia)	8	0.5	0.6	12.9
LDLR-ABCG8 double heterozygote	6	0.4	-	-
LDLR-APOE double heterozygote	2	0.1	-	-
LDLR heterozygote + *APOE* homozygote	2	0.1	-	-
ABCG8 compound heterozygote	1	0.1	0.1	1.6
ABCG8 homozygote	1	0.1	0.1	1.6
APOB-APOE double heterozygote	1	0.1	-	-
APOE compound heterozygote	1	0.1	0.1	1.6
LDLR-ABCG5 double heterozygote	1	0.1	-	-
LDLR-PCSK9 double heterozygote	1	0.1	-	-
LDLR compound heterozygote	1	0.1	-	-
LDLR homozygote	1	0.1	-	-

Supplementary Table 2: (Continued)

Genotype	n	% in all clinical FH patients	% of FH mutation-negative patients	% of FH mutation-negative patients with variant in minor FH gene
PCSK9-APOE double heterozygote	1	0.1	-	-
Total	1528	100	100	100

Supplementary Table 3: allele frequency of variants in minor FH genes in FH mutation-negative patients and gnomAD

	Allele Frequency FH mutation-negative	Allele Frequency European (non-Finnish) population gnomAD	Allele number of variants in this study not present in gnomAD (and excluded from allele frequency)	Number of novel variants in this study, not present in gnomAD (and excluded from allele frequency)
APOE	0.008	0.005	1	1
ABCG5	0.003	0.001	5	4
ABCG8	0.007	0.004	4	3

The allele frequencies of variants in *APOE*, *ABCG5*, and *ABCG8* in FH mutation-negative patients are reported. The E2/E2 genotype is excluded from this comparison, since it causes dysbetalipoproteinemia and not FH. The number of variants (and their allele frequency) identified in our cohort, but not earlier reported in gnomAD are also reported. The European (non-Finnish) subset was derived from gnomAD V2.1.1

Supplementary Table 4: Median LDL-C levels per mutation type

Mutation Type	n	LDL-C (mmol/L) – median [IQR]
LDLR		
Splicing	38	7.6 [6.5-8.3]
Frameshift	5	6.7 [6.2-6.9]
Missense	110	6.8 [6.0-7.6]
Nonsense	17	7.2 [6.6-9.1]
Promoter	3	7.4 [6.8-7.6]
Copy Number Variation	9	8.3 [6.1-10.2]
APOB		
Frameshift	1	5.5
Missense	31	6.7 [5.8-7.5]
Copy Number Variation	1	6.3
PCSK9		
Missense	12	6.7 [6.2-6.9]
FH mutation-negative	1298	6.0[5.5-6.6]

Mutation types of all identified FH causing variants and associated median [IQR] LDL-C levels. IQR, interquartile range.

Supplementary Table 5: Modified Dutch Lipid Clinic Network (DLCN) criteria

Criteria	Points	Used in modified DLCN
First degree relative with hypercholesterolemia (>95th percentile for age and gender) OR First degree relative with premature (<55 years, men; <60 years, women) coronary heart disease	1	Yes: parent with hypercholesterolemia (1 point)
First-degree relative with tendon xanthoma and/or corneal arcus OR Children (<18 years old) with hypercholesterolemia (>95th percentile for age and gender)	2	No
Subject has premature (<55 years, men; <60 years, women) coronary heart disease	2	Yes
Subject has premature (<55 years, men; <60 years, women) cerebral or peripheral vascular disease	1	Yes
Subject has tendon xanthoma	6	Yes
Subject has corneal arcus <45 years	4	Yes
LDL cholesterol of subject in mmol/L		Yes
• ≥8.5	8	
• 6.5-8.4	5	
• 5.0-6.4	3	
• 4.0-4.9	1	
• <4.0	0	

>8 points = definite FH; 6-8 points = probable FH; 3-5 points = possible FH; <3 points = unlikely FH

Supplementary Table 6: MedPed criteria

Age of patients	Clinical FH if LDL-C (mmol/L) is above:
<20	5.2
20-29	5.7
30-39	6.2
>=40	6.7

Supplementary Table 7: Yield modified Dutch Lipid Clinic Network (DLCN) score

	Full dataset DLCN score			Complete (minimum) DLCN score dataset		
	Possible FH	Probable FH	Definite FH	Possible FH	Probable FH	Definite FH
FH mutation-negative	90.1% (967)	76.4% (279)	59.8% (52)	75.0% (111)	49.1% (26)	50.0% (9)
FH mutation-positive	9.9% (106)	23.6% (86)	40.2% (35)	25.0% (37)	50.9% (27)	50.0% (9)
Total	100.0% (1073)	100.0% (365)	100.0% (87)	100.0% (148)	100.0% (53)	100.0% (18)

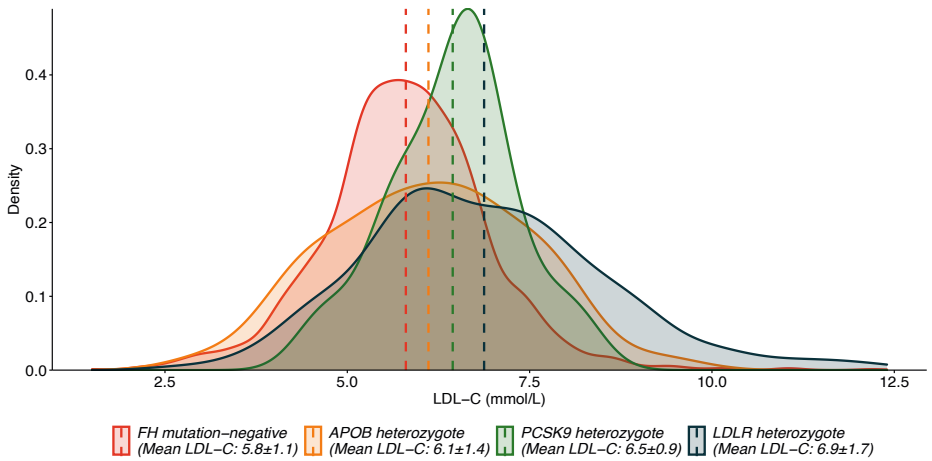
Supplementary Table 8: Yield MedPed criteria

	MedPed classification	
	FH	non-FH
FH mutation-negative	67.2% (336)	93.9% (962)
FH mutation-positive	32.8% (164)	6.1% (63)
Total	100.0% (500)	100.0% (1025)

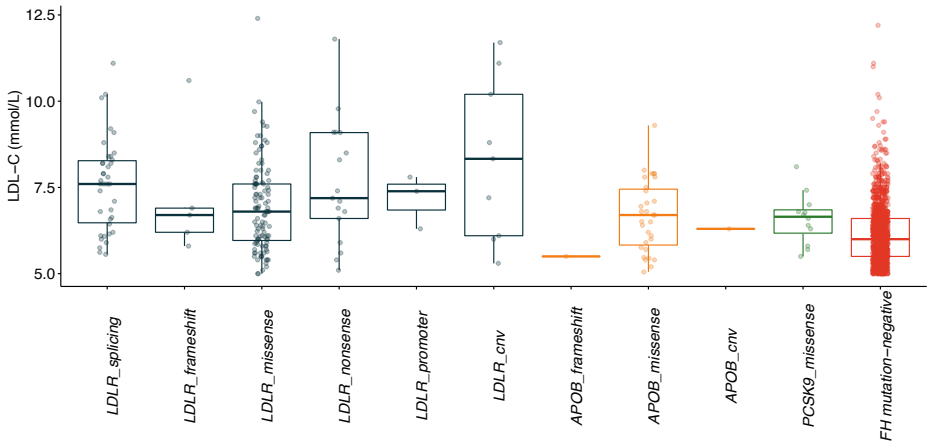
Supplementary Table 9: Logistic regression model for yield over time per age category

Variable	Estimate	P-value	Lower 95% Confidence Interval	Upper 95% Confidence Interval
(Intercept)	-0.04	0.75	-0.27	0.19
ns(Year, 3)1	-0.64	<0.01	-0.85	-0.43
ns(Year, 3)2	-1.44	<0.01	-2.00	-0.88
ns(Year, 3)3	-0.48	<0.01	-0.70	-0.26
age_cat2 (44, 57]	-1.19	<0.01	-1.57	-0.80
age_cat3 (57, 92]	-1.44	<0.01	-1.98	-0.93
ns(Year, 3)1:age_cat2 (44, 57]	0.20	0.24	-0.14	0.54
ns(Year, 3)2:age_cat2 (44, 57]	0.36	0.44	-0.55	1.29
ns(Year, 3)3:age_cat2 (44, 57]	0.43	0.01	0.09	0.77
ns(Year, 3)1:age_cat3 (57, 92]	-0.13	0.54	-0.54	0.29
ns(Year, 3)2:age_cat3 (57, 92]	0.40	0.52	-0.79	1.63
ns(Year, 3)3:age_cat3 (57, 92]	0.33	0.09	-0.06	0.71

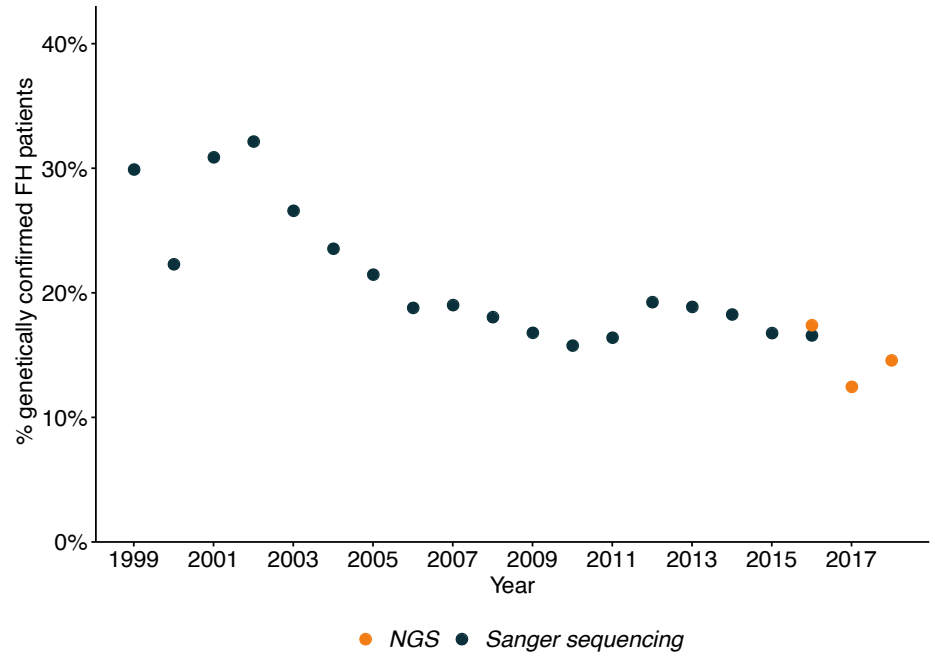
ns = natural spline, CI = confidence interval



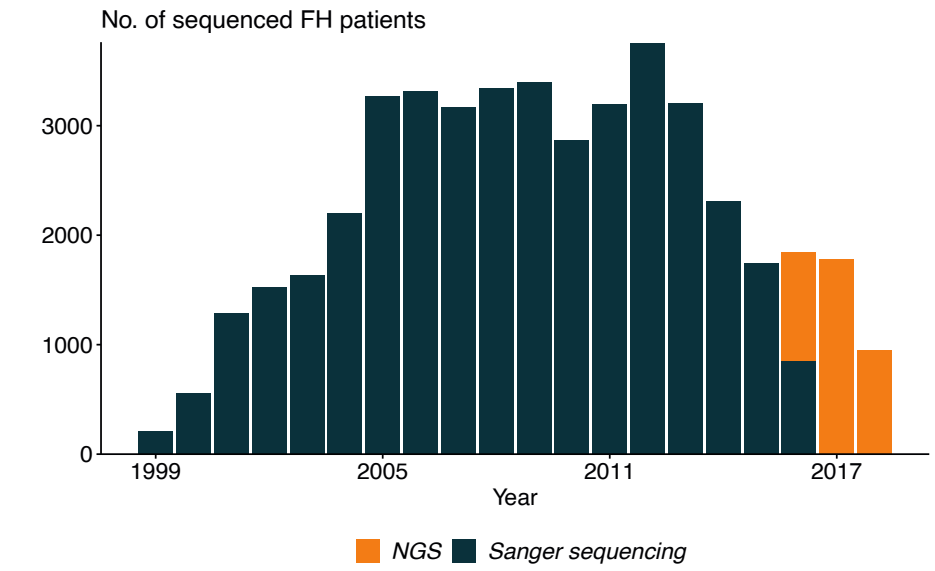
Supplementary Figure 1: Distribution of LDL-C levels per FH genotype
Distribution of LDL-C levels for all next-generation sequenced patients, including those with LDL-C <5 mmol/L.



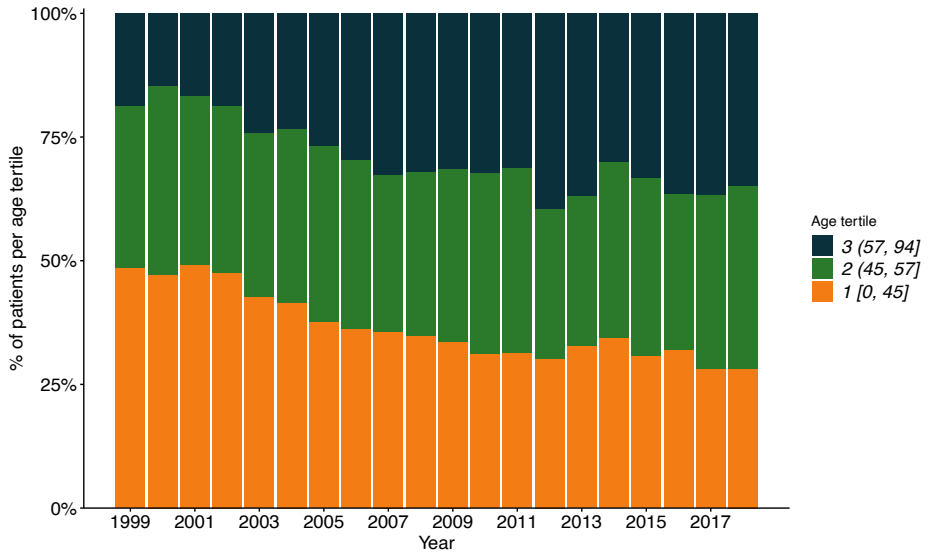
Supplementary Figure 2: Distribution of LDL-C levels per mutation type



Supplementary Figure 3: Percentage of genetically confirmed FH patients per year from 1999 to 2018.
Percentage of genetically confirmed FH patients per year from 1999 to 2018. NGS, next-generation sequencing.



Supplementary Figure 4: Number of sequenced patients per year
Number of sequenced patients per year since 1999 until July 2018. NGS, next-generation sequencing.



Supplementary Figure 5: Age tertile distribution of sequenced individuals per year
Age tertile distribution of sequenced subjects per year since 1999 until July 2018.

3

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 mutations was identified in the coding regions of these FH genes.

Methods: Whole genome sequencing was performed in 5 affected and 4 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 co-segregate with high LDL-C in 5 patients, but not in 4 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. Co-segregation analysis of a second family showed full penetrance of the novel variant with the FH phenotype over 3 generations.

Conclusions: The c.2140+103G>T mutation in *LDLR* is a novel intronic variant identified in FH that co-segregates 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 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 co-segregates 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.

Diagnostic procedures

The Academic Medical Center (AMC) is the national referral center for DNA diagnostics in dyslipidemia within the Netherlands. For this study we analyzed the DNA from patients in whom the referring physician requested molecular FH analysis based on their clinical judgement. An in house next-generation sequencing (NGS) capture covering 29 genes, including *LDLR*, *APOB*, and *PCSK9*, is used (SeqCap easy choice, Roche NimbleGen Inc., Pleasanton, USA). The intronic regions of these 29 genes are well covered (>30 times depth) up to over 150 base pairs distance from the exon/intron boundaries, but this method does not cover whole intronic regions of any of the sequenced genes. Since the implementation of NGS in 2016, DNA of a total of 2,154 patients have been analyzed using this array. Prior to the NGS strategy, a molecular diagnosis was made by means of Sanger sequencing of *LDLR*, *APOB*, and *PCSK9* and when negative, subsequently by Multiplex Ligation-dependent Probe Amplification (MLPA) of *LDLR*.

Families

The proband of family 1 is a 63 year old patient who was referred to our department of Vascular Medicine in 2011 and who was deemed to suffer from FH, as her DLCN score was 9, based on a LDL-C level of 348.0 mg/dL and a family history with the presence of LDL-C levels above 95th percentile for age and gender. Furthermore she suffered from a myocardial infarction at the age of 63 and had xanthelasmata.

However, no mutation in *LDLR* was identified upon routine Sanger sequencing and subsequent analysis of *APOB* and *PCSK9*. Family 1 was initially expanded and ultimately used for whole genome sequencing (WGS). Unlike proband 1, the proband of family 2 was analyzed by means of NGS, and no mutation was found in the coding region of any of the 3 putative FH genes. Subsequently she was classified as FH4, and was part of the NGS FH cohort described below. The families of both probands were expanded and clinically assessed. All participants gave written informed consent and the study was approved by the Institutional Review Board of the AMC and complies with the Declaration of Helsinki.

DNA extraction and lipid measurements

DNA was extracted from a 10 mL blood sample, collected in EDTA tubes, using a Gentra AutoPure LS (Gentra Systems, Minneapolis, MN). Total cholesterol, LDL-C, high density lipoprotein cholesterol (HDL-C), and triglycerides were measured using commercially available assays (Wako Chemicals, Neuss, Germany; and DiaSys Diagnostic Systems, Holzheim, Germany) on a Selectra analyzer. We adapted the lipid levels with the previously published correction factors in patients in whom no off-treatment lipid levels were available.[8]

Whole genome sequencing

The DNA of 9 individuals in family 1 was analyzed by WGS at the Hartwig Foundation (Amsterdam, the Netherlands) where the full genome was ascertained with the Illumina X Ten array with a minimal 30 times coverage per nucleotide. While assuming a full penetrant model, we selected rare variants (allele frequency <0.1% in reference database gnomAD[9]) that were present in the 5 family members with high LDL-C (> the 99th percentile for age and gender) and not in 4 unaffected relatives (see supplementals for the applied filter steps). Splicing was predicted *in silico* using 5 different algorithms: SpliceSiteFinder-like[10], MaxEntScan[11], NNSPLICE[12], GeneSplicer[13], and Human Splicing Finder[14].

Confirmation of splicing

To test the effect on splicing of the novel variant, RNA was isolated from white blood cells from the proband of family 1 according to the protocol of the TriPure Isolation Reagent (Roche Molecular Systems, Inc, Pleasanton, USA).

Subsequently, cDNA was generated using the SensiFAST™ cDNA Synthesis Kit (Bioline, London, United Kingdom) according to the manufacturer's protocol. With specific down- and upstream primers (5'-TGAACTGGTGTGAGAGGACC-3' and 5'-ACATTGTCACTATCTCCACCGT-3', respectively) spanning the region of the splice variant in exon 14 and 15 of *LDLR* PCR products were amplified and visualized on a 1% agarose gel. The bands were excised and Sanger sequenced.

Cohorts

The frequency of the novel variant was analyzed in 2,154 patients referred to the AMC for genetic analysis by NGS (NGS FH cohort) in whom a clinical diagnoses of FH was made by the referring physician. This cohort consists of all FH patients referred since 2016, and thus includes also patients with known pathogenic variants in *LDLR*, *APOB*, and *PCSK9*. Additionally, in 1,245 DNAs of patients in whom no mutations in the coding region of *LDLR*, *APOB*, and *PCSK9* were found with Sanger sequencing (FH4 cohort), we analyzed the presence of the novel variant using a custom made TaqMan SNP genotyping assay based on allelic discrimination with VIC-labeled and FAM-labeled probes (Applied Biosystems, Foster City, USA) for the wild-type (TCGTTGTAAGGACTCATG-VIC) and variant (TCGTTGTAAGTACTCATG-FAM), respectively. PCR conditions were as follows: denaturation for 10 min at 95°C, followed by 40 cycles (15 sec 92°C, 45 sec 60°C) and run on a CFX PCR system (BioRad Laboratories Inc, Herculus, CA). The same TaqMan assay was used in family 2 and to confirm the results of WGS in family 1.

Statistical analysis

Pedigrees were drawn in Progeny version 8.3.0.1 (Progeny, Delray Beach, FL). All statistical analysis were performed using R, version 3.4.3 (R Foundation, Vienna, Austria). Independent T-tests were used to compare normally distributed numeric data between groups, Mann-Whitney U test for not normally distributed numeric data, and Chi-squared tests in case of categorical data. A male of family 2 (ID 2) was excluded from statistical analysis, because of his married-in status. A P-value of <0.05 was considered statistically significant.

RESULTS

Whole genome sequencing of DNA derived form 9 subjects from family 1 retrieved 190 rare (exonic and intronic) variants, which were present in the affected, but not in the unaffected family members (Supplementary Table 1). 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*.

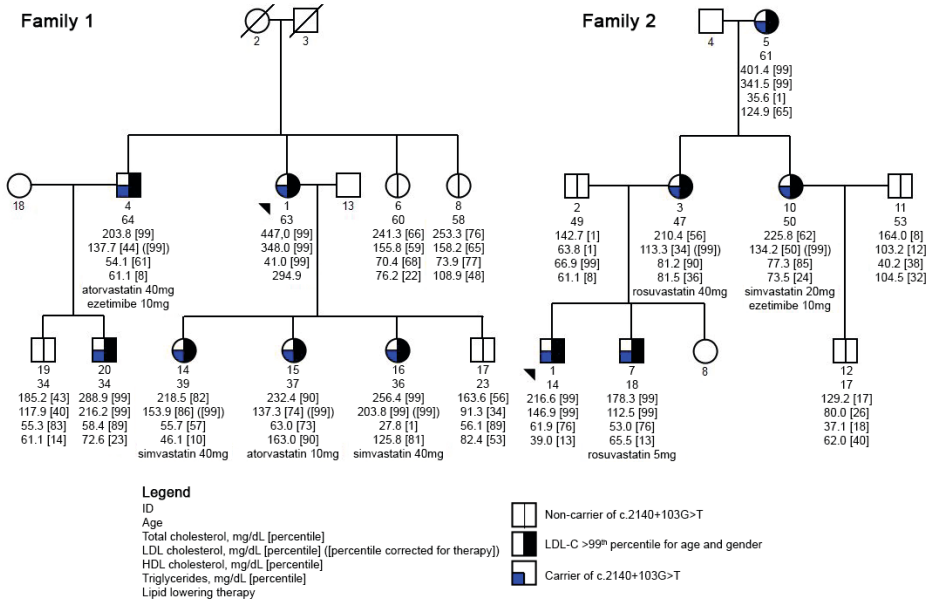

Figure 1: Pedigree of family 1 and family 2
100% penetrance of the c.2140+103G>T variant with LDL cholesterol above the 99th percentile for age and sex in two clinical FH families without another FH causing mutation. Males and females are depicted as squares and circles, respectively. LDL(-C) = low density lipoprotein (cholesterol), HDL = high density lipoprotein.

In silico analysis with 5 splicing prediction algorithms predicted an increase in splicing signal at a potential cryptic splice donor site consensus, 5 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.

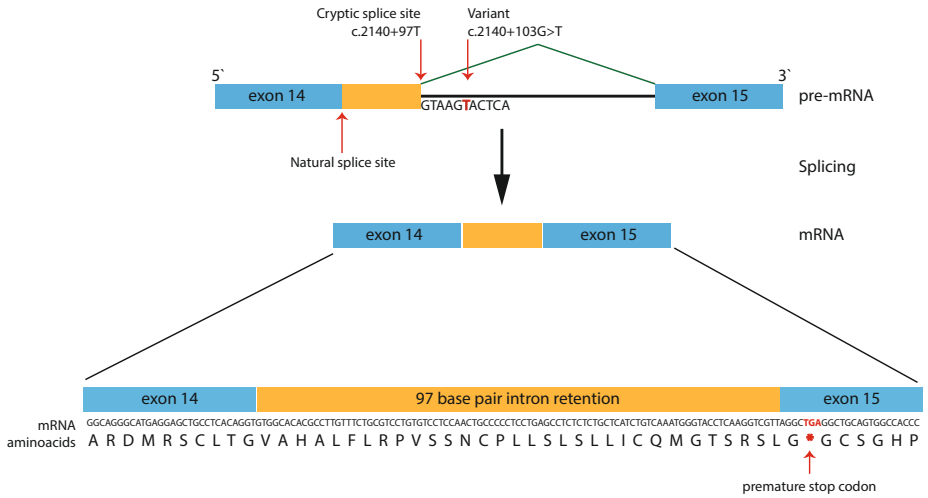

Figure 2: cryptic splicing of *LDLR* mRNA
The presence of the intronic variant c.2140+103G>T leads to a cryptic splice site between c.2140+97T and c.2140+98G, resulting in a 97 base pair intron retention in the mRNA after splicing of pre-mRNA. Subsequently, a frame shift leads to a premature stop codon 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.

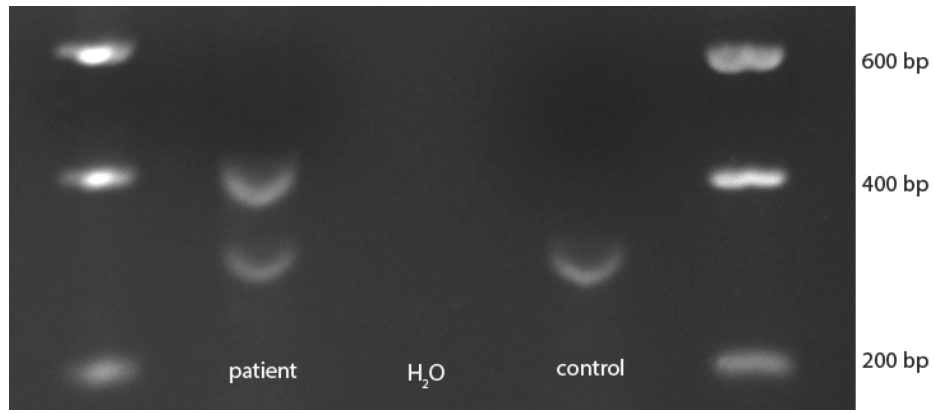

Figure 3: Agarose gel with cDNA PCR products from c.2140+103G>T carrier and control
cDNA PCR products of the proband of family 1 (patient) and a healthy control were run on a 1% agarose gel. PCR products were produced using primers covering the boundary between exon 14 and 15. The patient shows an additional PCR product of ~400 base pairs length, while the control only shows the expected PCR product. bp = base pairs.

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 5 heterozygous carriers (from 4 families) of the c.2140+103G>T variant, which translates in a prevalence of 0.23% (5/2154). The 5 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), 3 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 8 additional variant carriers (from 7 unrelated families) were identified.

We collected plasma and DNA from family members of one of the 8 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).

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.[8,15] *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, 8 individuals from 7 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.[7,16] Considering the visibility of cDNA containing the intron retention in the electrophoresis gel in Figure 2, 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 co-segregation 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 co-segregation 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 ommitance of functional defects in patients with FH. As a matter of fact; 8 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*.[17] 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.[18] *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.[19]

There is a number of examples of deep intronic mutations that are related to disease phenotypes.[19] 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[20]) up to thousands of bps into introns (e.g. 3849+10KbC>T in the Cystic Fibrosis Transmembrane conductance Regulator (*CFTR*) gene[21]) It can be hypothesized that, in resemblance to these 2 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.[22] As a consequence, mRNA analyses and co-segregation 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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DISCLOSURES

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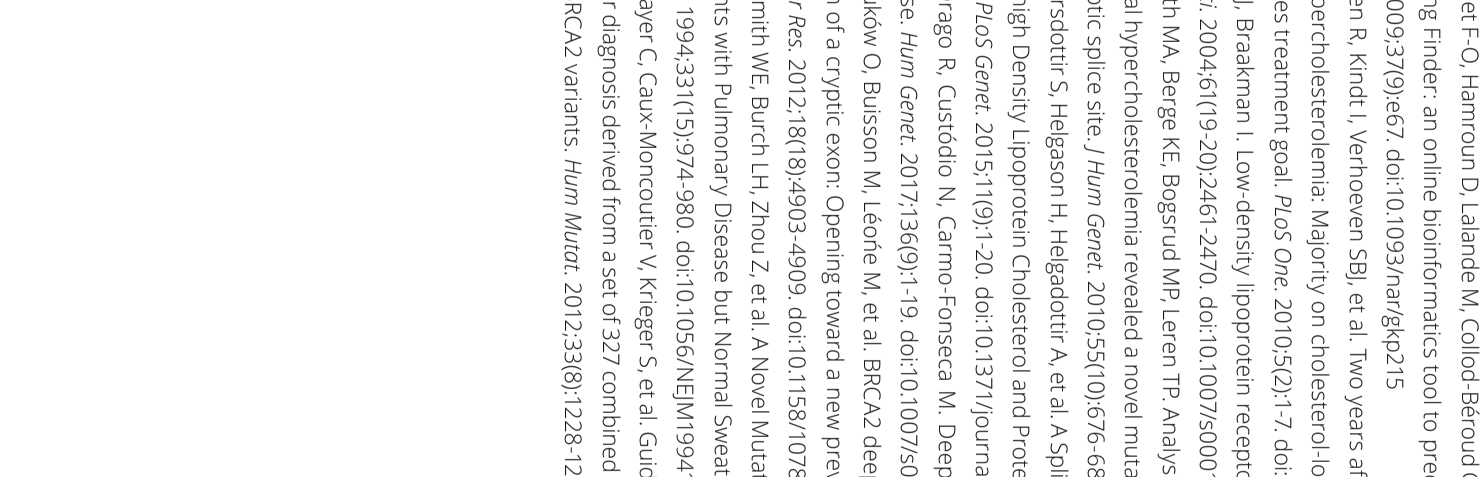
SUPPLEMENTAL MATERIAL



Supplementary Figure 1: Sanger sequencing trace of cDNA PCR products
Panel A shows the sequence of the intronic insert (upper sequence; "insert") and the reference genome (bottom sequence; "NM_000527"). The insert sequence was obtained by Sanger sequencing of the additional cDNA band found in the index patients shown in Figure 3 of the manuscript. The exact Sequence trace is shown in panel B.

Supplementary Table 1: genomic variant filtering steps whole genome sequencing in family 1

	Action	Number of variants left
1	Filtering variants of affected family members for unaffected relatives	10064
2	Selecting only heterozygote autosomal dominant variants versus wild type	7113
3	Selecting variants with an allele frequency of <0.5% in gnomAD database	426
4	Selecting variants with an allele frequency of <0.1% in gnomAD database	190
5	Selecting variants with an allele frequency of <0.05% in gnomAD database	159

Applied filtering steps on whole genome sequencing data from individuals of family 1. Affected individuals had LDL-C levels above the 99th percentile for age and gender. Reference database was the gnomAD database9 (http://gnomad.broadinstitute.org/). The c.2140+103G>T variant was found after manual exploration of the 190 identified variants in step 4.

4

Intronic variant screening with targeted next-generation sequencing reveals first pseudoexon in *LDLR* in Familial Hypercholesterolemia

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ABSTRACT

Background and aims: Familial Hypercholesterolemia (FH) is caused by pathogenic variants in *LDLR*, *APOB*, or *PCSK9* genes (designated FH+). However, a significant number of clinical FH patients do not carry these variants (designated FH-). Here, we investigated whether variants in intronic regions of *LDLR* attribute to FH by affecting pre-mRNA splicing.

Methods: *LDLR* introns are partly covered in routine sequencing of clinical FH patients using next-generation sequencing. Deep intronic variants, >20bp from intron-exon boundary, were considered of interest once a) present in FH- patients (n=909) with LDL-C >7mmol/L (severe FH-) or after *in silico* analysis in patients with LDL-C >5mmol/L (moderate FH-) and b) absent in FH+ patients (control group). cDNA analysis and co-segregation analysis was performed to assess pathogenicity of identified variants.

Results: Three unique variants were present in the severe FH- group. One of these was the previously described likely pathogenic variant c.2140+103G>T. Three additional variants were selected based on *in silico* analyses in the moderate FH- group. One of these variants, c.2141-218G>A, was found to result in a pseudo-exon inclusion, producing a premature stop codon. This variant co-segregated with the hypercholesterolemic phenotype.

Conclusion: Through a screening approach we identified a deep intronic variant causal for FH. This finding indicates that filtering intronic variants in FH- patients for absence in FH+ patients might enrich for true FH-causing variants and suggests that intronic regions of *LDLR* need to be considered for sequencing in FH- patients.

INTRODUCTION

Familial hypercholesterolemia (FH) is a common autosomal genetic disorder characterized by high levels of low-density lipoprotein cholesterol (LDL-C) resulting in an increased risk for premature cardiovascular disease. FH is caused by pathogenic variants in either the low-density lipoprotein receptor gene (*LDLR*), apolipoprotein B100 gene (*APOB*), or proprotein convertase substilisin kexin type 9 gene (*PCSK9*). However, depending on the severity of the phenotype an FH-causing mutation is not found in 12-60% of clinical FH patients.[1,2]

Multiple causes for the elevated LDL-C levels in these clinical FH variant-negative (FH-) patients have been suggested. For example, high levels of lipoprotein (a) or a polygenic predisposition for high LDL-C are known to mimic FH.[3,4] A third possible explanation for the missing inheritable part of FH are undiscovered genetic variants in known or novel genes involved in LDL-C metabolism. Despite advances in sequencing techniques that allow for large scale genomic analysis in many individuals, this has not yet resulted in the identification of novel FH candidate genes.[5]

In this context, sequencing of the usually neglected non-coding intronic regions of *LDLR* with second generation techniques (e.g. whole genome sequencing) has resulted in identification of new deep intronic variants causal for FH.[6,7] Although intronic variants reside in non-coding regions of the *LDLR* gene, they can affect regulatory processes such as splicing of *LDLR* pre-messenger RNA (pre-mRNA). This, in turn, can lead to (partial) exon skipping or(partial) intron retention. In most cases this results in a shift of the 3-letter coded reading frame that is used for translation of mRNA into proteins. In almost all cases, a frame shift ultimately results in a premature stop codon, leading to shorter, most often nonfunctional LDLR proteins or mRNA that is rapidly degraded by nonsense-mediated decay.

To further explore the role of deep intronic variants in FH causality, we developed a diagnostic algorithm that takes advantage of the available covered intron sequences generated during diagnostic targeted next-generation sequencing of FH patients. We selected rare deep intronic variants that were present in FH-

patients with markedly elevated LDL-C levels and were absent in FH patients with a pathogenic variant in *LDLR*, *APOB*, or *PCSK9* (FH+). Next, we applied clinical and *in silico* filtering tools to select those intronic variants that were most likely to cause FH and investigated their effect on mRNA splicing *ex vivo* and co-segregation with the hypercholesterolemic phenotype in the family of the proband.

METHODS

Study population and design

The Amsterdam UMC, location AMC, is the national referral center for DNA diagnostics in patients with different forms of dyslipidemia. For this study we analyzed the DNA from patients in whom the referring physician requested molecular FH analysis based on their clinical judgement. A molecular diagnosis was made with a targeted next-generation sequencing (NGS) capture (SeqCap easy choice version v.DLv2, Roche NimbleGen Inc., Pleasanton, USA) that covers 29 genes, including FH genes *LDLR*, *APOB*, and *PCSK9* (See Supplementary Table 1 for full covered gene list). Also copy number variant (CNV) analysis was performed for all 29 genes. Patients carrying an FH-causing CNV or variant in *LDLR*, *APOB*, or *PCSK9* (FH+ patients) formed the control group in this study. The patient group of interest consisted of clinical FH patients in whom no CNV or heterozygous or homozygous likely pathogenic (class 4) or pathogenic (class 5) variants in these genes were found (FH- patients). Supplemental Table 1 depicts whether variants needed to be heterozygous (dominant) or homozygous or compound heterozygous (recessive) for pathogenicity. Variants in *CYP7A1* and *SCLO1B1* were not taken into account since these are assumed not to affect lipid levels.

Additional exclusion criteria for the subjects in the FH- group were lipid values obtained during lipid-lowering treatment, LDL-C levels <5 mmol/L, triglycerides ≥3 mmol/L, or missing LDL-C or triglyceride values. Next, all eligible FH-patients were categorized into two groups. Patients with a severe FH phenotype (LDL-C levels ≥7 mmol/L and triglycerides <1.5 mmol/L) were defined as “severe FH” and all other clinical FH patients (LDL-C ≥5 mmol/L and triglycerides <3 mmol/L) were defined as “moderate FH”. The complete study design is summarized in Figure 1. All included

patients gave written informed consent for use of their clinical and genetic data for research purposes. The Medical Ethics Review Committee of the Amsterdam UMC, location AMC, provided a waiver for the re-use of the patients clinical and genetic data in the current study (reference ID: W20_490 # 20.542).

Genetic analysis

DNA was isolated from whole blood using EDTA blood withdrawal tubes and the Gentra Puregene kit (Qiagen, Hilden, Germany) according to the manufacturers protocols. Variants in exons and flanking nucleotides (20 base pairs) of 27 dyslipidemia-causing genes of the NGS panel were assessed for pathogenicity according to the American College of Medical Genetics and Genomics (ACMG) guidelines by two trained geneticists.[Richards 2015] For *LDLR*, reference genome NM_000527.4 (GrCH37) was used. Intronic regions of *LDLR* were partly covered by the NGS capture and a complete overview of the covered regions is provided in Supplementary Table 2 and Supplementary Figure 1.

Identification of variants of interest

Variants in the *LDLR* intronic regions (>20bp from exon) that were present in FH-patients, but not in the control group of FH+ patients, were selected for further analysis. We selected variants with a minor allele frequency (MAF) ≤0.3% [8] in all ethnic populations shown in the publicly available reference databases gnomAD (Both Exome and Genome datasets, release 2.1; gnomad.broadinstitute.org[9]) and GoNL (5th release; [10]). Variants passing internal sequencing quality filters were considered for this analysis, as were variants with low coverage (<30x coverage). In the severe FH group, all identified unique intronic variants were candidates for follow-up, irrespective of *in silico* splicing prediction. In the moderate FH- group, identified variants were first subjected to *in silico* splicing analyses and only variants that were strongly predicted to affect splicing were selected for cDNA analysis (Figure 1). One FH+ patient carrying the previously identified deep intronic *LDLR* variant (c.2140+103G>T) was annotated as being FH- to serve as positive control for our variant selection algorithm.[6]

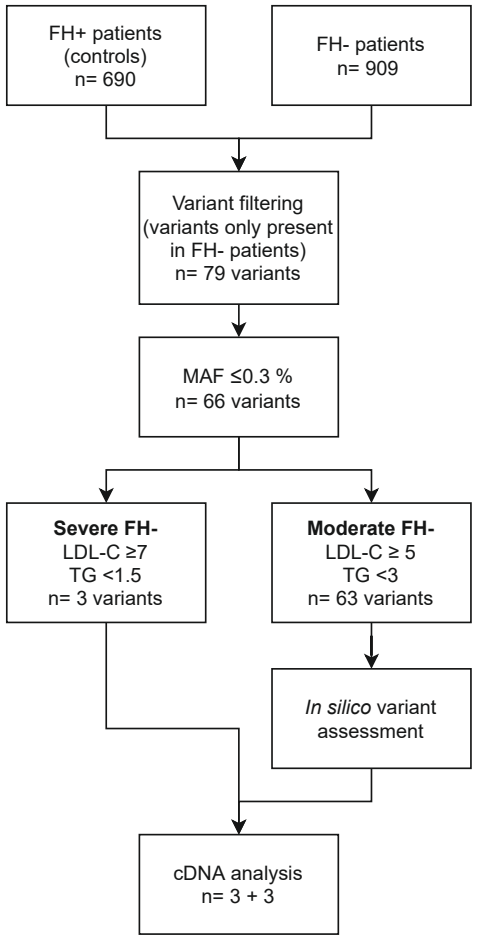

Figure 1: Flowchart of study design
Intronic variants present in FH patients without an FH-causing variant (FH-) that were not present in FH patients with an FH-causing variant (FH+) were filtered for having an minor allele frequency (MAF) of ≤0.3% in reference cohorts (e.g., gnomAD). Next, two groups of patients were selected, those with severe FH- (low-density lipoprotein cholesterol [LDL-C] ≥ 7 mmol/L and triglycerides [TG] levels ≤1.5 mmol/L) and those with moderate FH- (LDL-C ≥ 5 mmol/L and TG <3 mmol/L). Intronic variants in these two groups were further analyzed *in silico* and/or with cDNA analysis.

In silico splicing assessment

The identified intronic *LDLR* variants that were unique for the FH- group were assessed *in silico* for their effect on splicing of *LDLR* pre-mRNA using seven tools: SpliceSiteFinder-like (SSF)[11], MaxEntScan (ME)[12], NNSPLICE (NN)[13], and GeneSplicer (GS)[14], available in the Alamut Visual Software package (version 2.11; Interactive

Biosoftware, Rouen, France), and TraP (Version 2)[15], SPANR[16], and SpliceAI[17]. The SpliceAI *in silico* assessment was based on comparison of our identified variants with a predefined list of variants and scores provided by Illumina (San Diego, CA, USA; obtained through personal communication). A variant was annotated as being of interest when at least two of the four prediction tools in Alamut showed a score of ≥75% of the score range of that specific tool (SSF: 0-100; ME: 0-16; NN 0-1; GS 0-21, for acceptor splice sites and SSF: 0-100; ME: 0-12; NN 0-1; GS 0-24, for donor splice sites) and an increase of ≥2% compared to the wild type signal(Sangermano et al. 2019). A variant was also considered of interest when the splice score was above the given cut-offs of TraP (≥0.459), SPANR (≥5 for absolute difference of percentage spliced), or SpliceAI (≥0.2).

Splicing confirmation

RNA was isolated from whole blood using PAXgene tubes (BD Diagnostics, Franklin Lakes, NJ USA) and isolated using the PAXgene RNA isolation kit according to the manufacture protocol (BD Diagnostics, Franklin Lakes, NJ USA) to assess the effect of a variant on splicing. Subsequently, cDNA was generated using the SensiFAST™ cDNA Synthesis Kit (Bioline, London, United Kingdom) or with superscript III first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. *LDLR* PCR products were amplified using specific forward and reverse primers (see Supplementary Table 2) after which the splicing product was visualized on a 1% agarose gel. Bands of interest were excised, purified (QIAquick Gel Extraction Kit [Qiagen, Hilden, Germany]; BigDye™ Terminator v1.1 Cycle Sequencing Kit [Applied Biosystems, Foster City, CA, USA]) and Sanger sequenced.

Statistical analysis

All normally distributed data are presented as mean±SD, all non-normally distributed data as median [Inter Quartile Range], and counts as number(%). The mean number of variants per patient group was compared using two sample t-test or a one-way ANOVA. A p-value of <0.05 was considered statistically significant. All statistical analyses were performed in R (version 3.6.1; The R Foundation, Viena, Austria) and Rstudio (version 1.2.1335; RStudio, Inc. Boston, MA, USA)

RESULTS

Intronic variant detection

We included a total of 909 FH- patients in our analysis. 38 patients had severe FH (LDL ≥7 & TG <1.5) and 871 patients had moderate FH (LDL ≥5 & TG <3). The control group consisted of 690 patients who carried a likely pathogenic or pathogenic variant in either *LDLR*, *APOB*, or *PCSK9*. Clinical characteristics of all patients are depicted in Table 1.

Table 1: Characteristics of FH patient groups

	All FH- subjects	Severe FH- (LDL-C ≥7 & TG <1.5)	Moderate FH- (LDL-C ≥5 & TG <3)	FH+ controls
No. of patients	909	38	871	690
Females (n (%))	560 (62)	28 (74)	532 (61)	391 (57)
Age, years (mean (SD))	54.6 (11.6)	56.1 (9.57)	54.5 (11.7)	41.8 (16.9)
Total cholesterol, mmol/L (median [IQR])	8.2 [7.7-8.9]	9.7 [9.2-10.2]	8.2 [7.7-8.8]	8.7 [7.8-9.9]
LDL-C, mmol/L (median [IQR])	6.0 [5.5-6.6]	7.5 [7.2-7.9]	6.0 [5.5-6.5]	6.7 [5.6-7.8]
HDL-C, mmol/L (median [IQR])	1.7 [1.3-2.2]	1.2 [1.1-1.4]	1.7 [1.4-2.2]	1.2 [1.1-1.5]
Triglycerides, mmol/L (median [IQR])	1.4 [1.2-1.7]	1.52 [1.3-1.9]	1.4 [1.2-1.7]	1.5 [0.9-2.1]
BMI, kg/m^2 (mean (SD))	26.4 (4.0)	26.8 (5.4)	26.4 (3.9)	26.3 (5.0)
History of ASCVD* (n (%))	190 (20.9)	7 (18.4)	183 (21.0)	80 (11.5)
Diabetes Mellitus (n (%))	42 (4.6)	1 (2.6)	41 (4.7)	19 (2.7)
No. of unique deep intronic variants	67	3	64	0

*ASCVD = atherosclerotic cardiovascular disease (defined as history of myocardial infarction, angina pectoris, Percutaneous Coronary Intervention, Coronary Artery Bypass Grafting, peripheral artery disease, stroke).

On average 54±33% and 36±29% of the eighteen *LDLR* introns were covered ≥1 and ≥30 times, respectively, by our NGS capture (Supplementary Table 2). The average number of deep intronic *LDLR* variants per patient was 29±8.2. This number did not differ between FH+ and FH- patients (29.3±8.5 vs 28.7±8, p=0.185). A total of 64 and 196 different intronic variants were identified in severe FH- and moderate FH- patients, respectively. The average number of intronic variants in patients groups did not differ per FH+ genotype (i.e., *LDLR, APOB, PCSK9*) or FH- phenotype (i.e., severe FH- and moderate FH-); shown in Supplementary Table 3 & Supplementary

Figure 2. The highest number of intronic variants were identified in intron 11, 12 and 15 (Supplementary Figure 3).

Unique FH- intronic variant detection

Three unique intronic variants were detected in the severe FH- group, including the previously described c.2140+103G>T variant, which is considered to cause FH[6] and served as a positive control in our study. The other two novel variants were *in silico* predicted to be potentially damaging by only one of the 7 used tools (Table 2) and cDNA analysis confirmed that they had no effect on *LDLR* mRNA splicing.

Table 2: Intronic variants of interest

Variant	Group	Location	Allele frequency in total FH mutation-negative cohort (%)	Maximum allele frequency reference population (gnomAD)*	*In silico* analysis	cDNA sequencing results
c.313+277C>T (rs971920612)	Severe FH	Intron 3	0.0550	0.0009	Predicted 19.5% decrease of donor splice site at c.313+227 according to GS	No effect on splicing
c.694+25C>T (rs199540175)	Severe FH	Intron 4	0.1100	0.0022	1.4% increase of donor splice signal at c.694 according to GS	No effect on splicing
c.2140+103G>T	Severe FH	Intron 14	0.0550	-	63% chance of a donor splice signal at c.2140+97 according to SpliceAI. 7.1-100% increase in donor splice signal at c.2140+97 according to SSF, ME, NN, and GS	97 nucleotides insertion, leading to frameshift and premature stop codon exon 15 of *LDLR* [Reeskamp 2018]
c.1187-96C>T (rs931988884)	Moderate FH	Intron 8	0.0550	-	4.3 and 10.9% increase of acceptor splice signal at c.1187-84 according to SSF and NN, respectively.	No effect on splicing

Table 2: (Continued)

Variant	Group	Location	Allele frequency in total FH mutation-negative cohort (%)	Maximum allele frequency reference population (gnomAD)*	*In silico* analysis	cDNA sequencing results
c.1587-308C>T (rs866311080)	Moderate FH	Intron 10	0.0550	0.0001	13.9% increase of donor splice signal at c.1587-310 according to SSF. 62.4% chance of changing RNA transcript according to TraP	No participation
c.2141-218G>A (rs991805047)	Moderate FH	Intron 14	0.0550	-	3.8-100% increase of donor splice signal at c.2141-221 34% chance of a donor splice signal at c.2141-221 according to SpliceAI	132 nucleotides pseudo-exon with premature stop codon created in intron 14

Identified deep intronic variants in *LDLR* with potential effect on *LDLR* mRNA splicing. Three unique variants were identified in the severe FH mutation-negative group and three variants in the moderate FH mutation-negative group. *maximum allele frequency reported is the largest reported allele frequency in any ethnic population in gnomAD(Karczewski et al. 2020, ExAC(Karczewski et al. 2020, or GoNL(Francioli et al. 2014).FH, familial hypercholesterolemia; GS, GeneSplicer; SSF, SpliceSiteFinder; ME, MaxEntScan; NN, NNSPLICE;

Among moderate FH patients, a total of 63 unique variants were identified, of which 3 were identified as being of interest given the observed effect in 2 different *in silico* analyses (Table 2, Supplementary Table 4). The first variant, c.1187-96C>T, was deemed to affect splicing by two *in silico* tools by addition of an acceptor splice signal at c.1187-84. We performed cDNA analysis and found no effect on splicing. Unfortunately, the patient carrying the second variant, c.1587-308C>T, did not provide informed consent for RNA isolation for this study. The third variant, c.2141-218G>A, was predicted to have a 34% higher chance of causing a cryptic donor splice signal at position c.2141-221 compared to the wild type variant according to SpliceAI. The *in silico* prediction tools SSF, ME, NN, GS, reported a 5.5%, 45%, 3.8% and a 100% increase in donor splice signal at c.2141-221, respectively (Table 2). Subsequent electrophoresis of the cDNA derived from the carrier of this variant showed an additional larger band on agarose (Figure 2A). Sequencing of this band

showed an insertion of 132 base pairs between exon 14 and exon 15. This insertion starts at a predicted cryptic acceptor splice site at c.2141-352 and spans up to the predicted cryptic donor splice site that was created by the deep intronic variant at c.2141-221 (Figure 2B). Thus, the c.2141-218G>A deep intronic variant causes partial intron retention and thereby results in the transcription of a pseudoexon. This insertion included a premature stop codon near the end of the insertion, which likely leads to protein truncation. The patient carrying this variant presented with LDL-C levels of 5.7 mmol/L, corresponding with the 98th percentile for age and gender in the Netherlands (available via www.lipidtools.com).[18]

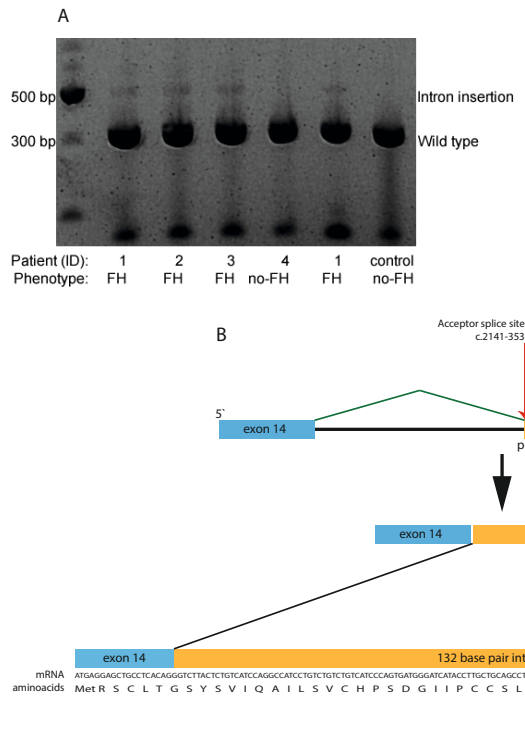

Figure 2: Agarose gel with cDNA polymerase chain reaction and schematic overview of pseudo-exon occurrence when c.2141-218G>A variant is present.
(A) Shown are the cDNA polymerase chain reaction (PCR) products for the patients in the pedigree in Figure 3. Patient (ID) corresponds to the IDs used in this pedigree. All patients with the FH phenotype (1-3) and carrying the c.2141-218G>A variant show an additional PCR product. Variant c.2141-218G>A creates a donor splice site at c.2141-211 and an acceptor splice site at c.2141-353 in intron 14, resulting in a pseudo-exon inclusion in the mRNA. This pseudo-exon has a length of 132 base pairs and predicts a premature stop codon near exon 15 (B). Patient 4, and a non-related no-FH control, did not carry this intronic variant and did not show an additional PCR product like her family members (A).

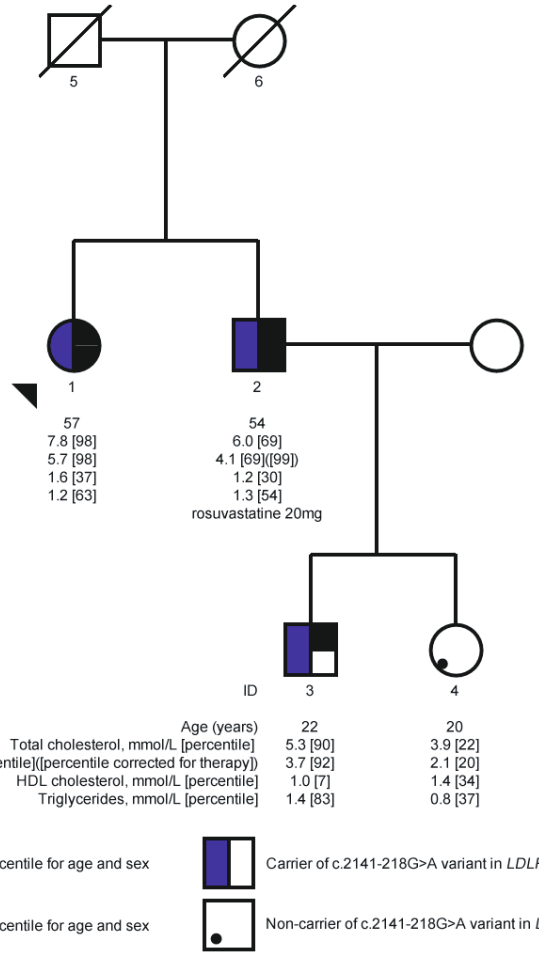

Figure 3: Pedigree of Family with c.2141-218G>A variant
The proband (ID: 1), brother (ID: 2), and nephew (ID: 3) carry the variant c.2141-218G>A and all have the FH phenotype (here defined as low-density lipoprotein levels > 95th percentile for age and sex). Her niece (ID: 4) was not found to carry this variant and did not have hypercholesterolemia.

Co-segregation analysis of the c.2141-218G>A variant

Next, we evaluated whether other carriers of this variant in the proband's family also presented with autosomal dominant hypercholesterolemia. The brother of the proband and his son were both carrier of the c.2141-218G>A variant and presented with a hypercholesterolemic phenotype (i.e., LDL cholesterol above 99th an 92th percentile for age and gender, respectively; Figure 3). In contrast, the niece of the

proband was not carrier of the identified variant and had no hypercholesterolemia. The proband had no children, and both her parents had deceased, which, unfortunately precluded us from further family analysis. We subsequently checked all dyslipidemic patients that were sequenced with our NGS platform from May 2016 (n=9675) and, although this variant was very well covered by our platform (minimal coverage of 20 times in all patients and mean±SD coverage of 56±15 times), we found no other index patients carrying this variant.

DISCUSSION

We used a novel approach to detect potential FH-causing deep intronic variants in *LDLR* and identified a variant, c.2141-218G>A, which causes the inclusion of a pseudoexon between exon 14 and exon 15 in the *LDLR* mRNA, including a premature stop codon. This variant fully segregated with the hypercholesterolemic phenotype in the small family of the identified proband. To our knowledge, the c.2141-218G>A variant described here is the deepest known FH-causing variant at 218 base pairs from the intron 14 – exon 15 boundary in *LDLR*, and currently the only variant described to result in a pseudoexon in this gene. Although we identified this specific variant in one proband only, our study suggests that intronic regions are of interest in patients with clinical but not yet genetically confirmed FH as these introns may harbor yet undiscovered FH-causing variants.

Disruption of the *LDLR* splicing machinery is a frequently observed pathogenic mechanism in FH (~10% of FH-causing variants[19], with almost all variants located in exons or near exon-intron boundaries[20]. Nonetheless, also deeper intronic variants can affect splicing or pre-mRNA secondary structure. Today, in the HGMD database (professional 2020.3) only two pathogenic variants are known beyond +/-20 base pairs, strikingly also in intron 14. Deep variants are however hard to investigate because of their size and numerous nonfunctional variants. For example, the size of intronic regions in *LDLR* are on average 2304 base pairs and while only achieving a mean coverage of 36% we already detected 29 variants on average in each patient.

To select potential deeper intronic pathogenic variants, we used a unique method that took advantage of all available intronic data that is generally not analyzed during diagnostic targeted next-generation sequencing. From these data we selected intronic variants that were absent in a control group of FH+ patients. This selection method yields several advantages. First, in our study, filtering against FH+ patients yielded greatly reduced numbers and minimizes the number of cDNA analyzes needed to confirm sequencing. Second, this will be an ever increasing refinement of this method with the increasing number of confirmed FH patients.

Our choice to define two groups of clinical FH patients (moderate and severe FH-) was based on the fact that those patients with the most severe FH phenotype have the highest chance of carrying an FH-causing variant.[1,2] Since *in silico* tools do not have a 100% sensitivity, we aimed not to miss potentially damaging variants in the severe FH- patients and decided to investigate all identified variants in these patients for their effect on splicing *ex vivo*. Vice versa, since hypercholesterolemia with LDL-C between 5 and 7 mmol/L is not always caused by pathogenic variants, but by other causes such as polygenic FH[4], secondary causes[21], or high lipoprotein (a) levels[3], we applied *in silico* tools to select variants of interest. The utility of these *in silico* tools was confirmed in our data, as in the severe FH- group, only the previously described probably pathogenic c.2140+103G>T variant[6] was *in silico* predicted to lead to aberrant splicing, in contrast to the two other variants (c.313+277C>T and c.694+25C>T).

Our study has several limitations. Firstly, the stringency of *in silico* criteria, remains elusive. In our study we used 7 different *in silico* tools and applied scoring cut-offs to identify variants of interest. This selection may have resulted in exclusion of functional variants. Moreover, these *in silico* tools may not be optimal for predicting the effect of deep intronic variants, as most tools are validated for variants close to the exon boundaries.[22,23] In our study, the most recent developed tool (SpliceAI[17]) was the only one to accurately predict the pathogenicity of the c.2141-218G>A variant. Secondly, it is possible that we discarded variants that caused compound heterozygous FH in the FH+ group. Thirdly, we still lack information on the deeper non-covered parts of the introns (Supplementary Table 2). Fourth, it is possible that our mRNA analysis was not sufficient to detect large pseudoexons or intron

retentions. Lastly, we did not examine whether the variant c.2141-218G>A indeed results in the absence or non-function of LDLR proteins. However, this variant was shown to result in a premature stop codon further downstream in the included pseudoexon, before the 5`-end of exon 15 (Figure 2B) and is therefore believed to result in truncation of the translated LDLR protein[24] or the *LDLR* mRNA will be targeted to the nonsense-mediated mRNA decay pathway.[25] The latter is suspected, as we were able to detect only small amounts of the mutated mRNA.

To assess deep intronic variants in a clinical setting and also assess currently uncovered regions by NGS of *LDLR*, NGS panels could be expanded by including all intronic LDLR regions. Next, additional filter steps, such as used in the current study (i.e. filtering for unique variants against FH+ patients and *in silico* assessment) are needed to reduce the number of variants that need to be assessed by mRNA sequencing (i.e., real time PCR or RNA-seq) This approach may result in identification of deep intronic variants, as well as an assessment of their effect on pre-mRNA splicing with only significantly increasing costs for RNA isolation and analysis.

In conclusion, we developed a novel selection method to identify deep intronic *LDLR* variants that potentially cause FH. We were able to discover a new variant in intron 14 (c.2141-218G>A) and describe for the first time a pseudoexon in the *LDLR* mRNA that likely results in FH. This finding emphasizes the need to consider more extensive *LDLR* analysis in patients in whom DNA sequencing fails to identify a molecular basis for their FH phenotype.

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CONFLICT OF INTERESTS

LFR is co-founder of Lipid Tools. GKH has served as consultant and speaker for biotechnology and pharmaceutical companies that develop molecules that influence lipoprotein metabolism, including Regeneron, Aegerion Pfizer, Merck, KOWA, Sanofi, and Amgen; has served as principal investigator for clinical trials conducted with a.o. Amgen, Sanofi, Eli Lilly, Novartis, Kowa, Genzyme, Cerenis, Pfizer, Dezima, and AstraZeneca; has received research grants from ZonMW (Vidi grant [016.156.445]), Klinkerpad fonds, the European Union, Amgen, Sanofi, AstraZeneca, Aegerion, and Synageva; has received honoraria and investigator fees (to the Department of Vascular Medicine) for sponsor- driven studies and lectures for companies with approved lipid- lowering therapy in the Netherlands; and is partly employed by Novo Nordisk AS, Copenhagen, Denmark (0.7FTE) and the Amsterdam UMC, Amsterdam, the Netherlands (0.3FTE).

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SUPPLEMENTAL MATERIALS

Supplementary Table 1: next-generation sequencing gene panel

Phenotype	Gene	Reference transcript	Dominant/recessive disease
Hypercholesterolemia	*LDLR*	NM_000527.4	Dominant
Hypercholesterolemia / hypocholesterolemia	*APOB*	NM_000384.2	Dominant
Hypercholesterolemia / hypocholesterolemia	*PCSK9*	NM_174936.3	Dominant
Hypercholesterolemia	*LIPA*	NM_000235.3	Recessive
Hypercholesterolemia	*LDLRAP1*	NM_015627.2	Recessive
Hypercholesterolemia	*ABCG5*	NM_022436.2	Recessive
Hypercholesterolemia	*ABCG8*	NM_022437.2	Recessive
Hypercholesterolemia	*STAP1*	NM_012108.2	Dominant
Hypo-alphalipoproteinemia	*ABCA1*	NM_005502.3	Dominant
Hypo-alphalipoproteinemia	*LCAT*	NM_000229.1	Dominant
Hypo-alphalipoproteinemia	*APOA1*	NM_000039.1	Dominant
Hyper-alphalipoproteinemia	*SCARB1*	NM_005505.4	Dominant
Hyper-alphalipoproteinemia	*CETP*	NM_000078.2	Dominant
Hyper-alphalipoproteinemia	*LIPG*	NM_006033.3	Dominant
Hyper-alphalipoproteinemia	*LIPC*	NM_000236.2	Dominant
Hyper-alphalipoproteinemia	*APOC3*	NM_000040.1	Dominant
Hypertriglyceridemia	*LPL*	NM_000237.2	Dominant
Hypertriglyceridemia	*APOC2*	NM_000483.4	Dominant
Hypertriglyceridemia	*APOA5*	NM_052968.4	Dominant
Hypertriglyceridemia	*GPIHBP1*	NM_178172.5	Dominant
Hypertriglyceridemia	*LMF1*	NM_001352018.1	Dominant/recessive
Hypertriglyceridemia	*APOE*	NM_001302688.1	Dominant/recessive
Chylomicron retention disease	*SAR1B*	NM_016103.3	Recessive
Hypolipoproteinemia	*ANGPTL3*	NM_014495.3	Dominant
Hypocholesterolemia	*MTTP*	NM_000253.3	Recessive
Hypocholesterolemia	*MYLIP*	NM_013262.3	Dominant
Cerebrotendineous xanthomatosis	*CYP27A1*	NM_000784.3	Recessive
Statin resistance	*CYP7A1*	NM_000780.3	Recessive
Myopathy on statins	*SCLO1B1*	NM_006446.4	Dominant

Supplementary Table 2: Percentage of intron with minimum 1x or minimum 30x coverage

Intron	% of intron with at minimum 1x coverage	% of intron with at minimum 30x coverage	Intron size (base pairs)
Intron1	18	10	10606
Intron2	29	14	2317
Intron3	33	19	2432
Intron4	68	36	963
Intron5	100	62	703
Intron6	24	14	3136
Intron7	99	57	741
Intron8	41	25	1637
Intron9	100	100	84
Intron10	28	17	2330
Intron11	100	61	645
Intron12	29	16	3092
Intron13	100	100	135
Intron14	30	18	2650
Intron15	25	16	4662
Intron16	47	25	1426
Intron17	42	23	1609
Mean±SD	54±33	36±29	2304±2460

Shown are the % of intronic base pairs that are covered with our in house next-generation sequencing capture at a minimum coverage. For example, in intron 1, 18% of the base pairs is at least covered 1x in all patients.

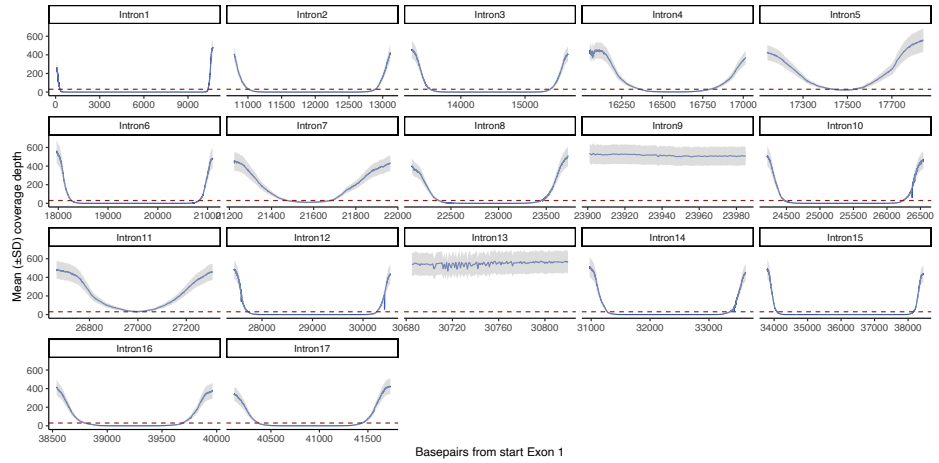
Supplementary Table 3: Primers used for cDNA analysis

Variant	Forward primer	Reverse primer
c.313+277C>T	GAAATTGCGCTGGACCGTC	GATTTGTCCTTGCAGTCGGG
c.694+25C>T	GCTGCATTCCTCAGTTCTGG	CAGAGCACTGGAATTCGTCA
c.1187-96C>T	CCGGGACTGGTCAGATGAAC	ATAGGAAGAGACGCCGTGGG
c.2141-218G>A	TGAACTGGTGTGAGAGGACC	ACATTGTCACTATCTCCACCGT

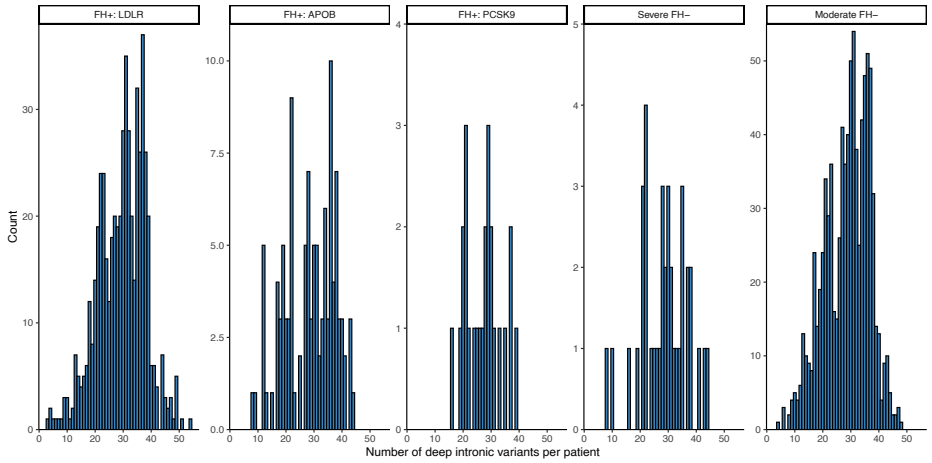
Supplementary Table 4: Average number of deep intronic variants per patient group

Patient group	Mean±SD number of deep intronic variants per patient
FH+	29.3±8.5
Caused by *LDLR* likely pathogenic or pathogenic variant	29.5±8.5
Caused by *APOB* likely pathogenic or pathogenic variant	28.6±8.8
Caused by *PCSK9* likely pathogenic or pathogenic variant	27.1±6.2
FH-	28.7±8
Severe FH-	28.7±8.0
Moderate FH-	28.5±8.3

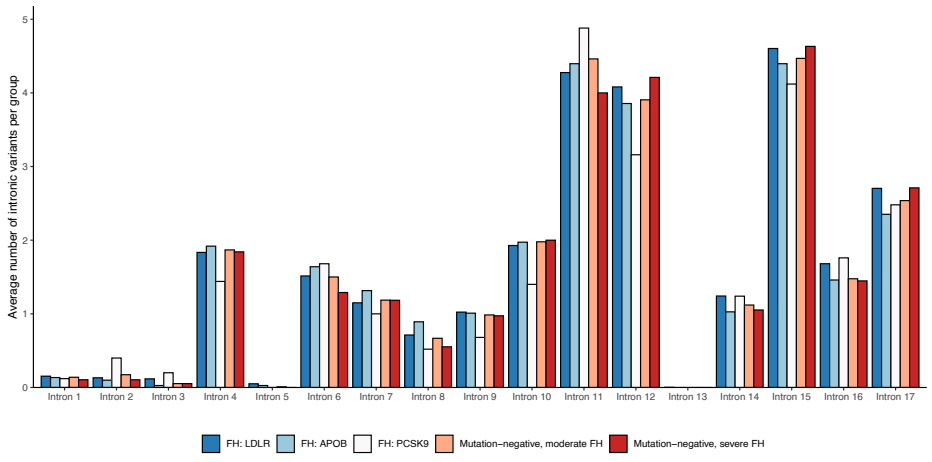
Shown are the average number of deep intronic variants per subject per patient group. These are not significantly different among groups: p=0.185 for FH mutation-positive vs FH mutation-negative and p=0.318 for the comparison of all subgroups (*LDLR*, *APOB*, *PCSK9*, Severe FH, and Moderate FH).



Supplementary Figure 1: Average coverage of intronic regions *LDLR* using targeted next-generation sequencing



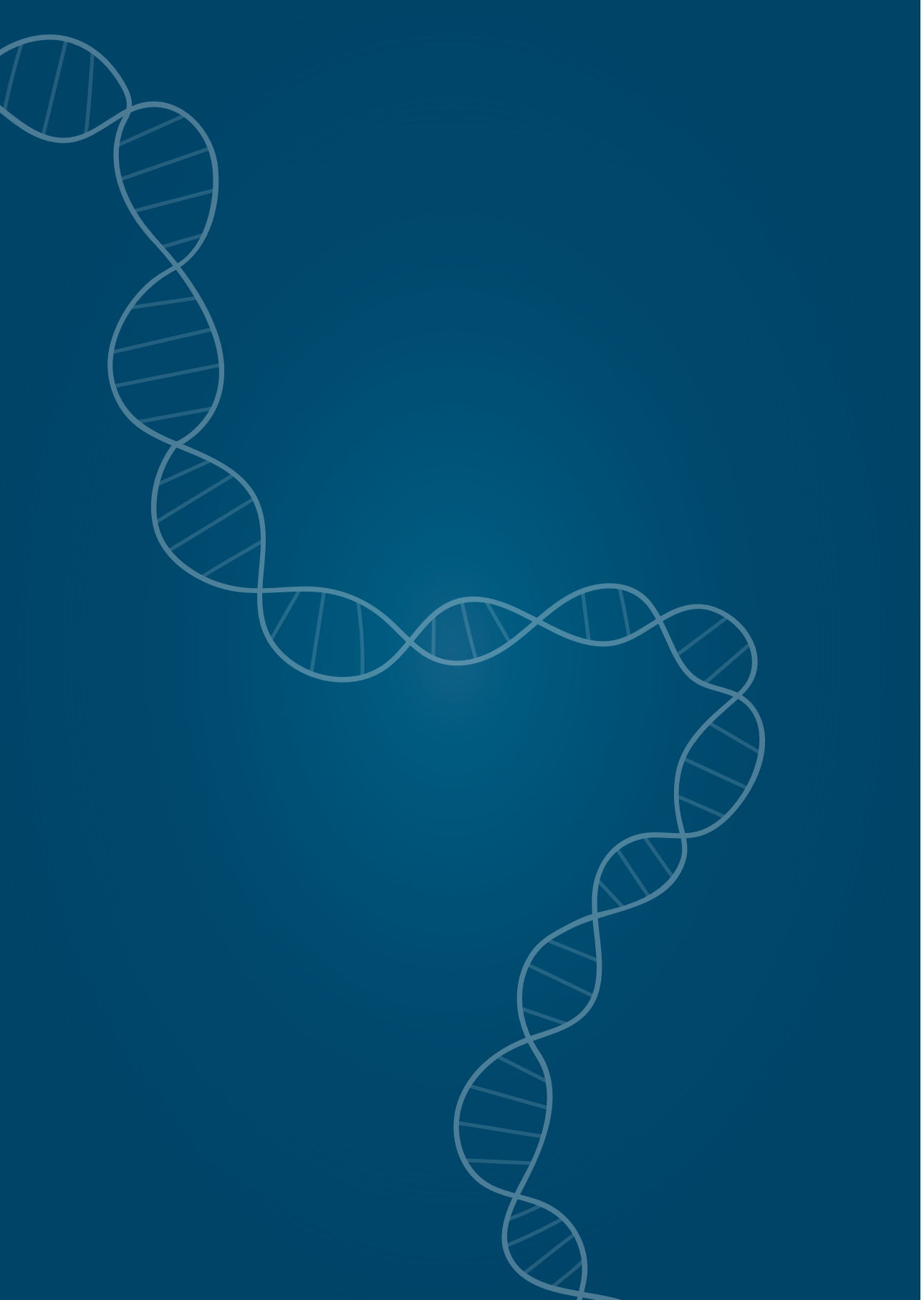
Supplementary Figure 2: Number of deep intronic variants per patient



Supplementary Figure 3: Distribution of variants over *LDLR* introns

PART I B

Novel diagnostic targets



5

ABCG5 and *ABCG8* genetic variants in Familial Hypercholesterolemia

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ABSTRACT

Background: Familial hypercholesterolemia (FH) is a common inherited disease characterized by elevated low-density lipoprotein cholesterol (LDL-C) plasma levels and increased cardiovascular disease risk. The majority of patients carry a mutation in the low-density lipoprotein receptor gene (*LDLR*). Common and rare variants in the genes encoding adenosine triphosphate-binding cassette transporters G5 and G8 (*ABCG5* and *ABCG8*) have been shown to affect LDL-C levels.

Objective: To investigate whether and to which extent heterozygous variants in *ABCG5* and *ABCG8* are associated with the hypercholesterolemic phenotype.

Methods: We sequenced *ABCG5* and *ABCG8* in a cohort of 3031 clinical FH patients and compared the prevalence of variants with a European reference population (gnomAD). Clinical characteristics of carriers of putative pathogenic variants in *ABCG5* and/or *ABCG8* were compared with heterozygous carriers of mutations in *LDLR*. Furthermore, we assessed the segregation of one *ABCG5* and two *ABCG8* variants with plasma lipid and sterol levels in three kindreds.

Results: The frequencies of (likely) pathogenic *LDLR, APOB, PCSK9, ABCG5* and *ABCG8* variants in our FH cohort were 11.42%, 2.84%, 0.69%, 1.48%, and 0.96%, respectively. We identified 191 *ABCG5* and *ABCG8* variants of which 53 were classified as pathogenic or likely pathogenic. Of these 53 variants, 51 were either absent from a reference population or more prevalent in our FH cohort than in the reference population. . LDL-C levels were significantly lower in heterozygous carriers of a (likely) pathogenic *ABCG5* or *ABCG8* variant compared to *LDLR* mutation carriers (6.2±1.7 vs 7.2±1.7 mmol/L, p<0.001). The combination of both an *ABCG5* or *ABCG8* variant and a *LDLR* variant was found not to be associated with significant higher LDL-C levels (7.8±2.3 vs 7.2±1.7 mmol/L, p=0.259). Segregation analysis in three families (nine carriers, in addition to the index cases, and 16 non-carriers) did not show complete segregation of the *ABCG5/G8* variants with high LDL-C levels and LDL-C levels were not different (3.9±1.3 vs 3.5±0.6mmol/L in carriers and controls respectively, p=0.295), while plasma plant sterol levels were higher in carriers compared to non-carriers (cholestanol: 10.2±1.7 vs 8.4±1.6 µmol/L, p=0.007; campesterol: 22.5±10.1 vs 13.4±3.5 µmol/L, p=0.008; sitosterol: 17.0±11.6 vs 8.2±2.6 µmol/L, p=0.024).

Conclusions: 2.4% of subjects in our FH cohort carried putative pathogenic *ABCG5* and *ABCG8* variants, but had lower LDL-C levels compared to FH patients who were heterozygous carriers of a *LDLR* variant. These results suggest a role for these genes in hypercholesterolemia in FH patients with less severely elevated LDL-C levels. We did not find evidence that these variants cause autosomal dominant FH.

INTRODUCTION

Familial Hypercholesterolemia (FH) is an autosomal dominant disorder of lipoprotein metabolism characterized by high LDL cholesterol (LDL-C) plasma levels with an estimated prevalence of 1 in 250 individuals in Western Europe.[1] Lifelong exposure to elevated plasma levels of LDL-C leads to atherosclerosis at an early age, and as such FH patients are at increased risk for cardiovascular disease (CVD).[2]

FH is primarily caused by genetic variants that affect the uptake of LDL particles from the circulation by the LDL receptor (*LDLR*). Variants in *LDLR, APOB*, and *PCSK9* result in clinical FH[3]: a mutation in one of these three genes can been found in 42.1% of patients with LDL-C levels between 5.00 and 5.99 mmol/L and in 88% of patients with an LDL-C >8 mmol/L.[4] It is widely acknowledged that mutations in other genes may be present in those FH patients who do not have a pathogenic variant in *LDLR*, *APOB* or *PCSK9*. Recently, mutations in the genes encoding for ATP-binding cassette sub-family G members 5 and 8 (*ABCG5* and *ABCG8)* have been described to result in FH.[5]

ABCG5 and ABCG8 form a heterodimer, responsible for transmembrane transport of sterols, in particular plant sterols. In the intestine, this heterodimer is located in the apical membrane and transports sterols from the enterocyte into the intestinal lumen while the hepatic heterodimer transports sterols into bile.[6] Homozygosity or compound heterozygosity for functional variants in *ABCG5* or *ABCG8* cause sitosterolemia, an autosomal recessive disorder characterized by severely elevated

plasma plant sterols and cholesterol levels.[7] Sitosterolemic patients also typically present with xanthomas, accelerated atherosclerosis and premature coronary artery disease, characteristics that closely mimic the clinical FH phenotype.[8] A recent study showed an association between hypercholesterolemia and sitosterolemia in the general population. Among the more than 200,000 subjects studied, plasma ß-sitosterol concentrations were above the 99th percentile in 4% of the subjects with plasma LDL-C levels above 4.9 mmol/L while such high plasma ß-sitosterol concentrations were presented in only 1% of those with plasma LDL-C levels below 3.3 mmol/L.[9]

The relationship between increased plasma lipid concentrations, CVD risk and genetic variations in *ABCG5* or *ABCG8* has been investigated in multiple studies in the general population.[10–13] However, the role of heterozygous (likely) pathogenic variants in the FH phenotype is less well understood, despite reports of the presence of variants in these two genes in hypercholesterolemic subjects and inclusion of these genes in FH sequencing panels.[5,14,15] Since variants in *ABCG5* and *ABCG8* are frequently discovered with the expansion of next-generation sequencing panels in FH patients, we set out to investigate the prevalence of these variants in a cohort of patients who were considered to suffer from FH based on clinical symptoms and to investigate their association with lipid levels. Moreover, we determined the co-segregation of one *ABCG5* and two *ABCG8* variants with plasma plant sterol and cholesterol levels in three families.

METHODS

Study population

The Amsterdam University Medical Center in Amsterdam is the national referral center for DNA diagnostics in dyslipidemia in the Netherlands. Patients are referred for genetic FH analysis based on the clinical judgement of the referring physician. An in house next-generation sequencing (NGS) capture covering, amongst others, *LDLR*, *APOB*, *PCSK9*, *ABCG5*, and *ABCG8* (SeqCap easy choice, Roche NimbleGen Inc., Pleasanton, USA) is used to establish the possible genetic origin FH. The full list of genes sequenced by means of this method is provided in Supplementary

Table 1. Genetic data was available for 3031 hypercholesterolaemic subjects, who underwent NGS analysis between May 2016 and July 2018. Written informed consent has been obtained from each patient.

Genetic analysis

Two trained molecular geneticists assessed all variants in the exons (+/- 20 base pairs) of *ABCG5* and *ABCG8* for their pathogenicity according to standard ACGM guidelines for variant classification.[16] Using this scoring system, class 4 variants were considered likely pathogenic and class 5 variants as pathogenic. The frequencies of all variants identified in the FH patients were compared to the general population of European descent (non-Finnish) in the Genome Aggregation Database (gnomAD, http://gnomad.broadinstitute.org;[17,18] which provides more than 120,000 exome sequences from unrelated individuals enrolled in various disease-specific and population genetic studies.

Clinical characteristics of *ABCG5/G8* variant carriers

Patients carrying a heterozygous (likely) pathogenic *ABCG5* or *ABCG8* variant of whom LDL-C levels were known (Group 2: heterozygous *ABCG5/G8* variant carriers, n = 72) were compared to an age- and sex-matched group of untreated FH patients who were heterozygous carriers of a deleterious *LDLR* mutation (Group 1: true FH patients, n = 72). *LDLR* variants were assessed for pathogenicity by two trained molecular geneticists according to the same guidelines as *ABCG5 and ABCG8* variants mentioned above. Additionally, four compound heterozygotes or homozygotes for *ABCG5* and/or *ABCG8* variants (Group 3: Compound heterozygote or homozygote for *ABCG5/G8* variants) and 13 FH patients who had a *LDLR* mutation and a (likely) pathogenic *ABCG5* or *ABCG8* variant (Group 4: True FH (*LDLR* mutation) + heterozygous for *ABCG5/G8* variant) were also compared to the True FH group. This group wise analysis was repeated after exclusion of patients carrying an *ABCG5* or *ABCG8* variant whom were on lipid lowering therapies (statins or ezetimibe). See Figure 1 for a flow diagram of the study.

Segregation analysis

Plasma and DNA were collected from family members of heterozygous carriers of a deleterious variant in *ABCG5* or *ABCG8* to perform co-segregation analysis of

the *ABCG5* or *ABCG8* variant with a FH phenotype (in this analysis defined as LDL-C levels above the 95th percentile for age and gender) as well as plasma sterol levels. The selected index patients had LDL-C levels above the 99th percentile for age and gender and were negative for mutations in *LDLR*, *APOB*, and *PCSK9*.

Laboratory analysis of *ABCG5* and *ABCG8* families

DNA was extracted from a 10 ml blood sample collected in EDTA tubes using a Gentra AutoPure LS (Gentra Systems, Minneapolis, MN). Variants were genotyped by polymerase chain reaction amplification (for the exact used primer sequences see Supplementary Table 2) followed by an ExoSap purification (Shrimp Alkaline Phosphatase, ThermoFisher Scientific; and Exonuclease I, ThermoFisher Scientific, Waltham, MA). The amplified regions were sequenced by Sanger method using the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and analysed by Sequencer software (Gene Codes Corporation, Ann Arbor, MI).

Total cholesterol, high density lipoprotein cholesterol (HDL-C), and triglycerides plasma levels were measured using commercially available assays (Wako Chemicals, Neuss, Germany; and DiaSys Diagnostic Systems, Holzheim, Germany) on a Selectra analyzer. LDL-C levels were calculated using the Friedewald formula.[19] In patients who were using lipid lowering agents, we calculated the lipid levels by means of previously published correction factors.[20]

Sterol levels were measured using gas chromatography-mass spectrometry (GCMS) and flame ionization detection (GC-FID). After an internal standard (Epicoprostanol; Sigma Aldrich, Saint Louis, USA) was added to 100 µL plasma, the sample was hydrolyzed by KOH (ethanol) at 80 °C for 2h and subsequently sterols were extracted with hexane. After evaporation to dryness (N_2, 40 °C) of the hexane fraction, sterols were derivatized with BSTFA (+1%TMCS) (80 °C, 30 min). Derivatized samples were ionized with Electron Impact and analyzed in parallel by GCMS for identification and GC-FID for quantitation.

Statistical analyses

The prevalence of (likely) pathogenic variants in *ABCG5/G8* in the FH group was numerically compared with a reference database (gnomAD). Pedigrees were drawn in

Progeny version 10.2.4.0 (Progeny, Delray Beach, FL). Independent T-tests were used to compare normally distributed numeric data between groups, Mann-Whitney U test for not normally distributed numeric data, and Fisher' Exact tests for categorical data. We used a Generalized Equations Estimates (GEE) analysis to correct for family relatedness to compare the lipid and sterol levels in carriers and non-carriers within the four families. All statistical analysis were performed using R, version 3.5.1 (R Foundation, Vienna, Austria). A P-value of <0.05 was considered to be statistically significant.

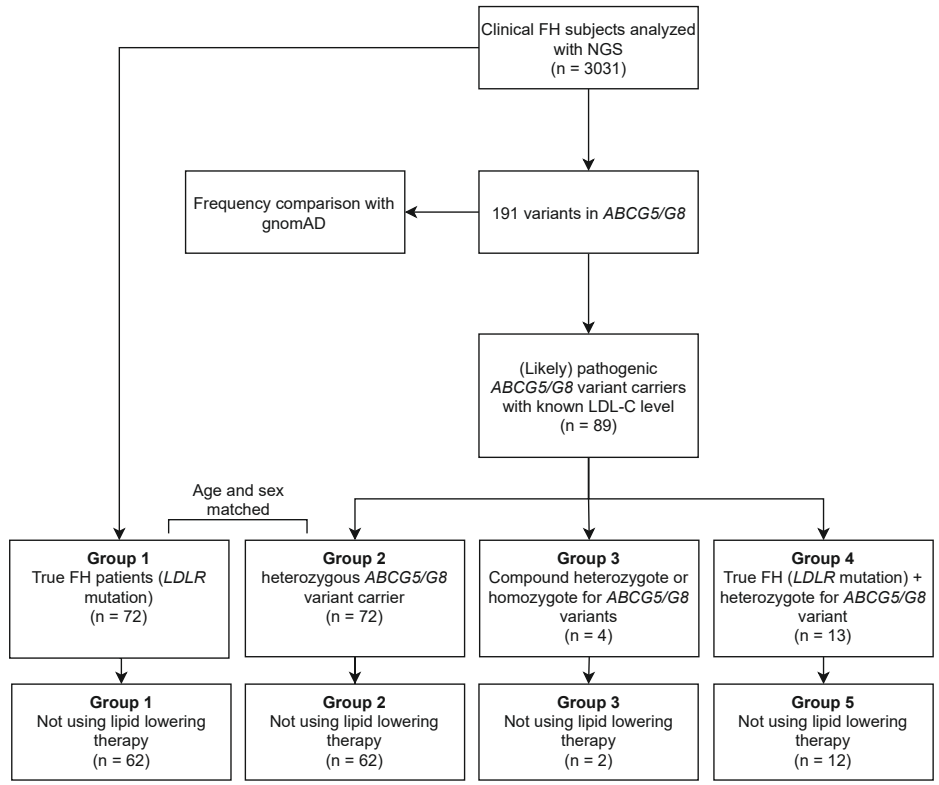

Figure 1: Flow diagram of analysis.
191 variants in *ABCG5* and *ABCG8* were identified in 3031 clinical FH patients and used for frequency comparison with *ABCG5* and *ABCG8* variants in Europeans present in the publicly accessible gnomAD. Next, lipid levels of heterozygous *ABCG5 and ABCG8* variant carriers (group 2), compound heterozygotes or homozygotes for *ABCG5* and/or *ABCG8* variants (group 3), and true FH patients (*LDLR* mutation) carrying an additional *ABCG5* or *ABCG8* variant (group 4) were compared to true FH patients carrying a *LDLR* mutation (group 1). True FH patients in group 1 were age and sex matched to *ABCG5* and *ABCG8* variant carriers in group 2.

RESULTS

Frequency of *ABCG5* and *ABCG8* variants

In our clinical FH cohort of 3031 subjects, the mean LDL-C level was 5.8±1.4 mmol/L. At least 14.2% of the FH patients was using lipid lowering therapy at moment of lipid profile measurement. 11.42%, 2.84%, and 0.69% was carrier of a FH causing mutation in *LDLR*, *APOB*, or *PCSK9*, respectively (Table 1). 2.44% of patients were heterozygous carrier of a (likely) pathogenic variants in either *ABCG5* or *ABCG8*. Clinical details are described in Supplementary Table 3. We found 79 variants in *ABCG5* and 112 variants in *ABCG8* of which 53 were classified as (likely) pathogenic. 31 of the (likely) pathogenic variants were more frequently observed among FH patients than in the publicly available genetic data of Europeans in the gnomAD database. 20 variants were new and not present in gnomAD. The location of the identified variants per gene are shown in Figure 2. The (likely) pathogenic variants are reported in Table 2, while the (likely) benign variants and variants of unknown significance (VOUS) are reported in Supplementary Table 4.

Clinical characteristics of *ABCG5* and *ABCG8* carriers

Plasma LDL-C levels were available for 89 subjects with at least one (likely) pathogenic variant in *ABCG5* and/or *ABCG8*. In order to assess whether plasma lipid profiles of *ABCG5* and *ABCG8* heterozygotes, compound heterozygotes and homozygotes were comparable to those of true FH patients with a deleterious *LDLR* mutation, we divided these 89 subjects over three groups (group 2 – 4) based on their genetic status. Group 2 consisted of heterozygous carriers of an *ABCG5* or *ABCG8* variant, group 3 comprised compound heterozygotes and homozygotes for *ABCG5* or *ABCG8* variants, and group 4 consisted of true FH subjects due to a deleterious *LDLR* mutation who also carried an additional *ABCG5* or *ABCG8* variant. Group 1 was age- and sex-matched to group 2 and contained true FH patients with a mutation in *LDLR* (Figure 1 and Table 3). Heterozygous *ABCG5* or *ABCG8* variant carriers (group 2) had significantly lower plasma LDL-C levels than the true FH patients in group 1 (Figure 3). LDL-C corrected for statin/ezetimibe use: 6.2±1.7 vs 7.2±1.7 mmol/L, p<0.001). Compound heterozygotes or homozygotes for *ABCG5* and *ABCG8* variants, and variant carriers on top of a *LDLR* mutation (Figure 3; group 3 and group 4) had similar LDL-C levels as true FH patients (6.2±2.5 and

7.8±2.3 vs 7.2±1.7 mmol/L, p=0.305 and p=0.259, respectively). After exclusion of those patients that were on lipid lowering therapies (LLT, statins and ezetimibe), *ABCG5* or *ABCG8* variant carriers (group 2) still had significant lower LDL-C levels compared to group 1 (5.9±1.4 vs 7.0±1.9 mmol/L, p<0.001; Supplementary Table 5 and Supplementary Figure 1).

Co-segregation between the FH phenotype and *ABCG5* or *ABCG8* variants

In order to investigate the causative role of a (likely) pathogenic variant in *ABCG5* or *ABCG8* in the FH phenotype, we analyzed the segregation of the FH phenotype as well as sterol levels with one *ABCG5* and two *ABCG8* heterozygous genetic variants in three families (Figure 4). In Family A we analyzed whether the p.Arg619Thr (c.1856G>C) variant in *ABCG5* resulted in a FH phenotype. The allele frequency (AF) of this variant was 0.017% in our FH cohort and is not present in gnomAD. Of the 6 tested family members, the two with a clinical FH phenotype (defined as an LDL-C >95th percentile for age and sex) were carriers of this likely pathogenic *ABCG5* variant. Further family expansion was not possible. *ABCG8*, p.Arg121* (c.361C>T) was one of the 31 variants that were more frequently observed in our FH cohort compared to Europeans in gnomAD (AF 0.099% vs 0.002%) and was found in three generations of Family B. This variant showed incomplete penetrance with the FH phenotype since two family members carrying the variant had normal LDL-C levels while one of the family members with an FH phenotype did not carry the variant. In Family C, five family members were heterozygous carriers of the p.Trp361* (c.1083G>A) *ABCG8* variant. The AF of this variant was 0.013% in FH patients compared to 0.004% in gnomAD. Three of these carriers were deemed not to be FH patients. Table 4 shows the lipid profiles and plant sterol levels of carriers and non-carriers of an *ABCG5* or *ABCG8* mutation of the 3 families. Index cases of the families were excluded from this analysis to minimize bias. LDL-C levels (corrected for use of lipid lowering therapies) were not significantly different between the two groups (3.9±1.3 vs 3.5±0.6 mmol/L, p=0.295), whereas the plant sterol levels (cholestanol, campesterol and sitosterol) were significantly higher in heterozygous variant carriers compared to family controls (cholestanol: 10.2±1.7 vs 8.4±1.6 µmol/L, p=0.007; campesterol: 22.5±10.1 vs 13.4±3.5 µmol/L, p=0.008; sitosterol: 17.0±11.6 vs 8.2±2.6 µmol/L, p=0.024). From two subjects (family B,

subjects 1 and 4) sterols were measured in blood drawn at a second visit, and thus does not corresponds with the lipid profile at visit 1, which is reported in Figure 4. However, while using the previously published classification of sitosterolemia, based on sitosterol levels above the 99th percentile compared to the general population (19.3 µmol/L[9]) no obvious co-segregation with an *ABCG5* or *ABCG8/G8* variants was observed. Only two subjects with an *ABCG8* c.361C>T (p.Arg121*) variant met this threshold.

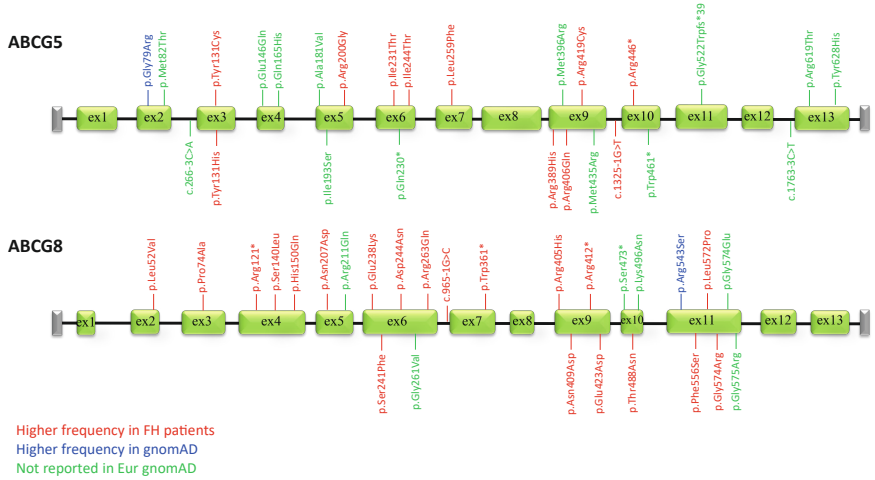

Figure 2: Identified (likely) pathogenic *ABCG5* and *ABCG8* variants in FH patients per gene.
Locations of identified (likely) pathogenic *ABCG5* and *ABCG8* variants in 3640 FH patients per *ABCG* gene. Those in red were significantly more frequently seen in FH patients than in the European general population. Those in green are newly identified variants not present in the European gnomAD dataset.

Table 1: Number of clinical FH patients with (likely) pathogenic variants in *LDLR, APOB, PCSK9, STAP1, LDLRAP1, ABCG5,* and *ABCG8*

FH genotype	No. of clinical FH patients with specific genotype	%
Mutation negative	2465	81.33
LDLR heterozygote	346	11.42
APOB heterozygote	86	2.84
ABCG8 heterozygote	45	1.48
ABCG5 heterozygote	29	0.96
PCSK9 heterozygote	21	0.69
LDLR/ABCG8 compound heterozygote	9	0.30
STAP1 heterozygote	7	0.23
LDLR/ABCG5 compound heterozygote	6	0.20

Table 1: (Continued)

FH genotype	No. of clinical FH patients with specific genotype	%
ABCG5/ABCG8 compound heterozygote or homozygote	4	0.13
LDLR homozygote	4	0.13
LDLR/PCSK9 compound heterozygote	4	0.13
LDLRAP1 homozygote	2	0.07
PCSK9 homozygote	2	0.07
LDLR/APOB compound heterozygote	1	0.03
Total	**3031**	**100**

Table 2: Pathogenic and likely pathogenic *ABCG5* and *ABCG8* variants in FH patients

Gene	Variant	Aminoacid change	In silico prediction	Allele frequency FH patients	Allele frequency gnomAD European (non-finish) population	Frequency highest in FH or gnomAD
ABCG5	c.1336C>T	p.Arg446*	Pathogenic	0.000660	0.000093	FH
	c.235G>A	p.Gly79Arg	Likely pathogenic	0.000495	0.000697	gnomAD
	c.1217G>A	p.Arg406Gln	Likely pathogenic	0.000330	0.000026	FH
	c.1255C>T	p.Arg419Cys	Likely pathogenic	0.000330	0.000053	FH
	c.1325-1G>T	NA	Pathogenic	0.000330	0.000009	FH
	c.391T>C	p.Tyr131His	Likely pathogenic	0.000330	0.000052	FH
	c.392A>G	p.Tyr131Cys	Pathogenic	0.000330	0.000069	FH
	c.1166G>A	p.Arg389His	Likely pathogenic	0.000165	0.000008	FH
	c.598C>G	p.Arg200Gly	Pathogenic	0.000165	0.000114	FH
	c.692T>C	p.Ile231Thr	Likely pathogenic	0.000165	0.000018	FH
	c.731T>C	p.Ile244Thr	Likely pathogenic	0.000165	0.000035	FH
	c.775C>T	p.Leu259Phe	Likely pathogenic	0.000165	0.000026	FH
	c.1882T>C	p.Tyr628His	Pathogenic	0.000330		
	c.542C>T	p.Ala181Val	Likely pathogenic	0.000330		
	c.1187T>G	p.Met396Arg	Likely pathogenic	0.000165		
	c.1304T>G	p.Met435Arg	Likely pathogenic	0.000165		
	c.1383G>A	p.Trp461*	Likely pathogenic	0.000165		
	c.1563dupT	p.Gly522Trpfs*39	Pathogenic	0.000165		
	c.1763-3C>T	NA	Likely pathogenic	0.000165		
	c.1856G>C	p.Arg619Thr	Likely pathogenic	0.000165		
	c.245T>C	p.Met82Thr	Likely pathogenic	0.000165		
	c.266-3C>A	NA	Likely pathogenic	0.000165		
	c.436G>C	p.Glu146Gln	Pathogenic	0.000165		
	c.495G>T	p.Gln165His	Likely pathogenic	0.000165		

Table 2: (Continued)

Gene	Variant	Aminoacid change	In silico prediction	Allele frequency FH patients	Allele frequency gnomAD European (non-finish) population	Frequency highest in FH or gnomAD
	c.578T>G	p.Ile193Ser	Likely pathogenic	0.000165		
	c.688C>T	p.Gln230*	Pathogenic	0.000165		
ABCG8	c.712G>A	p.Glu238Lys	Pathogenic	0.001815	0.001568	FH
	c.1225A>G	p.Asn409Asp	Likely pathogenic	0.001485	0.000658	FH
	c.1083G>A	p.Trp361*	Pathogenic	0.001320	0.001232	FH
	c.361C>T	p.Arg121*	Pathogenic	0.000990	0.000023	FH
	c.450C>A	p.His150Gln	Pathogenic	0.000495	0.000035	FH
	c.1269G>T	p.Glu423Asp	Pathogenic	0.000330	0.000023	FH
	c.722C>T	p.Ser241Phe	Likely pathogenic	0.000330	0.000225	FH
	c.1214G>A	p.Arg405His	Pathogenic	0.000165	0.000023	FH
	c.1234C>T	p.Arg412*	Pathogenic	0.000165	0.000116	FH
	c.1463C>A	p.Thr488Asn	Likely pathogenic	0.000165	0.000054	FH
	c.154C>G	p.Leu52Val	Likely pathogenic	0.000165	0.000023	FH
	c.1629G>T	p.Arg543Ser	Pathogenic	0.000165	0.000442	gnomAD
	c.1667T>C	p.Phe556Ser	Pathogenic	0.000165	0.000008	FH
	c.1715T>C	p.Leu572Pro	Likely pathogenic	0.000165	0.000124	FH
	c.1720G>A	p.Gly574Arg	Likely pathogenic	0.000165	0.000093	FH
	c.220C>G	p.Pro74Ala	Likely pathogenic	0.000165	0.000023	FH
	c.419C>T	p.Ser140Leu	Likely pathogenic	0.000165	0.000088	FH
	c.619A>G	p.Asn207Asp	Likely pathogenic	0.000165	0.000008	FH
	c.730G>A	p.Asp244Asn	Likely pathogenic	0.000165	0.000044	FH
	c.788G>A	p.Arg263Gln	Pathogenic	0.000165	0.000093	FH
	c.965-1G>C	NA	Pathogenic	0.000165	0.000009	FH
	c.1721G>A	p.Gly574Glu	Pathogenic	0.000660		
	c.1723G>C	p.Gly575Arg	Pathogenic	0.000660		
	c.782G>T	p.Gly261Val	Likely pathogenic	0.000495		
	c.1418C>A	p.Ser473*	Likely pathogenic	0.000165		
	c.1488G>C	p.Lys496Asn	Likely pathogenic	0.000165		
	c.632G>A	p.Arg211Gln	Likely pathogenic	0.000165		

Table 3: Characteristics and lipid profile of *ABCG5* and *ABCG8* variant carriers compared to true FH patients

	Group 1 True FH patients (*LDLR* mutation)	Group 2 Heterozygous *ABCG5* or *ABCG8* variant carrier		Group 3 Compound heterozygote or homozygote for *ABCG5* and/or *ABCG8* variants		Group 4 True FH (*LDLR* mutation) + heterozygote for *ABCG5* or *ABCG8* variant	
	n = 72	n = 72	P-value*	n = 4	P-value*	n = 13	P-value*
Age, years	52.1 (13.2)	53.0 (13.9)	0.682	54.6 (8.7)	0.710	41.8 (19.6)	0.020
No. of males	37 (51.4)	37 (51.4)	1	4 (100.0)	0.167	9 (69.2)	0.376
Total cholesterol, mmol/L	9.3 (1.8)	7.9 (1.7)	<0.001	8.0 (0.5)	0.219	10.0 (2.4)	0.223
LDL-C, mmol/L	7.2 (1.7)	5.7 (1.5)	<0.001	5.0 (1.5)	0.016	7.4 (2.5)	0.715
Corrected LDL-C, mmol/L	7.2 (1.7)	6.2 (1.7)	0.001	6.2 (2.5)	0.305	7.8 (2.3)	0.259
HDL-C, mmol/L	1.3 (0.4)	1.4 (0.5)	0.312	1.2 (0.1)	0.631	1.6 (0.4)	0.030
Triglycerides, mmol/L	1.6 [1.1, 2.3]	1.8 [1.1, 2.5]	0.290	2.5 [2.3, 2.7]	0.102	1.0 [0.8, 1.6]	0.104
No. of statin users	0 (0.0)	7 (9.7)	-	1 (25.0)	-	1 (7.7)	-
No. of ezetimibe users	0 (0.0)	5 (6.9)	-	1 (25.0)	-	0 (0.0)	-

FH1, Familial Hypercholesterolemia type 1; LDL-C, Low density lipoprotein cholesterol; HDL-C, High density lipoprotein cholesterol; *Compared with Group 1 (True FH patients), which was age and sex matched with Group 2. P-value for the statistical difference between a group and the reference group is determined with an independent t-test for normally distributed data and a Mann-Whitney U Test for non-normally distributed data (TG), and a Fisher Exact test for categorical data. All values are expressed as mean (standard deviation), except for No. of males, No. of statin users, and No. of ezetimibe users which are expressed as number (%), and TG which is expressed as median (interquartile range).

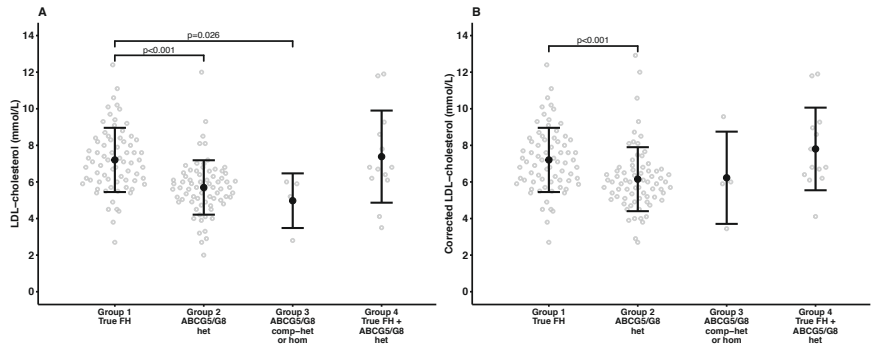

Figure 3: LDL-cholesterol levels of *ABCG5* and *ABCG8* variant carriers compared to true heterozygous FH patients.
LDL-cholesterol levels of *ABCG5* and *ABCG8* variant carriers compared to true FH patients (carrying a *LDLR* mutation). A) LDL-cholesterol levels are significantly lower in *ABCG5* and *ABCG8* heterozygote carriers (group 2) and compound heterozygotes and homozygotes for *ABCG5* and *ABCG8* variants (group 3) compared to true FH patients. B) LDL-cholesterol levels were corrected for statin and ezetimibe use as previously reported.[20] Corrected LDL-cholesterol levels were significantly lower in *ABCG5* and *ABCG8* variant carriers compared to true FH patients. FH: familial hypercholesterolemia, het: heterozygote, hom: homozygote.

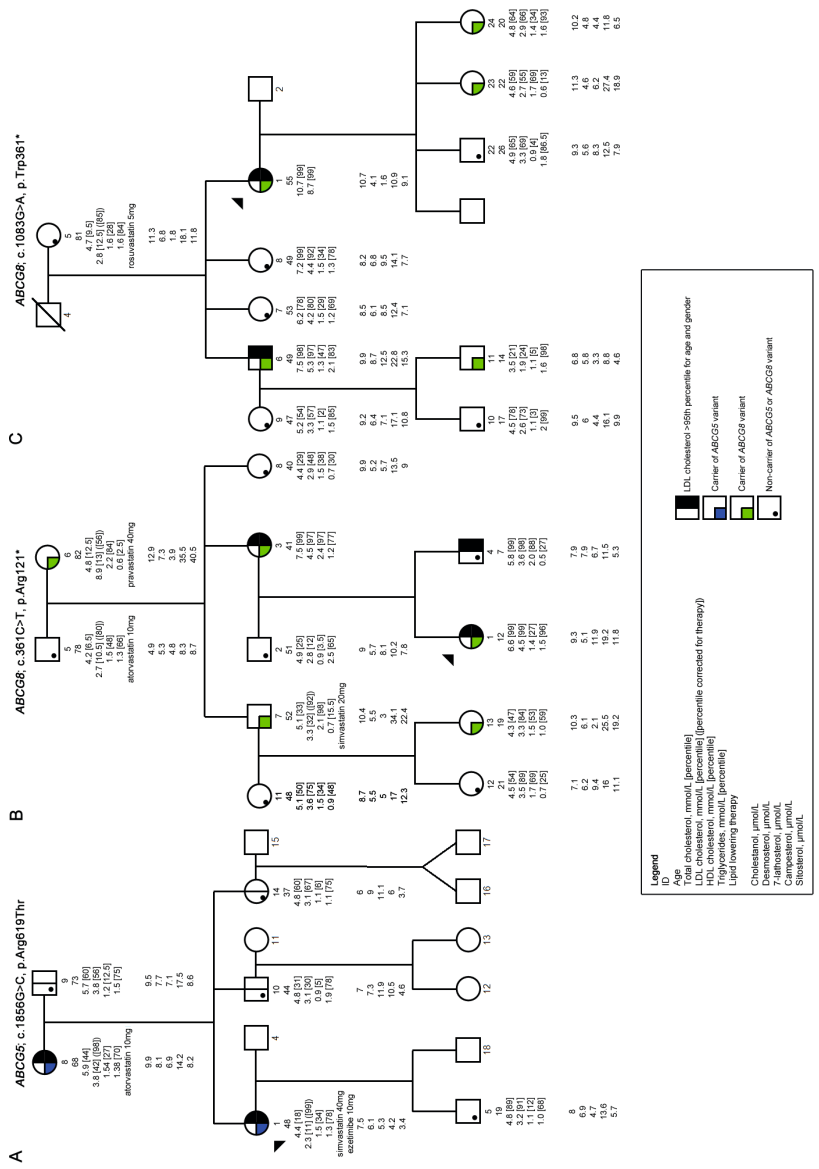

Figure 4: Cosegregation analysis of *ABCG5* and *ABCG8* variants with hypercholesterolemia in 3 FH families. Pedigrees of three FH families with *ABCG5* and *ABCG8* variants show that there is no cosegregation of the hypercholesterolemic phenotype (black color) with carrier status of *ABCG8* variants (c.361C>T and c.1083G>A; green colors) in families B and C. Possible cosegration of *ABCG5* variant c.1856G>C (blue color) in family A. Squares represent males, circles represent females. Triangles point to the index patients. For subjects 1 and 4 in family B, samples from a later visit were used for sterol measurements.

Table 4: Clinical characteristics of *ABCG5* and *ABCG8* variant carriers and non-carriers in three FH families

	Index patients (n = 3)	Carriers (n = 9)	Non-carriers (n = 16)	P-value (carriers vs non-carriers)
Age, years	38.6 (22.5)	41.2 (24.0)	43.7 (21.9)	0.782
No. of females	3 (100.0)	6 (66.7)	8 (50.0)	0.423
TC, mmol/L	7.2 (3.2)	5.3 (1.4)	5.1 (0.8)	0.623
LDL-C, mmol/L	5.2 (3.2)	3.4 (1.0)	3.3 (0.5)	0.800
corrected LDL-C, mmol/L	6.2 (2.2)	3.9 (1.3)	3.5 (0.6)	0.295
HDL-C, mmol/L	1.4 (0.1)	1.7 (0.4)	1.3 (0.3)	0.012
TG, mmol/L	1.4 [1.3, 1.4]	1.2 [0.7, 1.6]	1.3 [1.0, 1.7]	0.512
Statin users	1 (33.3)	3 (33.3)	2 (12.5)	0.236
Cholesterol, µmol/L	4567.0 (389.6)	4952.5 (1211.3)	4855.4 (755.1)	0.826
Cholestanol, µmol/L	9.2 (1.6)	10.2 (1.7)	8.4 (1.6)	0.007
Desmosterol, µmol/L	5.1 (1.0)	6.4 (1.5)	6.5 (1.1)	0.772
7-Lathosterol, µmol/L	6.3 (5.2)	5.3 (3.3)	7.1 (2.7)	0.149
Campesterol, µmol/L	11.4 (7.5)	22.5 (10.1)	13.4 (3.5)	0.008
Sitosterol, µmol/L	8.1 (4.3)	17.0 (11.6)	8.2 (2.6)	0.024

TC, Total cholesterol; LDL-C, Low density lipoprotein cholesterol; HDL-C, High density lipoprotein cholesterol; TG, Triglycerides. All values are expressed as mean (standard deviation), except for No. of females and No. of statin users which are expressed as number (%), and TG which is expressed as median (interquartile range). Index cases were excluded from the carrier group in the statistical analysis and are reported separately. Statistical difference between carriers and non-carriers was determined with an independent T-test for normally distributed data, Mann-Witney U Test for non-normally distributed data, and Chi squared test for categorical data. Corrected LDL-C levels were calculated by multiplying the LDL-C levels with previously reported correction factors for statin and/or ezetimibe use.[20]

DISCUSSION

This study provides an in-depth investigation into the consequences of heterozygosity for deleterious variants (class 4 and class 5) in *ABCG5* or *ABCG8* in an unprecedented large cohort of 3031 FH patients. We identified 191 variants in *ABCG5* or *ABCG8,* and performed frequency analysis and lipid profile comparison of variant carriers. Additionally, a co-segregation analysis in three FH families carrying an *ABCG5* or *ABCG8* variant was performed. Three major findings stand out from our research. Firstly, *ABCG5* and *ABCG8* variants are common among clinical FH patients; we identified 31 variants with a higher frequency in FH patients than the common European population, and identified 20 new (likely) pathogenic variants in this group. Secondly, heterozygous carriers of *ABCG5/G8* variants have lower

LDL-C levels than FH patients who are heterozygous carriers of a *LDLR* mutation. Thirdly, three (likely) pathogenic *ABCG5* and *ABCG8* variants did not co-segregate with the FH phenotype in three FH families. Altogether, these data show that *ABCG5* and *ABCG8* variants do not cause autosomal dominant FH, but probably have a small and inconsistent impact on plasma LDL-C levels in FH patients leading to the hypercholesterolemic phenotype.

Our analysis resulted in the identification of 20 (likely) pathogenic variants in *ABCG5/G8* that had not been annotated in the public database used for this study. Additionally, of the 33 variants that were present in both our cohort and in gnomAD, 31 were more frequently observed in our FH cohort. This indicates that our FH cohort might be enriched for specific *ABCG5* and *ABCG8* variants, suggesting a potential role for *ABCG5* and *ABCG8* contributing to the FH phenotype. However, a firm conclusion about enrichment would require the reverse comparison on *ABCG5* and *ABCG8* variants present in gnomAD that are not present in our FH cohort, which, unfortunately, cannot be performed due to the nature of the available data in gnomAD. Furthermore, sequencing of these genes in FH patients introduces potential bias in the association with hypercholesterolemia since any findings in a preselected FH population is automatically associated with LDL-C levels. Furthermore, it is possible that our FH cohort is not ethnically comparable to the (non-Finnish) European gnomAD population which was used as a reference population. This might influence the prevalence of certain ethnicity dependent genetic variants in *ABCG5 or ABCG8*.

A detailed analysis of the lipid profiles of 72 heterozygous carriers of an *ABCG5* or *ABCG8* variant revealed that plasma LDL-C levels were lower in those carriers compared to FH patients who were heterozygous carriers of a deleterious *LDLR* variant. Since *ABCG5* and *ABCG8* carriers can respond very well to LLT, especially ezetimibe treatment[21] and back calculation of LDL-C is a rough estimate, we also analyzed the lipid profiles of these carriers after exclusion of patients on lipid lowering therapies (Supplementary Table 5 and Supplementary Figure 1). The results confirm the earlier findings: heterozygous carriers of *ABCG5* or *ABCG8* have lower LDL-C levels compared to true FH patients. Thus, if variants in *ABCG5* or *ABCG8* causally contribute to elevated plasma LDL-C, their effect is associated with a less

severe phenotype than the effect of a *LDLR* mutation. The elevated plasma LDL-C levels might as well have been caused by genetic variations in other genes, by high plasma Lp(a) levels or have a polygenic or secondary cause.[22–24]

We also investigated the effect of an additional *ABCG5* or *ABCGG8* variant on top of a deleterious *LDLR* mutation. In contrast to Tada *et al.,* who showed that "accessory" variants in genes with a (potential) effect on LDL-C (e.g., *ABCG5* and *ABCG8*) in addition to a mutation in one of the three established FH genes have an effect on plasma LDL-C levels[25], we found that heterozygosity for mutations in *ABCG5* or *ABCG8* did not contribute to a significant effect on LDL-C on top of a *LDLR* mutation (p=0.259). However, this might be due to the low number of subjects and variance in LDL-C levels in this group.

To investigate whether heterozygous *ABCG5/G8* variants cause elevated LDL-C plasma levels, we studied three families from FH index patients with an *ABCG5* or *ABCG8* variant, but without a mutation in *LDLR*, *APOB*, or *PCSK9*. As is clear from the pedigrees shown in Figure 4, it is unlikely that the investigated variants of *ABCG8* (p.Arg121* and p.Trp361*) can solely explain the FH phenotype since no full co-segregation was found in these families. The p.Arg121* and p.Trp361* variants of *ABCG8* are both predicted to be pathogenic since they code for a part of the cytosolic region of the protein and result in proteins without the membrane spanning part. Interestingly, these variant were previously described in various sitosterolemic patients[26–28] and the latter variant (p.Trp361*) was also observed to cause sitosterolemia in a heterozygous carrier. We, also, detected higher plasma sterol levels in the family with the p.Trp361* variant but not in the family with the p.Arg121*variant (Supplementary Table 6). At the same time, plant sterols were increased by 2-fold at most, which is modest compared to the at least 50-fold increases normally seen in patients with sitosterolemia due to a (compound) homozygosity.[8]

In one family (Family A) we studied the co-segregation of the p.Arg619Thr variant in *ABCG5* that has never been associated with sitosterolemia or FH. The family consisted of only two subjects suffering from FH and both of them had the *ABCG5* variant. However, this family could not be expanded any further and the small size

of the family did not allow us to establish a definite association with autosomal dominant FH.

Although the presence of *ABCG5* and *ABCG8* variants in our FH cohort is not surprising since *ABCG8* is implicated to be involved in polygenic FH[24], it is still rather unknown how *ABCG5* or *ABCG8* heterozygous variants affect plasma LDL-C levels. Previous studies showed that *ABCG5* and *ABCG8* variants can attenuate plasma LDL-C, possibly by reducing the hepatic cholesterol efflux into bile.[10–13] It is possible that variants in *ABCG5* or *ABCG8* only lead to a hypercholesterolemic phenotype in the presence of a second stimulus, for example a specific diet characterized by high sterols levels or other factors that might affect cholesterol clearance from the body through the liver or via trans-intestinal cholesterol excretion[29], ultimately leading to hypercholesterolemia. The relation between diet and *ABCG5* variants is interesting, since an infant with hypercholesterolemia who had two nonsense mutations in *ABCG5* only showed sitosterolemia after weaning.[30]

Our study has several limitations. The subjects and data on their clinical characteristics (including lipid profiles) of our FH cohort are provided by referring physicians. Therefore, we cannot exclude the possibility that some of the included subjects are family members of each other, since two physicians could send in DNA from two family members around the same time. Additionally, we did not have all data (plasma lipid concentrations, the use of LLT, CVD history) available of all 3031 subjects and could not use the cohort's full potential. Secondly, the clinical diagnosis of FH is established by the refereeing physicians and this was probably not uniformly done, resulting a low detection rate of true FH patients. Thirdly, we observed that some variants in *ABCG5* and *ABCG8* were present in only 1 to 3 patients, making a statistical frequency comparison between our cohort and gnomAD impossible. Lastly, limited by various factors, we have investigated the co-segregation of only three *ABCG5* and *ABCG8* variants in three relatively small families. We might have accidentally selected three variants that were predicted to be pathogenic *in silico* but are not *in vivo*, at least not when expressed in a heterozygous fashion. This is however unlikely for the two *ABCG8* variants as they are predicted to induce a premature stop codon, resulting in the synthesis of only a fragment of the cytosolic part of the *ABCG8* protein.

In conclusion, in a large number of patients being screened for FH, (likely) pathogenic heterozygous variants at the *ABCG5* and *ABCG8* loci were found in approximately 2.4% of them. Carriers of these variants had lower LDL-C levels compared to FH patients with a *LDLR* mutation. Based on these results, and the lack of co-segregation of these variants with the hypercholesterolemic phenotype in two families, we conclude that these genes can (partly) explain the FH phenotype in some individuals, but that they might not cause autosomal dominant hypercholesterolemic inheritance patterns in FH families. Further research is needed to unravel the complicated relation between *ABCG5* and *ABCG8* genotype and hypercholesterolemia.

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CONFLICTS OF INTERESTS

Hovingh has served as consultant and speaker for biotech and pharmaceutical companies that develop molecules that influence lipoprotein metabolism, including Regeneron, Pfizer, MSD, Sanofi and Amgen. Until april 2019 Hovingh has served as PI for clinical trials conducted with a.o. Amgen, Sanofi, Eli Lilly, Novartis, Kowa, Genzyme, Cerenis, Pfizer, Dezima, Astra Zeneca.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1: Genes on next-generation sequencing panel

LDLR	*SCARB1*
APOB	*CETP*
PCSK9	*LIPG*
LIPA	*LIPC*
LDLRAP1	*APOC3*
ABCG5	*LPL*
ABCG8	*APOC2*
STAP1	*APOA5*
ANGPTL3	*GPIHBP1*
MTTP	*LMF1*
MYLIP	*APOE*
ABCA1	*SAR1B*
LCAT	*CYP27A1*
APOA1	*CYP7A1*

Supplementary Table 2: Primers used for genotyping of *ABCG5* and *ABCG8* variant carriers

ABCG5 Arg619Thr
F- AGCGCTTGGTAAATACTTGTTGA
R- CGGCAGCTTCACTTCCATTT

ABCG8 Arg121*
F- CTCCGCTCTCAGGGATATCC
R- GTTGTTCAAACTCGACCTGC

ABCG8 Trp361*
F- AGGGGTGATCAGCATTGTGA
R- GTTGGTGTCTAGTGGGGTCA

Supplementary Table 3: Characteristics of FH patients

Total n=	3031
No. Of females (%)	1732 (57.1)
Age (mean (SD))	51.2 (14.4)
BMI (mean (SD))	26.6 (4.4)
Total cholesterol (mean (SD))	8.1 (1.6)
LDL cholesterol (mean (SD))	5.8 (1.4)
HDL cholesterol (mean (SD))	1.4 (0.4)
Triglycerides (median [IQR])	1.8 [1.3, 2.6]
LLT at time of lipid measurements (%)	
Yes	425 (14.2)
No	2140 (71.2)
Unknown	445 (14.7)

Supplementary Table 3: (Continued)

Father with CVD (%)	
Yes	1505 (49.7)
No	800 (26.4)
Unknown	726 (24)
Mother with CVD (%)	
Yes	1051 (34.7)
No	1268 (41.8)
Unknown	712 (23.5)
History of MI (%)	
Yes	349 (11.5)
No	2374 (78.3)
Unknown	308 (10.2)
History of AP(%)	
Yes	303 (10.0)
No	2384 (78.7)
Unknown	344 (11.4)
History of PCI (%)	
Yes	323 (10.7)
No	2359 (77.8)
Unknown	349 (11.5)
History of CABG(%)	
Yes	129 (4.3)
No	2552 (84.2)
Unknown	350 (11.5)
History of PAD (%)	
Yes	115 (3.8)
No	2545 (84.0)
Unknown	371 (12.2)
History of CVA (%)	
Yes	154 (5.1)
No	2512 (82.9)
Unknown	365 (12.0)
Any CVD history (%)	
Yes	758 (25.0)
No/unknown	2273 (75.0)

Supplementary Table 4: Variants of unknown significance (VOUS) and (likely) benign *ABCG5* and *ABCG8* variants in FH patients

Gene	Variant	Aminoacid change	In silico prediction	Allele frequency FH patients	Allele frequency gnomAD European (non-finish) population	Frequency highest in FH or gnomAD
ABCG5	c.1810C>G	p.Gln604Glu	Benign	0.159518	0.163487	gnomAD
	c.148C>T	p.Arg50Cys	Benign	0.046684	0.063636	gnomAD
	c.1864A>G	p.Met622Val	Benign	0.009238	0.006937	FH
	c.80G>C	p.Gly27Ala	Benign	0.006598	0.005532	FH
	c.1567A>G	p.Ile523Val	Benign	0.003629	0.002161	FH
	c.696C>T	p.=	Benign	0.002804	0.002129	FH
	c.1870T>C	p.Phe624Leu	Benign	0.001320	0.000879	FH
	c.293C>G	p.Ala98Gly	Benign	0.001320	0.002223	gnomAD
	c.593G>A	p.Arg198Gln	VOUS	0.001320	0.002248	gnomAD
	c.785A>G	p.Lys262Arg	Benign	0.001320	0.000155	FH
	c.27C>T	p.=	Benign	0.000825	0.000070	FH
	c.719G>A	p.Arg240His	Benign	0.000660	0.000039	FH
	c.775-18C>T	NA	Likely benign	0.000495	0.000562	gnomAD
	c.827C>T	p.Thr276Met	VOUS	0.000495	0.000015	FH
	c.897C>T	p.=	Benign	0.000495	0.000333	FH
	c.1228A>C	p.Asn410His	Likely benign	0.000330	0.000031	FH
	c.1550C>G	p.Thr517Ser	Benign	0.000330	0.000085	FH
	c.1570G>A	p.Val524Ile	Benign	0.000330	0.000046	FH
	c.1763-7T>G	NA	Benign	0.000330	0.000016	FH
	c.1806C>T	p.=	Benign	0.000330	0.000250	FH
	c.775-10G>T	NA	Likely benign	0.000330	0.000039	FH
	c.1153G>A	p.Ala385Thr	VOUS	0.000165	0.000009	FH
	c.1218G>A	p.=	Benign	0.000165	0.000009	FH
	c.1251G>A	p.=	Benign	0.000165	0.000279	gnomAD
	c.1284C>T	p.=	Likely benign	0.000165	0.000085	FH
	c.1358G>C	p.Ser453Thr	VOUS	0.000165	0.000009	FH
	c.1398C>T	p.=	Benign	0.000165	0.000106	FH
	c.139G>T	p.Val47Phe	VOUS	0.000165	0.000052	FH
	c.1410C>T	p.=	Likely benign	0.000165	0.000116	FH
	c.1528C>A	p.His510Asn	Likely benign	0.000165	0.000031	FH
	c.1744G>A	p.Gly582Arg	VOUS	0.000165	0.000016	FH
	c.238C>A	p.Gln80Lys	VOUS	0.000165	0.000149	FH
	c.265+10G>A	NA	Benign	0.000165	0.000039	FH
	c.78G>T	p.Glu26Asp	Likely benign	0.000165	0.000112	FH
	c.804C>T	p.=	Likely benign	0.000165	0.000018	FH

Supplementary Table 4: (Continued)

Gene	Variant	Aminoacid change	In silico prediction	Allele frequency FH patients	Allele frequency gnomAD European (non-finish) population	Frequency highest in FH or gnomAD
	c.810G>C	p.Glu270Asp	VOUS	0.000165	0.000009	FH
	c.915G>A	p.=	Likely benign	0.000165	0.000015	FH
	c.774+215delA	NA	Benign	0.331244		
	c.1463+319C>G	NA	Benign	0.284065		
	c.*380T>G	NA	Benign	0.184098		
	c.501+16_501+17insC	NA	Benign	0.000825		
	c.1118+8G>A	NA	Likely benign	0.000165		
	c.1180C>A	p.Leu394Met	VOUS	0.000165		
	c.1292C>T	p.Pro431Leu	VOUS	0.000165		
	c.1324+8C>T	NA	Benign	0.000165		
	c.219C>G	p.=	Benign	0.000165		
	c.33G>T	p.=	Likely benign	0.000165		
	c.37A>G	p.Met13Val	Likely benign	0.000165		
	c.388T>C	p.Ser130Pro	VOUS	0.000165		
	c.501+11C>A	NA	Likely benign	0.000165		
	c.501+16delC	NA	Likely benign	0.000165		
	c.775-18C>G	NA	VOUS	0.000165		
	c.900C>G	p.Phe300Leu	VOUS	0.000165		
ABCG8	c.1895T>C	p.Val632Ala	Benign	0.750247	0.780218	gnomAD
	c.1412-8C>T	NA	Benign	0.591389	0.593099	gnomAD
	c.64-7C>T	NA	Benign	0.395744	0.384383	FH
	c.161A>G	p.Tyr54Cys	Benign	0.392775	0.384308	FH
	c.64-21C>A	NA	Likely benign	0.392115	0.382646	FH
	c.-19T>G	NA	Benign	0.333388	0.318282	FH
	c.1695C>T	p.=	Benign	0.156384	0.168933	gnomAD
	c.1199C>A	p.Thr400Lys	Benign	0.152590	0.190133	gnomAD
	c.1412-9_1412-8insT	NA	Benign	0.142032	0.148413	gnomAD
	c.675G>A	p.=	Benign	0.056747	0.048672	FH
	c.55G>C	p.Asp19His	Benign	0.048499	0.065083	gnomAD
	c.453G>A	p.=	Benign	0.045200	0.061406	gnomAD
	c.165+13C>T	NA	Benign	0.012702	0.011410	FH
	c.1506G>A	p.=	Benign	0.004949	0.005085	gnomAD
	c.1941C>G	p.=	Benign	0.002474	0.001433	FH
	c.1201A>T	p.Thr401Ser	VOUS	0.002309	0.002278	FH
	c.-15A>C	NA	Benign	0.001485	0.000253	FH
	c.239G>A	p.Cys80Tyr	Benign	0.001320	0.000201	FH

Supplementary Table 4: (Continued)

Gene	Variant	Aminoacid change	In silico prediction	Allele frequency FH patients	Allele frequency gnomAD European (non-finish) population	Frequency highest in FH or gnomAD
	c.1160C>T	p.Pro387Leu	Likely benign	0.001155	0.000714	FH
	c.1436A>G	p.Tyr479Cys	VOUS	0.000825	0.000550	FH
	c.1785C>T	p.=	Benign	0.000825	0.001974	gnomAD
	c.628G>A	p.Val210Met	Likely benign	0.000825	0.000140	FH
	c.1266G>A	p.=	Benign	0.000660	0.000018	FH
	c.1839T>C	p.=	Benign	0.000660	0.000550	FH
	c.1386C>T	p.=	Benign	0.000495	0.000349	FH
	c.1756+15C>T	NA	Benign	0.000495	0.000127	FH
	c.1924G>A	p.Ala642Thr	Benign	0.000495	0.001262	gnomAD
	c.1365C>T	p.=	Benign	0.000330	0.000101	FH
	c.1922A>T	p.Tyr641Phe	VOUS	0.000330	0.000472	gnomAD
	c.1963A>G	p.Met655Val	Benign	0.000330	0.000070	FH
	c.561+14C>T	NA	Likely benign	0.000330	0.000827	gnomAD
	c.576C>T	p.=	Benign	0.000330	0.000218	FH
	c.94A>G	p.Ser32Gly	Likely benign	0.000330	0.000062	FH
	c.1036G>A	p.Ala346Thr	VOUS	0.000165	0.000016	FH
	c.1093A>G	p.Thr365Ala	VOUS	0.000165	0.000008	FH
	c.1094C>T	p.Thr365Met	VOUS	0.000165	0.000008	FH
	c.1117T>C	p.Cys373Arg	Likely benign	0.000165	0.000009	FH
	c.1127+14C>T	NA	VOUS	0.000165	0.000035	FH
	c.1170G>A	p.=	Benign	0.000165	0.000008	FH
	c.1186G>C	p.Val396Leu	Likely benign	0.000165	0.000018	FH
	c.1210C>T	p.Arg404Cys	VOUS	0.000165	0.000009	FH
	c.1211+7A>T	NA	Likely benign	0.000165	0.000036	FH
	c.1285A>G	p.Met429Val	Benign	0.000165	0.000139	FH
	c.1488+9G>A	NA	Benign	0.000165	0.000018	FH
	c.1717G>A	p.Ala573Thr	VOUS	0.000165	0.000016	FH
	c.1782C>T	p.=	Benign	0.000165	0.000031	FH
	c.1837T>C	p.Tyr613His	Likely benign	0.000165	0.000054	FH
	c.29G>C	p.Gly10Ala	Likely benign	0.000165	0.000017	FH
	c.342G>A	p.=	Benign	0.000165	0.000242	gnomAD
	c.369C>T	p.=	Benign	0.000165	0.000070	FH
	c.381C>T	p.=	Likely benign	0.000165	0.000079	FH
	c.451G>A	p.Val151Met	VOUS	0.000165	0.000044	FH
	c.51C>A	p.=	Benign	0.000165	0.000040	FH
	c.611G>A	p.Arg204His	VOUS	0.000165	0.000217	gnomAD

Supplementary Table 4: (Continued)

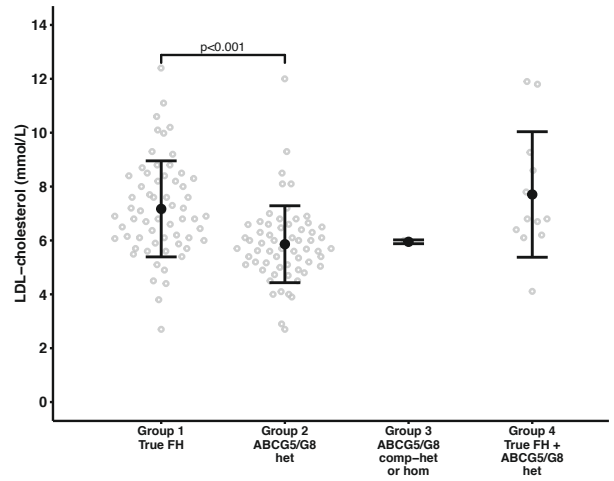
Gene	Variant	Aminoacid change	In silico prediction	Allele frequency FH patients	Allele frequency gnomAD European (non-finish) population	Frequency highest in FH or gnomAD
	c.641C>T	p.Ser214Leu	VOUS	0.000165	0.000035	FH
	c.76A>G	p.Arg26Gly	Likely benign	0.000165	0.000026	FH
	c.887C>T	p.Ala296Val	Likely benign	0.000165	0.000039	FH
	c.1411+90delA	NA	Benign	0.798416		
	c.1412-152A>G	NA	Benign	0.733256		
	c.1488+27_1488+28insCCC	NA	Likely benign	0.209667		
	c.322+431T>C	NA	Benign	0.143847		
	c.1757-499A>G	NA	Likely benign	0.116628		
	c.1757-545A>G	NA	Benign	0.072748		
	c.1756+465T>C	NA	Benign	0.051798		
	c.64G>A	p.Gly22Ser	Benign	0.000825		
	c.695-17_695-16delCT	NA	Benign	0.000660		
	c.1763C>A	p.Ala588Glu	VOUS	0.000495		
	c.1568C>T	p.Pro523Leu	VOUS	0.000330		
	c.734G>C	p.Ser245Thr	VOUS	0.000330		
	c.1200G>A	p.=	Benign	0.000165		
	c.1211+8_1211+9delTC	NA	Likely benign	0.000165		
	c.1226A>G	p.Asn409Ser	VOUS	0.000165		
	c.1402A>G	p.Ile468Val	Benign	0.000165		
	c.1619T>C	p.Phe540Ser	VOUS	0.000165		
	c.1689C>G	p.Ser563Arg	VOUS	0.000165		
	c.169G>C	p.Asp57His	VOUS	0.000165		
	c.1700A>C	p.Tyr567Ser	VOUS	0.000165		
	c.1718C>T	p.Ala573Val	VOUS	0.000165		
	c.171C>A	p.Asp57Glu	VOUS	0.000165		
	c.2011C>G	p.Gln671Glu	VOUS	0.000165		
	c.379A>G	p.Ile127Val	Benign	0.000165		
	c.406G>T	p.Gly136Trp	VOUS	0.000165		
	c.51_63dupCCAGGATACCTCG	p.Gly22Profs*9	VOUS	0.000165		
	c.561+19T>C	NA	Likely benign	0.000165		
	c.928C>G	p.Pro310Ala	VOUS	0.000165		

VOUS, Variant of uncertain significance

Supplementary Table 5: Characteristics and lipid profile of *ABCG5* and *ABCG8* variant carriers not on lipid lowering therapies compared to true FH patients

	Group 1 True FH patients (*LDLR* mutation)	Group 2 Heterozygous *ABCG5* or *ABCG8* variant carrier		Group 3 Compound heterozygote or homozygote for *ABCG5* and/or *ABCG8* variants		Group 4 True FH (*LDLR* mutation) + heterozygote for *ABCG5* or *ABCG8* variant	
	n = 62	n = 62	P-value*	n = 2	P-value*	n = 12	P-value*
Age, years	52.5 (13.8)	53.4 (14.4)	0.741	48.2 (8.2)	0.66	41.8 (20.5)	0.026
No. of males	30 (48.4)	30 (48.4)	1	2 (100.0)	0.472	8 (66.7)	0.399
Total cholesterol, mmol/L	9.1 (2.0)	8.1 (1.6)	0.006	8.1 (0.6)	0.501	9.9 (2.5)	0.181
LDL-C, mmol/L	7.0 (1.9)	5.9 (1.4)	<0.001	6.0 (0.1)	0.432	7.7 (2.3)	0.263
HDL-C, mmol/L	1.3 (0.4)	1.4 (0.6)	0.344	1.2 (0.1)	0.62	1.5 (0.4)	0.162
Triglycerides, mmol/L	1.5 [0.9, 2.2]	1.8 [1.1, 2.5]	0.112	2.4 [2.3, 2.6]	0.159	1.2 [0.8, 1.8]	0.422

FH1, Familial Hypercholesterolemia type 1; LDL-C, Low density lipoprotein cholesterol; HDL-C, High density lipoprotein cholesterol. *Compared with Group 1 (True FH patients), which was age and sex matched with Group 2. P-value for the statistical difference between a group and the reference group is determined with an independent t-test for normally distributed data and a Mann-Witney U Test for non-normally distributed data (TG), and a Fisher Exact test for categorical data. All values are expressed as mean (standard deviation), except for No. of males, No. of statin users, and No. of ezetimibe users which are expressed as number (%), and TG which is expressed as median (interquartile range).



Supplementary Figure 1: LDL-cholesterol levels of *ABCG5* and *ABCG8* variant carriers not on lipid lowering therapies compared to true heterozygous FH patients.
LDL-cholesterol levels are significantly lower in *ABCG5* and *ABCG8* heterozygote carriers (Group 2) compared to true FH patients (Group 1; carrying a *LDLR* mutation). FH: familial hypercholesterolemia, het: heterozygote, hom: homozygote.

Supplementary table 6: Clinical characteristics, lipid and sterol data per family

	Family A *ABCG5*; c.1856G>C, p.Arg619Thr		**Family B** *ABCG8*; c.361C>T, p.Arg121*			**Family C** *ABCG8*; c.1083G>a, p.Trp361*		
	Carriers (n=1)	**Non-carriers** (n=4)	**Carriers** (n=4)	**Non-carriers** (n=6)	**P-value**	**Carriers** (n=4)	**Non-carriers** (n=6)	**P-value**
Age, years	68.4	43.8 (22.7)	26.7 (15.7)	45.9 (22.3)	0.075	48.9 (26.0)	41.6 (24.9)	0.616
Males	0	3 (75.0)	2 (50.0)	2.0 (33.3)	0.600	1 (25.0)	3 (50.0)	0.437
TC, mmol/L	5.9	5.0 (0.4)	5.1 (1.7)	5.4 (1.1)	0.688	5.4 (1.4)	4.8 (0.6)	0.371
LDL-C, mmol/L	3.8	3.3 (0.4)	3.2 (1.5)	3.4 (0.7)	0.718	3.5 (0.7)	3.2 (0.4)	0.343
corrected LDL-C, mmol/L	6.0	3.3 (0.4)	3.2 (1.5)	3.8 (0.8)	0.416	4.2 (0.7)	3.4 (0.5)	0.037
HDL-C, mmol/L	1.5	1.1 (0.1)	1.4 (0.3)	1.2 (0.3)	0.412	2.0 (0.4)	1.5 (0.4)	0.015
TG, mmol/L	1.4	1.3 [1.1, 1.6]	1.6 [1.3, 1.7]	1.5 [1.3, 1.8]	0.754	0.9 [0.7, 1.0]	0.8 [0.7, 1.2]	0.509
Statin users	1	0	0	1 (16.7)	-	2.0 (50.0)	1 (16.7)	0.278
Cholesterol, µmol/L	5900.0	4965.3 (415.8)	4836.0 (1755.0)	5151.0 (1033.4)	0.712	4792.0 (30.2)	4486.7 (511.0)	0.110
Cholestanol, µmol/L	9.9	7.6 (1.5)	9.6 (1.9)	9.3 (1.1)	0.815	11.2 (1.5)	7.9 (1.8)	<0.001
Desmosterol, µmol/L	8.1	7.7 (0.9)	6.0 (1.9)	6.3 (0.5)	0.713	6.3 (0.9)	6.0 (1.0)	0.561
7-Lathosterol, µmol/L	6.9	8.7 (3.4)	6.6 (4.1)	6.6 (2.9)	1.000	3.0 (0.9)	6.6 (1.8)	<0.001
Campesterol, µmol/L	14.2	11.9 (4.9)	17.7 (8.8)	15.1 (2.4)	0.500	31.7 (5.4)	12.8 (3.4)	<0.001
Sitosterol, µmol/L	8.2	5.7 (2.1)	11.3 (6.9)	9.1 (2.0)	0.468	27.4 (11.5)	9.0 (2.5)	0.001

TC, Total cholesterol; LDL-C, Low density lipoprotein cholesterol; HDL-C, High density lipoprotein cholesterol; TG, Triglycerides; SD, standard deviation; IQR, interquartile range. All values are expressed as mean (standard deviation), except for No. of females and No. of statin users which are expressed as number (%), and TG which is expressed as median (interquartile range). Statistical difference between carriers and non-carriers was determined with an independent T-test for normally distributed data, Mann-Witney U Test for non-normally distributed data, and Chi squared test for categorical data. Corrected LDL-C levels were calculated by multiplying the LDL-C levels with previously reported correction factors for statin and/or ezetimibe use.[20]

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Taking One Step Back in Familial Hypercholesterolemia: *STAP1* does not alter plasma LDL cholesterol in mice and humans

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ABSTRACT

Objective: *STAP1*, encoding for STAP1 (signal transducing adaptor family member 1), has been reported as a candidate gene associated with familial hypercholesterolemia. Unlike established familial hypercholesterolemia genes, expression of *STAP1* is absent in liver but mainly observed in immune cells. In this study, we set out to validate *STAP1* as a familial hypercholesterolemia gene.

Approach and results: A whole-body Stap1 knockout mouse model (*Stap1*$^{-/-}$) was generated and characterized, without showing changes in plasma lipid levels compared with 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 or atherosclerotic lesions. To functionally assess whether *STAP1* expression in B cells can affect hepatic function, HepG2 cells were cocultured with peripheral blood mononuclear cells isolated from heterozygotes carriers of *STAP1* variants and controls. The peripheral blood mononuclear cells from *STAP1* variant carriers and controls showed similar *LDLR* mRNA and protein levels. Also, LDL (low-density lipoprotein) uptake by HepG2 cells did not differ upon coculturing with peripheral blood mononuclear cells 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 (high-density lipoprotein) cholesterol, triglycerides, and lipoprotein(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 the UK Biobank do not show association between *STAP1* rare gene variants and LDL cholesterol.

Conclusions: Our combined studies in mouse models and carriers of *STAP1* variants indicate that *STAP1* is not a familial hypercholesterolemia gene.

INTRODUCTION

Familial hypercholesterolemia (FH) is a common genetic disorder characterized by lifelong elevated levels of LDL (low-density lipoprotein) cholesterol (LDL-c) and increased risk for premature atherosclerotic cardiovascular disease. In ~30% of patients with extreme LDL-c (LDL >4.9 according to DLCN [Dutch Lipid Clinic Network] score), a genetic cause can be found,[1–3] with 95% accounted for mutations in the genes encoding the *LDLR* (LDL receptor), *APOB* (apolipoprotein B), and *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 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 1 Spanish FH patient who experienced an acute myocardial infarction.[21] A p.Thr47Ala variant was furthermore found in 2 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 (BM)[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 VLDL (very-low-density lipoprotein) 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 2 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 readdress the association of *STAP1* gene variants with plasma lipid and lipoproteins in 4 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 on 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 *Stap1*$^{-/-}$ were generated using CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/clustered regularly interspaced short palindromic repeat–associated 9) technology as described previously[28] (technical details provided in Materials and Methods in the 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 of fasting in the morning, before the start of the high-fat–high-cholesterol diet and after 2 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 nitro- gen and stored at −80°C. The processing and analysis of mouse tissues was performed as indicated below.

BM Transplantation and diet-induced atherosclerosis

Stap1$^{-/-}$ mice were backcrossed to C57BL/6J mice for 8 generations. BM transplantations were performed as described elsewhere.[29] In brief, 5×10^6 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 Materials and Methods in the Data Supplement). After a recovery period of 5 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-hour fasted mice before the initiation of the WTD and after 8 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 Materials and Methods in the 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, the Netherlands). The hearts were dehydrated and embedded in paraffin and cut into 4-µm cross sections throughout the aortic root area. Hematoxylin-eosin staining was performed on the sections, and the average from 6 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).

Protein analyses by targeted quantitative proteomics

Tissue homogenates were prepared at 10% w/v in NP-40 buffer supplemented with Roche cOmplete Protease Inhibitor Cocktail 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 (13C-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 mmol/L dithiothreitol and alkylation with 55 mmol/L iodoacetamide, followed by solid-phase extraction (SPE C18-Aq 50 mg/1 mL, Gracepure; ThermoFisher Scientific) for sample cleanup.

Liquid chromatography on a nano-ultra high-performance liquid chromatography system (Ultimate UHPLC focused; Dionex, ThermoFisher 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; ThermoFisher Scientific), 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 ≤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 (TC) 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 stan- dard (Roche Molecular Biochemicals) as reference.

Fast-performance liquid chromatography in mice

As part of the initial characterization of the whole-body *Stap1*$^{-/-}$ 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). An extra PU-2080i Plus pump (Jasco) 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 performed with ChromNav chromatographic software, version 1.0 (Jasco). The plots for individual fast-performance liquid chromatography profiles were generated with R, version 3.6.1 (2019-07-05), and RStudio, using ggplot2_3.2.1, RColorBrewer_1.1-2, dplyr_0.8.3, and tidyr_0.8.3.

For the BM transplantation (BMT) study, fast-performance liquid chromatography profiles were obtained using pooled plasma samples (350 µL) from 12 animals of the corresponding genotype, collected before starting WTD diet and after 8 weeks. These fast-performance liquid chromatography profiles were run using 2

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 v235 and SIFT[34] (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) cholesterol, 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.[35] LDL-c concentrations in humans were corrected for the use of lipid-lowering drugs.[36,37]

Immunologic profiling in patients

White blood cell counts and blood cell types were determined using 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 944g for 20 minutes at RT with slow acceleration and no brake. The PBMC-containing interphases was

collected, washed 3× with cold PBS +2 mmol/L EDTA and centrifuged at 563g for 10 minutes at 4°C. Cells were counted and sample volume was adjusted with cold PBS +1% BSA to 1 million PBMCs per 100 µL. A proportion of the PBMCs was stored in TriPure Isolation Reagent (Roche Applied Sciences, Almere, the Netherlands) at −80°C for RNA isolation and gene expression analysis. Three million PBMCs were incubated for 30 minutes at 4°C protected from light with antibodies against CD3 (cluster of differentiation 3), CD19, CD24, CD27, IgD, and CD43 with or without an antibody against CD38 (see Major Resources in the Data Supplement for information about the antibodies). Subsequently, the PBMCs were washed twice with cold PBS +1% BSA and centrifuged at 281g 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.[38] (Figure VI in the Data Supplement). In short, non-B lymphocytes are CD19−, naive 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 American Type Culture Collection (Manassas, VA) and maintained in DMEM with 4.5 g/L glucose, GlutaMAX, and pyruvate (Gibco; Invitrogen, Breda, the Netherlands) supplemented with 10% fetal bovine serum (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 Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Leibniz, Germany) and maintained in RPMI 1640 with GlutaMAX and HEPES (Gibco) supplemented with 10% fetal bovine serum, 100 IU/ mL penicillin, and 100 µg/mL streptomycin.

Coculture experiments

For cocultures, 125000 HepG2 cells per well were plated in 24-well plates, allowed to proliferate for ~70 hours, 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, United Kingdom) according to the manufacturer's instructions. Quantitative RT-PCR was performed using SensiFAST SYBRgreen (Bioline) with a CFX384 Real-Time PCR System (Bio-Rad Laboratories, Hercules, CA). Sequences of the used primers are listed in Table IV in the Data Supplement. The expression of each gene was expressed in arbitrary units after normalization to the average expression level of the housekeeping genes *RN18S*, *HPRT1*, and *RPLP0* using the $2^{-\Delta\Delta Ct}$ method.[39]

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 (catalog 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) and analyzed using FlowJo (BD Life Sciences).

LDL uptake studies

LDL with a density of 1.019 to 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 1 hour according to the manufacturer's protocol and dialyzed against PBS overnight.[40] After 24 hours of coculture (HepG2 and PBMCs or HepG2 and B-cell precursor acute lymphoblastic leukemia cells), 4 µg DyLight apoB-labeled 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 13523g for 15 minutes at 4°C. The fluorescence at 488 nm in the supernatant was determined and compared with 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 t test for normally distributed data or a Mann-Whitney U test for not-normally distributed data was performed when 2 different groups were compared. When >2 groups were compared, Kruskal-Wallis test or 2-way ANOVA was performed with Tukey 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 (Figure IA and IB in the Data Supplement). Mouse line A has a deletion of 5 base pairs (Del5bp), and mouse line B carriers a 14-bp deletion (Del14bp). Both defects introduced premature stop codons as illustrated in Figure IC in the 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 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 Data Supplement).

Stap1$^{-/-}$ mice present no alterations in plasma lipid levels

Compared with 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 4 weeks on a high-fat–high-cholesterol diet (Figure 1C through 1F; similar data for line B in Figure IIA through IID in the Data Supplement). In addition, plasma lipoprotein profiles of *Stap1*$^{-/-}$ mice did not show significant differences compared with wild-type littermates (Figure 1E and 1F).

Irradiated female *Ldlr*$^{-/-}$ mice transplanted with BM of *Stap1*$^{-/-}$ donors do not show changes in plasma lipid levels or atherosclerosis compared with 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 that 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,[11] 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 BM 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 Data Supplement). As in the whole-body *Stap1*$^{-/-}$ mice, no differences in plasma cholesterol or triglyceride concentrations were observed on a standard laboratory diet or after 8 or 12 weeks of WTD (Figure 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 2G and 2H), 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 Data Supplement).

Stap1 depletion in BM causes minor changes in lymphocytes and monocytes in mice

As BMT 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 small increase in lymphocytes and B cells in the *Ldlr*$^{-/-}$BM*Stap*$^{-/-}$ mice compared with *Ldlr*$^{-/-}$BM*Stap*$^{+/+}$ (Figure VA through VC in the 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*Stap*$^{-/-}$ animals compared with controls (Figure VD in the Data Supplement). This difference appeared to specifically involve the Ly6Clow subpopulation (Figure VE and VF in the 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.

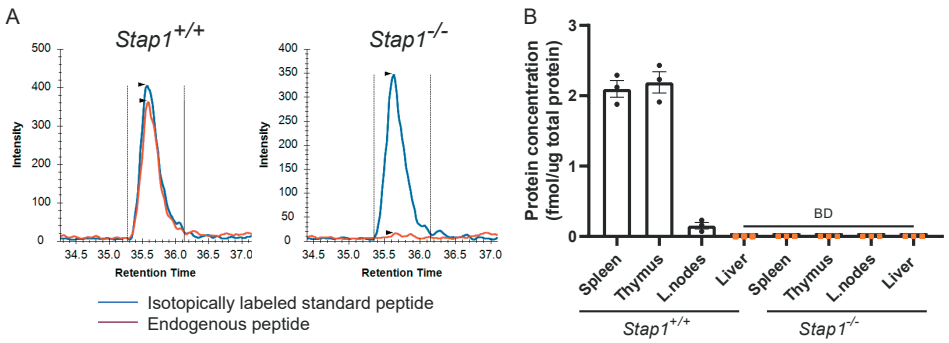

Figure 1: Characterization of whole-body *Stap1*$^{-/-}$ (mouse line A) on a standard laboratory diet and after 2 and 4 wk on high-fat–high-cholesterol diet (HFCD)
A, Quantification of STAP1 protein in spleen using a mass spectrometry–based targeted proteomics assay. The black peak indicates the stable (heavy) isotope-labeled standard, and the gray 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 and D, Total cholesterol plasma levels in male (C) and female (D) *Stap1*$^{+/+}$ and *Stap1*$^{-/-}$ mice on a standard laboratory diet and after 2 and 4 wk on HFCD. E and F, Triglyceride plasma levels for *Stap1*$^{+/+}$ and *Stap1*$^{-/-}$ male (E) and female (F) mice on a standard laboratory diet and after 2 and 4 wk on HFCD. C–F, Two-way ANOVA with Sidak multiple comparisons test; *P<0.05, **P<0.01; n=8 animals per genotype. G and H, Fast-performance liquid chromatography profiles for plasma cholesterol of individual mice for *Stap1*$^{+/+}$ and *Stap1*$^{-/-}$ males (G) and females (H) at termination after 4 wk on HFCD. The dark line indicates the mean, and the light shades indicate SEM; n=7 to 8 per genotype. Data shown as mean±SEM.

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 well as white blood cell counts did not differ between the groups. ɣGT (gamma-glutamyltransferase) 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 among these groups (Figure 3A through 3E). STAP1 mRNA expression appeared lower in PBMCs from carriers compared with controls, but this difference did not reach significance (Figure 3F).

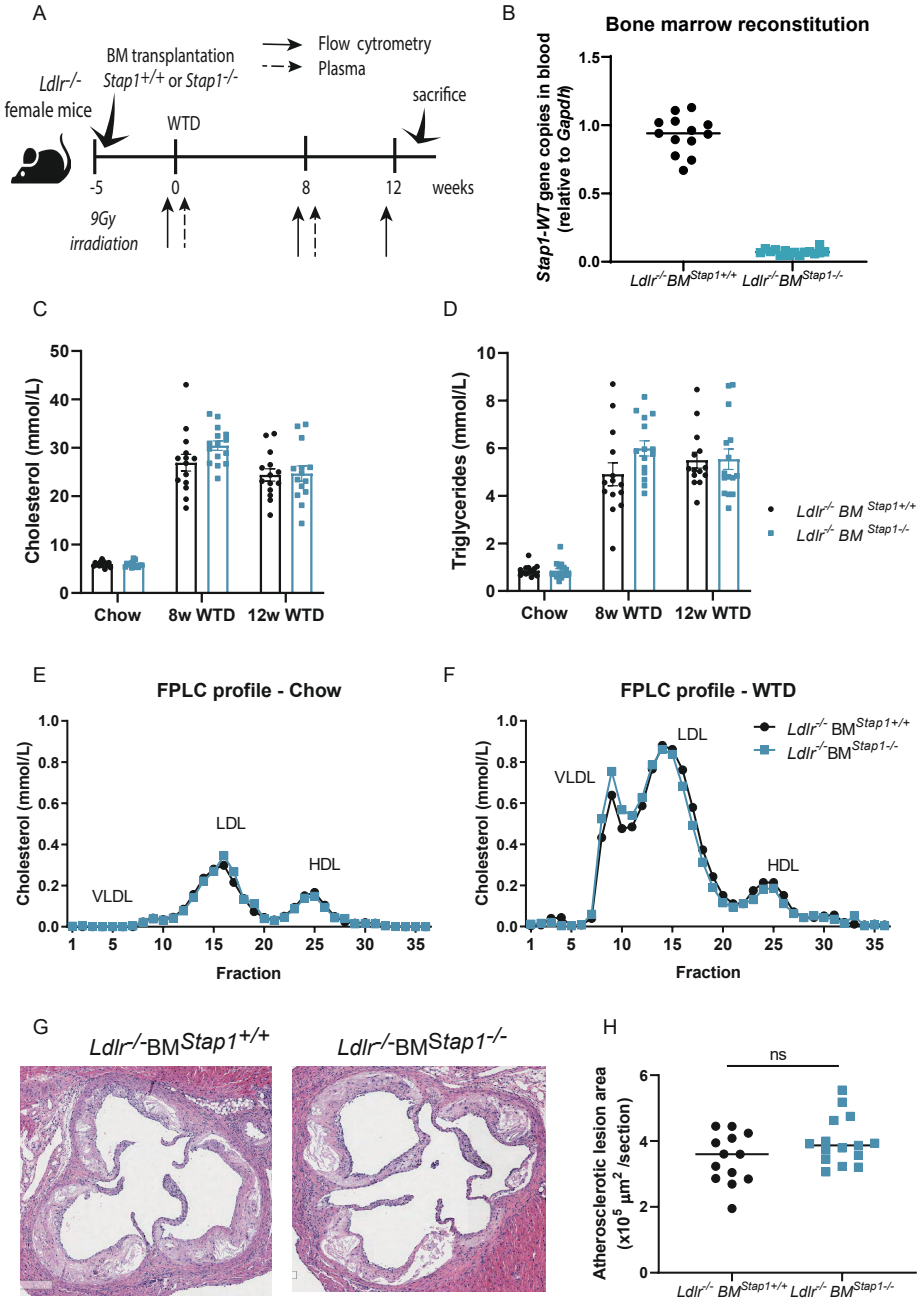

Figure 2: Bone marrow (BM) deficiency of *Stap1* in *Ldlr*$^{-/-}$ female mice does not induce changes in plasma lipids and does not affect the development of atherosclerosis plaques
A, Experimental design to evaluate BM Stap1 deficiency on lipid metabolism and atherosclerosis in *Ldlr*$^{-/-}$ mice. 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 BM transplantation assessed by qPCR. C, Plasma cholesterol and (D) triglyceride levels of *Ldlr*$^{-/-}$ transplanted with BM from *Stap1*$^{-/-}$ compared with those that received *Stap1*$^{+/+}$ BM. C and D, Two-way ANOVA with Sidak correction for multiple comparisons test; n=13 to 16 animals per genotype. E, Fast-performance liquid chromatography (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 wk on Western type diet (WTD). G, Representative example for hematoxylin-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*$^{+/+}$ (H; Student t test). Data shown as mean±SEM. HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; ns, nonsignificant; and VLDL, very-low-density lipoprotein.

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, y	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

LDL-c concentrations were calculated by the Friedewald formula.[35] Values are mean±SD or median with interquartile range (TG and Lp[a]). ɣGT indicates gamma-glutamyltransferase; AF, alkalic phosphatase; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; Lp(a), lipoprotein(a); TC, total cholesterol; and TG, triglycerides. *P<0.05.

Hematopoietic cells of carriers of *STAP1* gene 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 2 variants are predicted to negatively affect *STAP1* protein function, based on 2 predictive algoritms[34,41] (Table I in the 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* gene variant and controls (Figure 3G and 3H). Finally, HepG2 cells were cocultured with 2 different B-cell precursor leukemia cell lines Kasumi-2 and Nalm6, which have previously been reported to have low and high STAP1 mRNA expression, respectively.[42] We could confirm this (Figure VIIA in the Data Supplement) but did not observe significant changes in the expression of *APOB*, *LDLR*, *MTTP*, *PCSK9*, and *SREBP2* mRNA in HepG2 cells upon coculturing with these 2 cell lines (Figure VIIB in the Data Supplement). In line, there was no effect on cell surface LDLR protein or LDL uptake (Figure VIIC and VIID in the 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* For this, we compared lipid profiles in newly collected plasma of 39 carriers of *STAP1* gene variant carriers with those of 71 family controls. Levels of TC and LDL-c were not different between groups, which was also true for HDL cholesterol and triglyceride levels (Table 2). Also, when stratifying for the 3 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).

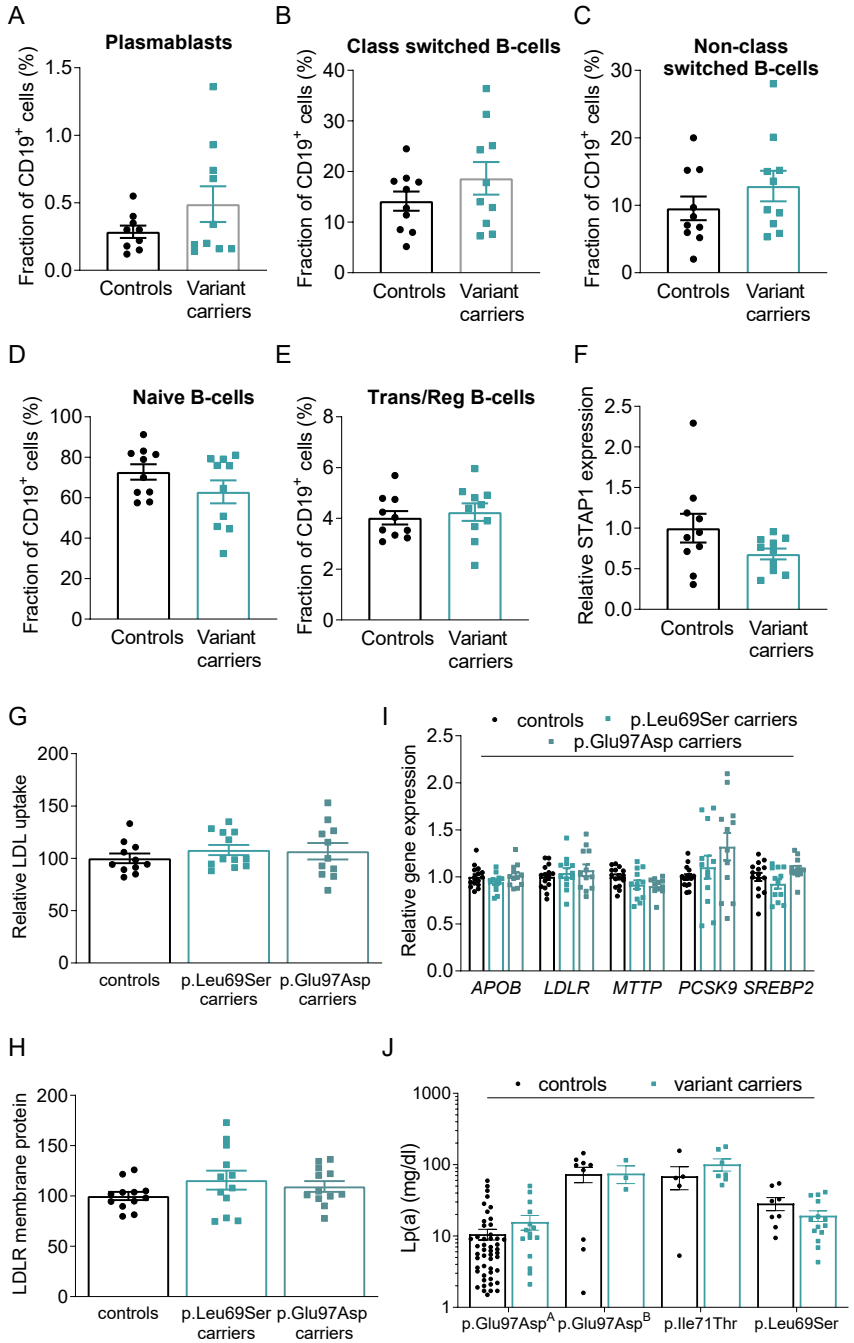

Figure 3: Characterization of blood-derived cells from 10 selected carriers of *STAP1* gene variants and age- and sex-matched family controls (Table 1)
A–E, Relative amount of different B-cell subtypes in *STAP1* variant carriers and controls: plasmablasts (A), class-switched B cells (B), non–class-switched B cells (C), naive B cells (D), and transitional (Trans) and regulatory (Reg) B cells (E), depicted as percentage of the total CD19+ (cluster of differentiation) cells. Data shown as mean±SEM, n=10 per group. F, Relative *STAP1* mRNA expression in peripheral blood mononuclear cells (PBMCs) from *STAP1* variant carriers and family controls, normalized to *RN18S*, *HPRT1*, and *RPLP0* with data from controls set to 1. Mann-Whitney U test was used in 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 (low-density lipoprotein) by the HepG2 cells after coculturing. Uptake is corrected for cellular protein content and data from HepG2 cells cocultured with control PBMCs set at 100% (n=12–15). H, Relative LDLR (LDL receptor) protein on the surface of the HepG2 cells after coculturing as determined by fluorescence-activated cell sorting analysis. Data are corrected for the amount of cells, and data from HepG2 cells cocultured with control PBMCs were set at 100% (n=12–15; Mann-Whitney U test was used in 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 PBMCs defined as 1 (n=12–15). J, Comparison of plasma lipoprotein(a) (Lp[a]) concentrations between *STAP1* variant carriers and their control family members in 4 different families (2 families in which a p.Glu97Asp variant was found, 1 family with a p.Ile71Thr variant, and 1 family with a p.Leu69Ser variant). Values shown as mean±SEM; 1-way ANOVA and Kruskal- Wallis test were used in G–J. *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, y	48.2±16.7	44.7±18.8	39.6±17.7	40.2±21.9	53.8±18.5
BMI, kg/m2	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,* 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)

LDL-c concentrations were calculated by the Friedewald formula.[35] BMI indicates body mass index; HDL, high-density lipoprotein; LDL-c, low-density lipoprotein cholesterol; Lp(a), lipoprotein(a); NA, not assessed; TC, total cholesterol; and TG, triglycerides. *Off-treatment LDL-c levels are calculated based on the 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 and studies with PBMCs of carriers of *STAP1* gene variants, we were unable to find a role for *STAP1* in controlling plasma LDL-c concentrations. 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 5 years following its identification in 2 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 (eg, 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 sup- port for *STAP1* as a lipid gene.[43,44] Finally, a recent study reported no association between lipid traits in carriers and non-carriers 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 >50000 UK Biobank participants, also aligned with our findings.[45] In this data set, 150 rare variants (minor allele frequency, <0.1%) affecting coding regions of *STAP1* were found in 37889 individuals. Carriers of these variants did not present with statistically significant changes in LDL-c values (summary statistics: β=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 UK Biobank data set and did not show association with LDL-c levels (Table II in the Data Supplement).

Retrospectively, LDL-c levels in carriers of *STAP1* gene variants in the original publication were only 11% higher compared with 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 used 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.[2,3,46–48] 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 1 case of nonpenetrance (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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DISCLOSURES

G.K. Hovingh has received lecturing fees and is on the advisory boards at Amgen, Sanofi-Aventis, Regeneron, Pfizer, and The Medicines Company. The other authors report no conflicts.

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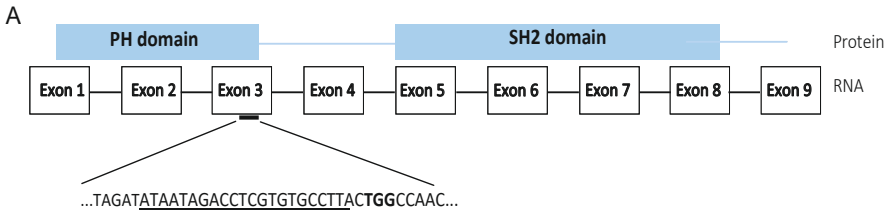
SUPPLEMENTAL MATERIALS

A



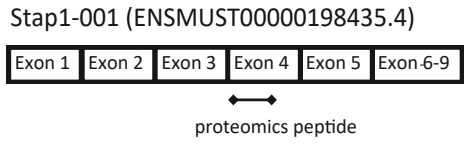
B

Stap1 Variant	Nucleotide sequence
WT	AGATATAATAGACCTCGTGTGCCTTACTGGCCAAC
Mutant line A	AGATATAATAGACCTCGTGTGC_ _ _ _ _ TGGCCAAC (Del5nt)
Mutant line B	AGATATAATAGACCTCGTG _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ AC (Del14nt)

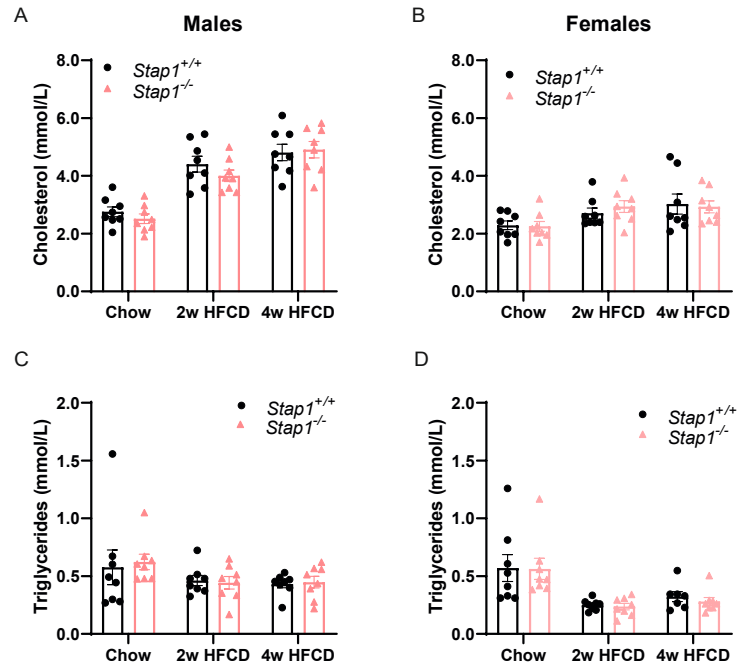
C

Stap1 Variant	Amino acid sequence
WT	MMAKKPPKPAPRRIFQERLKITALPLYFEGFLLVKRSDHQEYKHYTELRGTTLFFYTDK KSTIYVGKLDIIDLVCLTGQHSTEKNCAKFTLVLPKEEVHVKTENTESGEEWRGFILTVT ELTVPQHVSLLPGQVIRLHEVLEREKRRIETDQLPLMPPEKEKEPVQDYADVLNPLPEC FYAVS RKEATAMLEKNPSWGNMILRPGSDSKNYSITIRQEIEMPRIKHFKVTRTGNNY TIELEKPVTLPNLFSVIDYFVKETRGNLRPFIHSADDNFGQDPNIEDRSEKFKKNPHNA*
Mutant line A	MMAKKPPKPAPRRIFQERLKITALPLYFEGFLLVKRSDHQEYKHYTELRGTTLFFYTDK KSTIYVGKLDIIDLVC**WPTFN***
Mutant line B	MMAKKPPKPAPRRIFQERLKITALPLYFEGFLLVKRSDHQEYKHYTELRGTTLFFYTDK KSTIYVGKLDIIDLV**TFN***

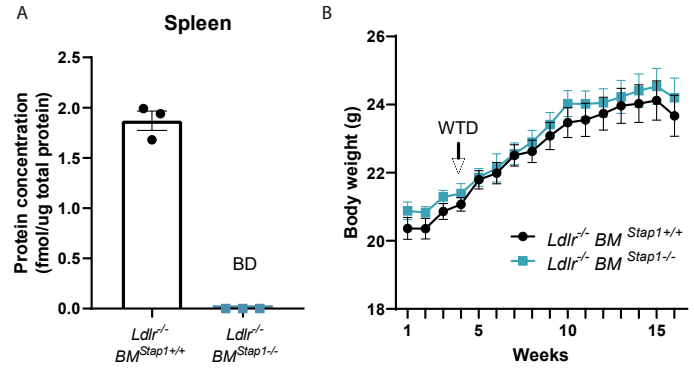
D



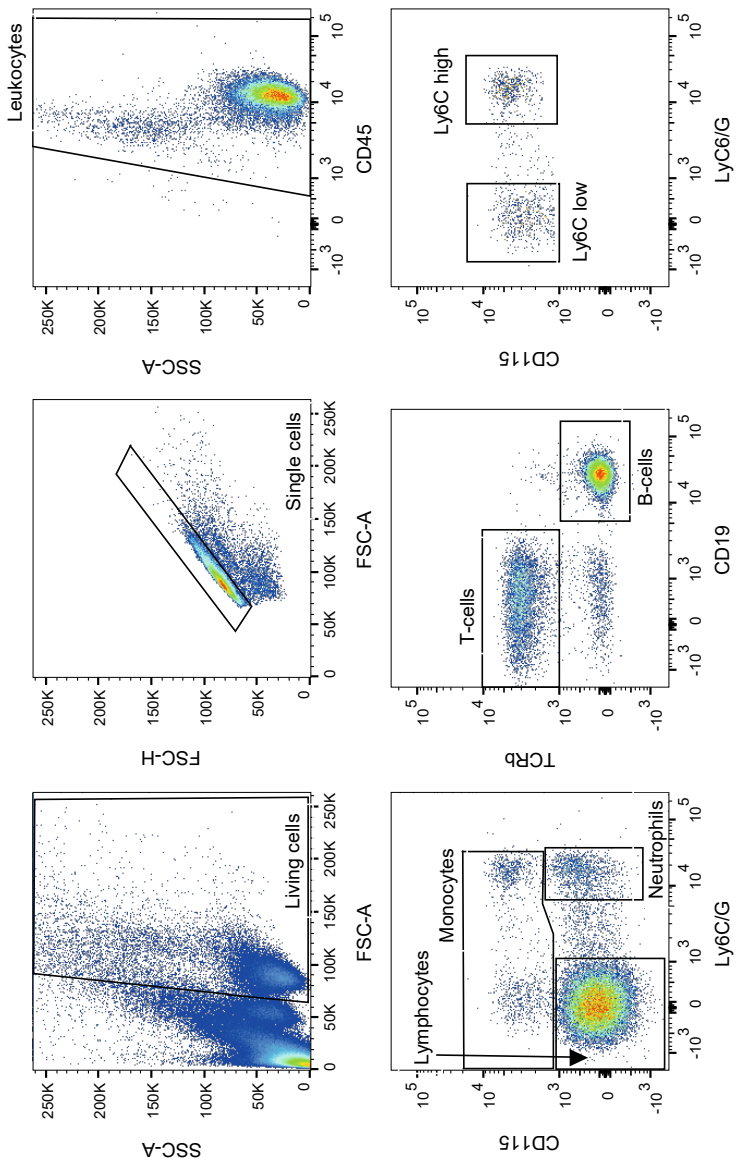
Supplemental Figure I: Generation of whole-body *Stap1*$^{-/-}$ mouse lines using CRISPR/Cas9
(A) Schematic structure for murine STAP1 protein and RNA. sgRNA were designed to target exon 3 of murine *Stap1*. The sgRNA sequence is underlined and the PAM sequence is shown in bold. (B) *Stap1* nucleotide sequence in wild-type mice and the mutated mouse lines. Mouse line A presents a deletion of 5 nucleotides (Del5bp) while line B has a deletion of 14 nucleotides (Del14bp). (C) Both mutant mouse lines present out-of-frame mutations, which result in a truncated STAP1 protein due to an early stop codon (*). (D) Localization of the peptide used to quantify STAP1 protein in mouse tissues by targeted proteomics.



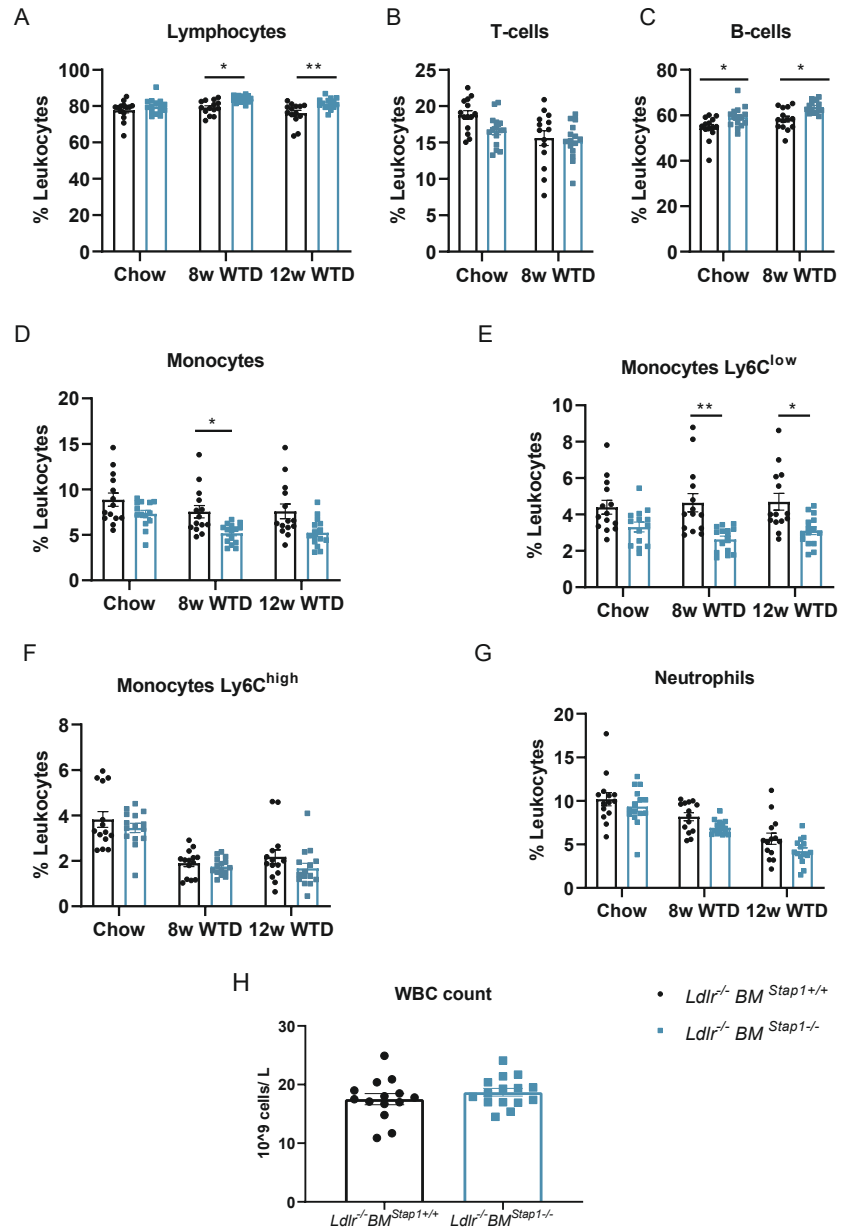
Supplemental Figure II: Plasma lipid profile of the additional *Stap1*$^{-/-}$ mouse line developed (Del14bp, mouse line B) on a standard laboratory diet and after 4 weeks on HFCD, for male and female animals (A) Plasma cholesterol levels of *Stap1*$^{-/-}$ (Del14) males on a standard laboratory diet and HFCD. (B) Plasma cholesterol levels of female mice *Stap1*$^{-/-}$ (Del14) on a standard laboratory diet and HFCD. (C) Plasma triglyceride levels of male mice *Stap1*$^{-/-}$ (Del14) on a standard laboratory diet and HFCD. (D) Plasma triglyceride levels of *Stap1*$^{-/-}$ (Del14) females on a standard laboratory diet and HFCD. Two-way ANOVA with Sidaks correction for multiple comparisons, n=8 animals per genotype.



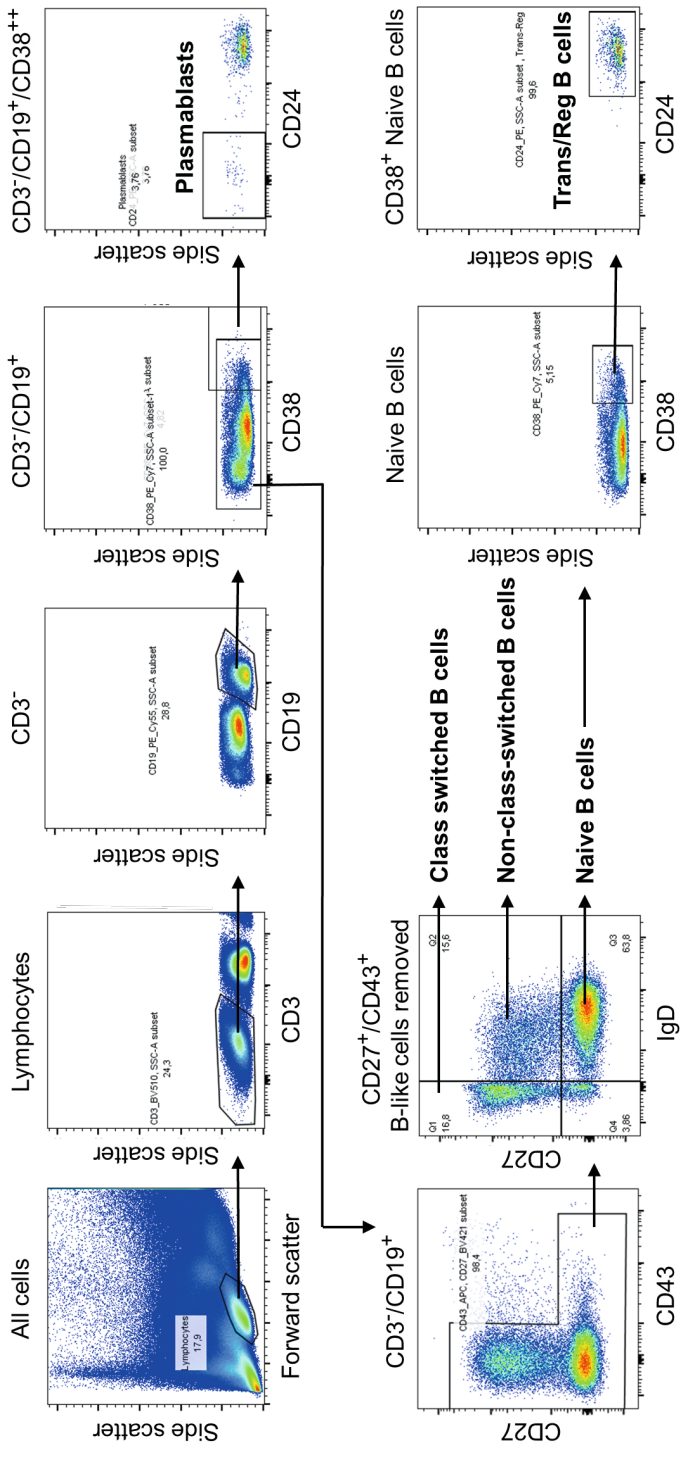
Supplemental Figure III: STAP1 protein levels and body weight evaluation of *Ldlr*$^{-/-}$ mice during bone marrow transplantation study
(A) STAP1 protein expression in spleen determined by mass-spectrometry in transplanted animals after sacrifice. Stap1 protein cannot be detected in the spleen of *Stap1*$^{-/-}$ bone-narrow transplanted mice, indicating that *Stap1* deficiency induced by BM transplantation was sustained over time. (B) Body weight during the bone marrow transplantation study in *Ldlr*$^{-/-}$ female mice which were housed individually. No significant differences were observed with Two-way ANOVA. Data are shown as mean ± SEM. n= 13-16 animals per genotype.



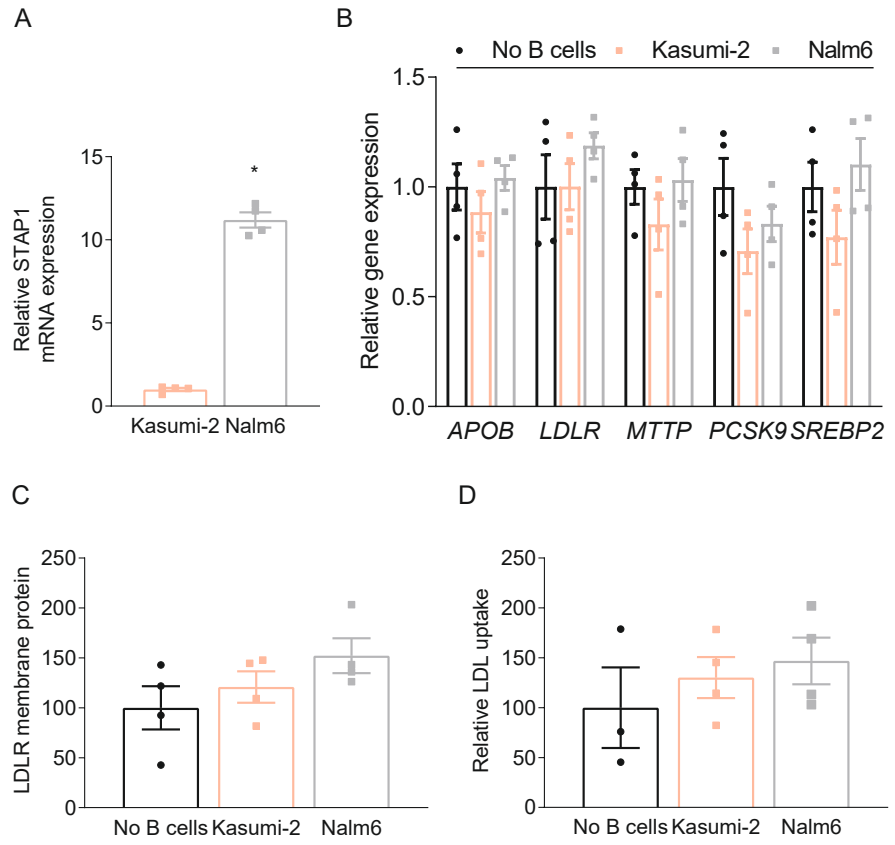
Supplemental Figure IV: Gating examples for the evaluation of leukocyte populations in peripheral blood for the bone marrow transplantation study
Representative examples are shown for *Ldlr*$^{-/-}$BM*Stap1*$^{-/-}$ (upper) and *Ldlr*$^{-/-}$BM*Stap1*$^{+/+}$ mice (lower). The different immune cell subpopulations evaluated were: leukocytes (CD45+), monocytes (CD45+/CD115+), neutrophils (CD45+/Ly6C/G+/CD115-), lymphocytes (CD45+/CD115-/Ly6C/G-/TCRb+/CD19-) and B-cells (CD45+/CD115-/Ly6C/G-/TCRb-/CD19+). Monocyte subpopulations Ly6C/Ghi and Ly6C/Glo were also analyzed.



Supplemental Figure V. Flow cytometry analysis of immune population changes in peripheral blood on a standard laboratory diet and during WTD feeding in bone marrow transplanted female mice, *Ldlr*$^{-/-}$BM$^{Stap1+/+}$ and *Ldlr*$^{-/-}$BM$^{Stap1-/-}$
(A) Lymphocytes, (B) T-cells, (C) B-cells, (D) monocytes, (E) Ly6Clow, (F) monocytes Ly6Chigh, (G) neutrophils. Figures A-G: Two-way ANOVA with Sidaks's correction for multiple comparisons. (H) White blood cell count after 12 weeks on WTD in *Ldlr*$^{-/-}$BM$^{Stap1+/+}$ and *Ldlr*$^{-/-}$BM$^{Stap1-/-}$. Figure H: T-test. *p<0.05, **p<0.01, ***p < 0.001. n=13-15 animals per genotype. Data shown as mean ± SEM.



Supplemental Figure VI: The FACS selection of different B cell subtypes from the PMBC fraction of *STAP1* variant carriers and controls
The different B cell subtypes are plasmablasts (CD19+/CD27+/IgD−/IgM−/CD20−/CD38++), class- switched memory B cells (CD19+/CD27+/IgD−/IgM−/CD20+/CD38+/−), non-class-switched memory B cells (CD19+/CD27+/IgD+), naïve B cells (CD19+/CD27−/IgD+), and transitional and regulatory (Trans/Reg) B cells (CD19+/CD24++/CD38++).



Supplemental Figure VII: The effect of the human B cell precursor leukemia cell-lines Kasumi-2 and Nalm6 on HepG2 LDL homeostasis
The human B cell precursor leukemia cell-lines Kasumi-2 and Nalm6 were co-cultured for 24 hours with HepG2 cells. (A) Relative *STAP1* mRNA expression in Kasumi-2 and Nalm6 cells. The expression was normalized to *RN18S*, *HPRT1*, and *RPLP0* with data from Kasumi-2 cells set as '1' (Mann-Whitney test, *, $p<0.05$, n=4). (B) Relative mRNA expression in the HepG2 cells after co-culturing. Expression is normalized to *RN18S*, *HPRT1*, and *RPLP0* with data from HepG2 cells not co-cultured with B cells defined as '1'. (C) Relative LDLR protein on the surface of the HepG2 cells after co-culturing as determined by FACS analysis. Data are corrected for amount of cells and data from HepG2 cells not co-cultured with B cells set to 100%. (D) 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 not co-cultured with B cells set at 100%. Values are mean ± SEM (n=4-5 per group; one-way ANOVA and Kruskal-Wallis test, *, $p<0.05$).

Supplemental Table I: Frequency of *STAP1* gene variants and their predicted impact on protein function.

	RefSNP	Frequency (a)	PolyPhen-2 prediction (b)	SIFT prediction (c)
p.Thr47Ala	rs793888522	1.22×10^{-5}	0	0.15
p.Leu69Ser	rs938523789	1.58×10^{-5}	0.921	0.01
p.Ile71Thr	rs141647940	2.16×10^{-5}	0.249	0
p.Glu97Asp	rs779392825	1.65×10^{-5}	0.944	0.07
p.Pro176Ser	rs199787258	3.27×10^{-4}	0.997	0.01
p.Asp207Asn	rs146545610	1.34×10^{-4}	0.046	0.1

(a) Population frequency according to the Genome Aggregation Database (gnomAD) at http://gnomad.broadinstitute.org. (b) Polymorphism Phenotyping v2 (PolyPhen-2) is a tool that predicts the impact of an amino acid substitution on the structure and function of a protein.[41] Higher values indicate a stronger impact on protein function. (c) Sorting Intolerant From Tolerant (SIFT) predicts whether an amino acid substitution affects protein function based on the sequence homology and physical properties of the amino acids. Lower values indicate higher prediction of impact on protein function.[34]

Supplemental Table II: Summary statistics for *STAP1* variants included in our study and also present in the exome sequencing data from the UK Biobank (2019) and association with LDL-c

***STAP1* variants**		**Allele count**	**Allele number**	**Beta**	**Standard error**	**p.value**
Genome (Hg19)	**Protein**					
chr4:68441122, T>C	p.Ile71Thr	9	75778	-0.311173	0.329947	0.32
chr4:68447185, C>T	p.Pro176Ser	45	75778	-0.107893	0.3147616	0.41

Note: *STAP1* variants Glu97Asp, Thr47Ala, Leu69Ser and Asp207Asn were not found in this released of the UK biobank[45]

Supplemental Table III: Sequences of the primers used for PCR and qPCR for mouse samples

Gene	**Primer sequence**	**Reverse**
Surveyor Stap1	TACTGAGCACGGCATGTGAC	GCCTGAGTTCCAGTCCTGAC
T7addsgRNA	TTAATACGACTCACTATAGGATAGACCTCG TGTGCCTTAC	TTTAAAAGCACCGACTCGGTGCC
T7addCas9	TAATACGACTCACTATAGGGAGAATGGAC TATAAGGACCACGAC	GCGAGCTCTAGGAATTCTTAC
Stap1 WT BMT	GTGTGCCTTACTGGCCAA	GTTGCTATTTCTCCTCGCTCA
Stap1 genotyping	TACTGAGCACGGCATGTGAC	GCCTGAGTTCCAGTCCTGAC
Gapdh	CCTGCTTCACCACCTTCTTG	GAGAGCTGGGCTTGTCTCTG

Supplemental Table IV: Sequences of the primers used for real time PCR for human samples

Gene	**Forward Primer**	**Reverse Primer**
APOB	GACGACTTTTCTAAATGGAACTTCTAC	CTCAGTTTTGAATATGGTGAGTTTTT
HPRT1	TGACCTTGATTTATTTTGCATACC	CGAGCAAGACGTTCAGTCCT
LDLR	AAGGACACAGCACACAACCA	CCCAGAGCTTGGTGAGACAT
MTTP	ATACCTGCAGCCTGACAACC	GCCAGGAAGTTTCTGACAGC
PCSK9	CTCAACTGCCAAGGGAAGGG	GCTGGCTTTTCCGAATAAACTCC
RN18S	GAGGGAGCCTGAGAAACGG	GTCGGGAGTGGGTAATTTGC
RPLP0	ACGGGTACAAACGAGTCCTG	GCCTTGACCTTTTCAGCAAG
SREBP2	GGCTCATCTTTGACCTTTGC	AGGCTGGCTTCTCTCCCTAC
STAP1	CGGTCAGGATACCGGGAGTA	GCTCAGTAAGGCATGTGAGGT

SUPPLEMENTAL MATERIALS AND METHODS

Designing and validating sgRNA

3 sgRNA sequences to target murine *Stap1* were selected using the online tool "Crispr.mit.edu". The gRNAs were cloned into pX459 vector and transfected into Neuro2A (ATCC; CCL-131) cells. The most efficient gRNA, analyzed by a Surveyor assay, according to Ran et al, (2013)1 (Supplemental Table III), was then used as template in a PCR reaction to add the T7 RNA promoter (Supplemental Table III). The PCR product of 124 bp was cloned into a pCR-blunt vector (Thermo Fisher Scientific, #K270020) and the sequence was verified by Sanger Sequencing. After, EcoR-I/Dra-I digestion and purification, 200 ng DNA template was used as template for in-vitro transcription (MEGAshortscript kit, Thermo Fisher Scientific, AM1354), according manufacturers protocol. Subsequently, the spCas9 from pX459 with T7 addition was amplified and cloned into pCR-blunt. After sequence verification, 1 ug of this Hind-III linearized plasmid was used as template for in vitro transcription (mMESSAGE mMACHINE T7 ULTRA kit, Thermo Fisher Scientific, #AM1345) according manufacturers protocol. Cas9 RNA, as well as sgRNA, was purified using the MEGAclear kit (Thermo Fisher Scientific, #AM1908) and eluted in RNAse free water.

Zygote injections

Female FVB mice were super ovulated by injection with 5 IU Folligonan (0.2 ml i.p.) and 48 h later with 5 IU Chorulon (0.2 ml i.p.). The next day, zygotes were isolated

from the infundibulum and injected with 100 ng/ul Cas9 RNA + 50 ng/ul sgRNA *Stap1*. Injected zygotes were incubated overnight at 37ºC and transferred to the infundibulum of pseudopregnant female FVB mice.

Genotyping

To assess whether CRISPR/Cas9 introduced a mutation in the *Stap1* gene of F0 mice, DNA was isolated from the ear cuts. The ear-pieces were incubated in 500 µl 1x SE supplemented with 50 µl 10% SDS and 5 µl proteinase k (20 mg/ml) at 55 ºC until dissolved. To each tube, 222 µl 6M saturated NaCl and 777 µl chloroform was added. After centrifugation for 10 min at 12,000 g (RT), the upper phase was transferred to a new tube and diluted 1:1 with isopropanol. Subsequently, DNA was centrifugated for 10 min at 12,000 g (RT). The pellet was air-dried and dissolved in distilled RNAse/DNAse free water (Gibco) before measuring the DNA concentration. A surveyor PCR was performed as described above. The F1 offspring was Sanger sequenced and analyzed for mutations. Next, F0 mice were mated with C57Bl/6J mice to generate F1 mice. Subsequently, *Stap1*$^{+/-}$ F1 animals were mated with each other to obtain *Stap1*$^{-/-}$ mice. DNA was isolated from the ears by adding a solution of 17.8 µl mQ, 2.0 µl buffer Gold and 0.2 µl prepGEM. The sample was heated for 15 minutes at 75 ºC and subsequently at 95 ºC for 5 minutes. PCR reaction was performed with specific primers for murine Stap1 (see sequences in Supplemental Table III) and the amplification product was digested overnight with BglI (New England Biolabs) at 37 ºC overnight and visualized on a 2% wt/vol agarose gel with Midori Green. The restriction enzyme cuts a specific nucleotide sequence only present on alleles without mutation, thus the length and the number of PCR fragments determines the genotype. A single product of 760 bp corresponds to *Stap1*$^{-/-}$ mice while two fragments of 478 and 278 bp orginates from *Stap1*$^{+/+}$ mice, and *Stap1*$^{+/-}$ mice present all three PCR products of 760, 478 and 278 bp.

Bone marrow isolation

Donor *Stap1*$^{-/-}$ and *Stap1*$^{+/+}$ female mice of 10 weeks of age (backcrossed into C57Bl6/J background for 8 generations) were sacrificed by cervical dislocation and BM was isolated from tibia, femur, hip and vertebras by bone crushing. After lysis of red blood cells, BM cells were passed through a cell strainer, counted and adjusted at a concentration of 500,000 cells/µl in buffer.

Bone Marrow transplant

Female *Ldlr*$^{-/-}$ mice (B6.129S7-Ldlrtm1Her/J) were purchased from The Jackson Laboratory (Germany) and group-housed in individually-ventilated cages (IVC). Prophylactic ciprofloxacin treatment (0.1mg/L) was provided in the drinking water during 10 days, starting the day before irradiation. At 8 weeks of age the mice were irradiated with 9 Gy and injected i.v. in the orbita with 5x106 BM cells from donor female mice of different genotypes (*Stap1*$^{-/-}$ or *Stap1*$^{+/+}$). After a recovery period of 5 weeks, the transplanted animals were moved to single-house cages in the conventional specific- pathogen-free facility.

Verification of bone marrow reconstitution

5 weeks after transplantation (see main text materials and methods), blood was collected by orbita bleeding under anesthesia with isoflurane, and DNA was isolated using the QIAamp DNA Blood Mini Kit (QIAGen) according to manufacture instructions. The DNA was diluted 1:10 and amplified by qPCR using Stap1-WT specific primers (for sequences see Supplemental Table III) targeting the mutated region in the Stap1-/- mice and FastStart Universal SYBR Green Master (Roche). Amplification results were normalized to Gapdh gene, as endogenous control. Gapdh primers were designed to target an intron-exon junction to be specific for DNA amplification. Data were analyzed with QuantStudio Real- Time PCR software version 1.2 (Applied Biosystems). Bone marrow reconstitution was calculated based on the ratio of *Stap1*$^{+/+}$ copies in blood in animals transplanted with *Stap1*$^{-/-}$ bone marrow, compared to animals transplanted from *Stap1*$^{+/+}$ donors.

Flow cytometry analysis in BM transplanted mice

For the bone marrow transplantation study, total blood was obtained by tail bleeding and anticoagulated with EDTA, before the diet intervention and at 8 and 12 weeks after WTD feeding. Red blood cells were lysed with lysis buffer (BD Pharm Lyse, BD Bioscience) during 5 min, washed and suspended in FACS buffer (0.1% BSA, 5 mM EDTA). Cell surface staining was performed with monoclonal antibodies against CD45-APC-Cy7, Ly6-C/G-PerCP-Cy5.5 (BD Pharmingen), CD115-APC, CD44-PE-Cy7, CD62L-APC, CD8-FITC (eBioscience), CD19-PE and TCRβ-PB (Biolegend) (See complete information in Major Resourses). Data was acquired in the LSR-II using FACS DiVA Software. Immune cell populations were defined as follows: leukocytes

were identified as CD45+, monocytes as CD45+CD115+, neutrophils as CD45+Ly6C/G+CD115-, lymphocytes as CD45+CD115-Ly6C/G-, T-cells as CD45+CD115-Ly6C/G-TCRb+CD19- and B-cells as CD45+CD115-Ly6C/G-TCRb-CD19+. Gating examples can be found in Supplemental Figure IV. After 12 weeks on WTD, white blood cell counts were performed with the Medonic Hematology analyzer (Medonic CA 620) to confirm earlier findings. The staining at this time point was performed with a different cocktail containing Ly6C/G-PerCP, CD115-PE, CD45-APC-Cy7, and only leukocytes, lymphocytes, monocytes and neutrophils populations were analyzed. Flow-cytometry data were analyzed with FlowJo version 10.5.3 (FlowJo, LLC), Excell (Microsoft Office Proffesional Plus, 2016) and GraphPad Prism 8.2.0 (GraphPad Software, Inc).

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Differential DNA methylation in familial hypercholesterolemia

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ABSTRACT

Background: Familial hypercholesterolemia (FH) is a monogenic disorder characterized by elevated low-density lipoprotein cholesterol (LDL-C). A FH causing genetic variant in *LDLR*, *APOB*, or *PCSK9* is not identified in 12-60% of clinical FH patients (FH mutation-negative patients). We aimed to assess whether altered DNA methylation might be associated with FH in this latter group

Methods: In this study we included 78 FH mutation-negative patients and 58 FH mutation-positive patients with a pathogenic *LDLR* variant. All patients were male, not using lipid lowering therapies and had LDL-C levels >6mmol/L and triglyceride levels <3·5mmol/L. DNA methylation was measured with the Infinium Methylation EPIC 850K beadchip assay. Multiple linear regression analyses were used to explore DNA methylation differences between the two groups in genes related to lipid metabolism. A gradient boosting machine learning model was applied to investigate accumulated genome-wide differences between the two groups.

Findings: Candidate gene analysis revealed one significantly hypomethylated CpG site in *CPT1A* (cg00574958) in FH mutation-negative patients, while no differences in methylation in other lipid genes were observed. The machine learning model did distinguish the two groups with a mean Area Under the Curve (AUC)±SD of 0·80±0·17 and provided two CpG sites (cg26426080 and cg11478607) in genes with a possible link to lipid metabolism (*PRDM16* and *GSTT1*).

Interpretation: FH mutation-negative patients are characterized by accumulated genome wide DNA methylation differences, but not by major DNA methylation alterations in known lipid genes compared to FH mutation-positive patients.

RESEARCH IN CONTEXT

Evidence before this study

A causal pathogenic variant in one of the Familial Hypercholesterolemia (FH) genes (i.e. *LDLR*, *APOB*, *PCSK9*) is not found in a large proportion of patients with clinical FH. We hypothesized that differential DNA methylation, a form of epigenetic regulation, contributes to the FH phenotype in these FH mutation-negative patients. We performed a PubMed search with the following terms: "Familial Hypercholesterolemia" AND "DNA methylation" and found 11 studies. None of the studies investigated the DNA methylation pattern in FH mutation-negative patients. Next, we searched PubMed with the terms ("dna methylation" OR "methylation" OR "cpg islands" OR "ewas" OR "CpG Islands"[MeSH Terms] OR "DNA Methylation"[MAJR]) AND ("ldl" OR "low-density lipoprotein") Filters: Humans. This yielded 370 articles, and in 5 of these, epigenome wide association studies showed an association between DNA methylation in multiple genes and LDL cholesterol levels. None of the studies investigated DNA methylation patterns in FH mutation-negative patients.

Added value of this study

This study was the first large scale study in FH mutation negative patients. In order to control for confounding due to high lipid levels we studied two unique FH patient groups: FH mutation-negative patients, and a group comprising FH mutation-positive patients. Although classical candidate gene analysis did, except for *CPT1A*, not reveal major DNA methylation differences in known lipid genes, a machine learning approach showed that FH mutation-negative patients are characterized by a different genome wide DNA methylation pattern compared to FH mutation-positive patients, with important model features for the genes *PRDM16* and *GSTT1*.

Implications of all the available evidence

Despite extensive sequencing efforts, a causative genetic variant is not found in a large proportion of patients with a clinical FH diagnosis. Hence efforts to find novel factors causing the FH phenotype are deemed of great relevance. Additional studies to further investigate DNA methylation and its causal role in (familial) hypercholesterolemia are warranted and might benefit from focusing on accumulation of genome-wide methylation differences instead of single gene or CpG site methylation.

INTRODUCTION

Familial hypercholesterolemia (FH) is a common inherited autosomal dominant disease characterized by high plasma levels of low-density lipoprotein cholesterol (LDL-C) and high risk for premature cardiovascular disease (CVD). Pathogenic variants in the genes coding the low-density lipoprotein receptor (*LDLR*), apolipoprotein B (*APOB*), and proprotein convertase subtilisin/kexin type 9 (*PCSK9*) have been shown to cause FH. However, no pathogenic variant in any of these three FH genes is identified in a large proportion of patients who are diagnosed with FH based on clinical signs and symptoms[1], fuelling an ongoing search for novel pathogenic pathways causing FH.

Differential epigenetic regulation of the genes involved in lipid metabolism may be such a factor causing FH. DNA methylation, in which a methyl group is covalently bound to the fifth carbon atom of the nucleotide cytosine when it is followed by guanine (CpG site) is the most studied form of epigenetic gene expression regulation.[2] In general, methylation of CpG sites in promoter regions of genes results in low expression of the gene, while methylation of CpG sites within the gene typically results in high expression of the gene.[2]

The role of DNA methylation in lipid metabolism is relatively under investigated, but some studies have shown that DNA methylation of multiple genes is associated with plasma LDL-C as well as other lipid levels.[3–6] The expression of known genes involved in LDL-C metabolism (i.e. *APOE*, *NPC1L1*) has been found to be regulated by CpG methylation.[7,8] Moreover, DNA methylation of multiple genes (i.e. *ABCA1*, *ABCG1*, *LIPC*, *PLTP*, *CETP*, and *LPL*) were associated with lipid traits[9–11] and coronary artery disease outcomes (i.e. *ABCA1*)[9] in patients with molecularly proven FH. However, the impact of methylation of lipid genes has not been investigated in FH patients in whom no variant in the coding region of the three major FH genes is found.

In the current study we analysed the methylation pattern in patients with and without FH causing variants. A potential confounding factor is the effect of elevated lipid levels on DNA methylation itself. To overcome this issue, we compared DNA methylation in FH patients without FH causing variant (FH mutation-negative)

to group of FH patients with a known pathogenic variant in *LDLR* (FH mutation-positive). We not only investigated methylation differences in single genes using classical regression analysis, but also used an unbiased machine learning approach to identify whole genome differences in DNA methylation between the two groups.

MATERIALS AND METHODS

Study population

In this study we investigated DNA methylation differences between FH mutation-negative patients and FH mutation-positive patients. The Amsterdam UMC, location Academic Medical Center (AMC) in Amsterdam, is the national referral centre for the genetic analysis of all Dutch patients with various forms of dyslipidaemias. For this study we analysed the DNA derived from index patients for whom the referring physician, after clinical evaluation (laboratory results, family history, and physical examination) based on national guidelines[12,13], requested molecular testing for FH causing genetic variants between 2012 and 2017. In the samples collected before 2016 (n = 122), molecular analysis was performed by Sanger sequencing of *LDLR*, *APOB*, and *PCSK9* and was followed by multiplex ligation-dependent Probe Amplification (MLPA) of *LDLR* when no pathogenic variants in these three genes were found. In samples collected from 2016 onwards (n = 14), a targeted next-generation sequencing (NGS) capture covering 27 lipid genes (including *LDLR*, *APOB*, and *PCSK9*) was used (Supplementary Table 4). Subsequent genetic cascade screening within families of index patients is done in a separate diagnostic program. These patients were not included in the current study.

The DNA of male patients was used for the current study when the patients were not a carrier for any known FH causing variant in *LDLR*, *APOB*, and *PCSK9* (FH mutation-negative) or had a FH causing variant in *LDLR* (FH mutation-positive). We selected patients who had plasma LDL-C levels above 6 mmol/L, which corresponds to the >99th percentile for males from all ages in The Netherlands.[14] Moreover, patients whose triglycerides levels were above 3·5 mmol/L and those who were using lipid lowering therapies (i.e. statins) at the time of DNA sampling were excluded. Females were excluded from this study because of the influence of sex

differences on DNA methylation.[15] The study size was based on the availability of DNA samples of patients meeting these criteria. All included subjects gave written informed consent for re-use of their DNA samples for research into novel causes of hypercholesterolemia. The Medical Ethics Review Committee of the Amsterdam UMC, location AMC, provided a waiver for the re-use of the patients clinical data and DNA samples in the current study (reference ID: W20_246 # 20.281).

DNA methylation measurements

The Gentra Puregene kit was used to isolate DNA from whole blood collected in EDTA containing tubes according to standard protocols. Samples were stored at 4°C until analysis. DNA concentrations were measured using Qubit standard methodology. DNA was treated with bisulfite using the EZ DNA Methylation kit of ZYMO® according to the standard protocol recommended by Illumina. DNA methylation of the bisulfite treated DNA was analysed with the Illumina Infinium Methylation EPIC 850K beadchip (Illumina, California, USA) at GenomeScan (Leiden, The Netherlands). Samples of FH mutation-negative and FH mutation-positive patients were randomly assigned to different slides to avoid potential confounding batch effects.

Statistical analysis

We analysed the methylation data in a two-step approach. First, linear regression models for each CpG site were constructed to test for major difference in DNA methylation between FH mutation-positive and FH mutation-negative patients. Next, a gradient boosting machine learning technique was used to investigate unbiased subtle genome-wide DNA methylation differences between the two FH groups.

Quality control and normalization of methylation data

Quality control of the obtained data was performed using the R-package MethylAid (version 1.30.0), conform default settings.[16] Concordance between sex chromosome probes and self-reported sex were evaluated using principal component analyses (PCA). Next the data was normalized using the *Funnorm* function from the Minfi R package (version 1.30.0).[17] Probes susceptible to cross-hybridization(12), probes previously described include single nucleotide polymorphisms (SNPs) with a minor allele frequency (MAF) >0.01 in either the CpG dinucleotide itself or at the position

of the single base extension, and probes which included SNPs in the probe binding position were excluded (according to the Illumina manifest).

Candidate gene analysis

In the candidate gene analysis, CpG methylation was the dependent variable with group (FH mutation-negative or FH mutation-positive), age and leukocyte cell distributions incorporated as independent variables in this model. Leukocyte cell distributions were estimated using the obtained data according to the method of Houseman *et al.*, resulting in information on relative cell counts of CD8+ and CD4+ T cells, natural killer cells, B cells, monocytes and granulocytes.[18] Quality of the epigenetic profiles was further evaluated using density plots of raw and normalized data and PCA. Correlations of the principal components one to eight with all available variables were evaluated upon entering our statistical model. For differential methylated positions (DMPs), we applied the *LMfit* function in the R package Limma (version 3.40.2). Cell distribution was determined with the R package FlowSorted.Blood.EPIC. To control for multiple testing the false discovery rate (FDR) method was used, where an FDR <0.05 was defined to be significant. We corrected for inflation using the BACON package for R (version 1.12.0).[19]

We generated four groups of genes according to the grade of impact on lipid metabolism (Supplementary Table 1). Tier 1 and 2 comprised the major (*LDLR*, *APOB*, *PCSK9*), and minor (*LDLRAP*, *STAP1*, *ABCG5*, *ABCG8*, *APOE*, *LIPA*) FH genes, respectively. Tier 3 comprised all genes that were shown to be significantly associated with plasma LDL-C or total cholesterol levels in a large genome wide association study.[20] Tier 4 included eighteen cytosine-guanine dinucleotide positions that have been shown to be associated with LDL-C and total cholesterol levels in previous studies.[3,5,6,21] All CpG probes within 3000 base pairs surrounding the candidate genes on either side were analysed in order to cover CpG sites in the 5` promoter region and possible downstream regulatory regions that were not annotated to a gene by the Illumina manifest.

Machine learning analysis

Statistical machine learning analysis was used to identify differentially methylated CpG sites that could discriminate between FH mutation-negative and FH mutation-

positive subjects on a unbiased genome wide level. In brief, we used a combination of multiple gradient boosting classifiers to improve prediction accuracy.[22,23] To avoid over-fitting, we used a 5-fold stratified cross-validation over the training partition of the data (80%) while the remaining data (20%) was used as the test dataset.[24] The latter set was not used for the construction of the machine learning models. We conducted a rigorous stability selection procedure to ensure the reliability and robustness of the biomarker signatures.[25] This was repeated 50 times and Receiver Operating Characteristics (ROC) Area Under Curve (AUC) scores were computed each time and averaged for the final test ROC AUC. A permutation (randomization test) was used to evaluate statistical validity of the results.[26] In the permutation test, the outcome variable (i.e., the FH group, either FH mutation-negative or FH mutation-positive) was randomly reshuffled 1000 times while the corresponding epigenetic profiles were kept intact. By evaluating the distribution of all the results obtained in these simulations and comparing it to the outcome variable, we computed statistical significance associated with the joint panel of the selected CpG sites. To gain insight into the features that contributed the most to the model we also report relative feature importance scores for each of the CpG sites that demonstrate preferences in the model for predicting the outcome variable in the gradient boosting model. To gain insight into the biological relationship between the top features of this model and lipid metabolism, we searched for publications listed in PubMed that described a relationship between the genes identified in the top 20 contributing CpG sites and hypercholesterolemia. We used Python version 3.8 (www.python.org), with packages Numpy, Scipy and Scikits-learn for implementing the model and R version 3.5.3 (R Foundation, Vienna, Austria) for visualizations.

Correlation methylation and gene expression

Significantly differentially methylated CpG sites identified in the candidate gene analysis and the top 20 CpG sites that contributed the most in the machine learning model, were submitted for *in silico* validation by exploring their correlation with gene expression data in two publicly accessible liver hepatocellular carcinoma datasets; accessible via the webtools *SMART*[27] and *MEXPRESS*[28]. These datasets were based on the smaller 450K Illumina Infinium Beadchip assay, implying that only EPIC/450K overlapping CpG sites were investigated. Spearman's and Pearson's correlation were retrieved from both databases. Correlations between DNA-methylation and

gene expression showing a P-value < 0·05 and a correlation coefficient (R) > 0·1 were suggestive to be biological relevant.

Data statement:

All individual normalized DNA methylation data are available via https://dx.doi.org/10.6084/m9.figshare.12334586.

RESULTS

Subjects for this study were diagnosed with clinical FH by the physician, who requested genetic analysis for FH in our centre. The analysed cohort comprised of 78 FH mutation-negative and 58 mutation positive patients. Characteristics of the cohort are shown in table 1. FH mutation-negative patients were older (50·7±12·3 *vs.* 39·1±12·0 years old, p < 0·05 [Student's t-test]) had slightly lower LDL-C levels (median[IQR] 6·7 [6·4-7·2] mmol/L *vs.* 7·4 [6·7-8·4] mmol/L, p < 0·05 [Mann-Whitney U test]) and higher TG levels (1·3 [1·1-2·0] mmol/L *vs.* 1·8 [1·3-2·3] mmol/L, p = 0·011 [Mann-Whitney U test]) compared to the FH patients with a *LDLR* mutation.

Table 1: characteristics of study population

	FH mutation-positive	FH mutation-negative	P-value*
n	58	78	-
Age in years (mean (SD))	38·1 (12·0)	50·7 (12·3)	<0·001
Males (n (%))	58 (100)	78 (100)	-
Total cholesterol, mmol/L (mean (SD))	9·6 (1·3)	9·0 (1·4)	0·022
LDL cholesterol, mmol/L (median [IQR])	7·4 [6·7-8·4]	6·7 [6·4-7·2]	0·001
HDL cholesterol, mmol/L (mean (SD))	1·3 (0·8)	1·3 (0·4)	0·668
Triglycerides, mmol/L (median [IQR])	1·3 [1·1-2·0]	1·8 [1·3-2·3]	0·011

SD, Standard Deviation; IQR, interquartile range; LDL, low-density lipoproteins; HDL, high-density lipoproteins. *normally distributed values (age, total cholesterol, HDL cholesterol) were compared using student's t-test, non-normally distributed values (LDL cholesterol and triglycerides) were compared using a Mann-Whitney U test.

Quality control of data

No major inflation was observed after BACON inflation correction of the data (lambda = 0·9546; Supplementary Figure 1).

Candidate gene analysis

To investigate the association between CpG sites related to genes involved in lipid metabolism and the FH group, we performed a candidate gene analysis according to the four predefined tiers of genes (Supplementary Table 1). Tier 1 consisted of the three major FH genes: *LDLR*, *APOB*, and *PCSK9*. None of the studied CpG sites in these genes were significantly differently methylated in FH mutation-negative patients compared to FH-mutation positive patients (see Figure 1, panel A). Also, in tier 2, consisting of so called "minor" FH genes, no differences between the two groups were observed (Figure 1, panel B). Next, we investigated methylation differences in genes that were previously shown to be associated with LDL-C and total cholesterol in a large GWAS study.[20] Again, no significantly differently methylated CpG sites between FH mutation-negative and FH mutation-positive patients was found(Figure 1, panel C). Lastly, in tier 4, consisting of CpG sites previously associated with LDL-C or total cholesterol, one CpG site (cg00574958 in the gene *CPT1A*) showed a significant 1·3% lower methylation in FH mutation-negative patients compared to FH mutation-positive patients (β -0·013, FDR = 0·001; see Figure 1, panel D). Methylation of the identified C*PT1A* CpG site is associated with decreased expression of the *CPT1A* gene according to MEXPRESS (Supplementary Table 3) and negatively associated with triglyceride levels in our study (r = -0·27, p = 0·001 [Spearman Rank Correlation Test]).

Machine learning analysis

Clearly, methylation of single genes is not likely to account for the FH phenotype in FH mutation-negative patients. To investigate whether methylation changes in multiple genes may cause the defect we applied machine learning on the whole genome methylation data set. Next, a gradient boosting machine learning analysis was applied on the whole dataset for the discovery of genome wide differences in methylation between FH mutation-positive and FH mutation-negative patients. A hierarchical structure was generated based on the effect size and the top 20 probes with the highest relative feature importance in this model are reported in Table 2 and shown in Figure 2. Fifty percent of the top 20 CpG sites were hypermethylated with the biggest median methylation difference between the two groups for the genes *PRDM16*, *GSTT1*, and *LOC728743* (Figure 2A). In contrast, *DOCK11* and *KCNMA1* were most differentially hypomethylated in FH mutation-negative compared to FH mutation-positive patients. All probes with a Relative Feature Importance >10% are listed in Supplementary Table 2.

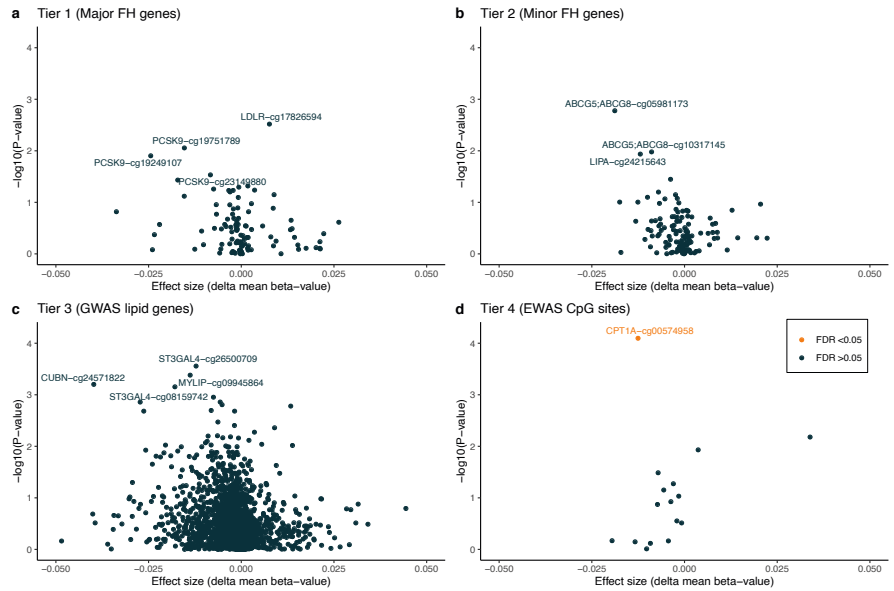

Figure 1: Candidate gene analysis.
Four tiers of genes were constructed based on literature (genes are listed in Supplementary Table 1). Shown are the difference in methylation (effect size) between FH-mutation negative and FH-mutation positive patients for the four tiers (panels A-D) Only in tier 4 (panel D), one CpG site (CPT1A-cg00574958) was significantly less methylated in FH-mutation negative patients. Significance was defined as a False Discovery rate (FDR) of <0·05. FH, Familial Hypercholesterolemia; GWAS, genome-wide association study; EWAS, epigenome-wide association study.

Most of the top 20 CpG sites were located within introns or exons of known genes, and none are located in promotor regions of genes. Of the top 20 CpG sites, five were not located in close proximity of a gene. Eleven of the top 20 CpG sites were hypomethylated in FH mutation-negative patients compared to FH mutation-positive patients. Boxplots of the methylation per top 20 CpG site per patient group are shown in Supplementary Figure 2 and their correlation with gene expression in Supplementary Table 3.

The model generated by machine learning distinguishes methylations landscape in FH mutation-negative and FH mutation-positive patients with an average Area Under the Curve (AUC) of 0·80±0·17 over 50 repeat runs with different validation and test sets(Figure 3A). A principle component analysis showed an explained variance of 11·33% for component 1 and 9·52% for component 2 (Figure 3B). Permutation analysis revealed that the observed AUC was statistically significant (p < 0·05).

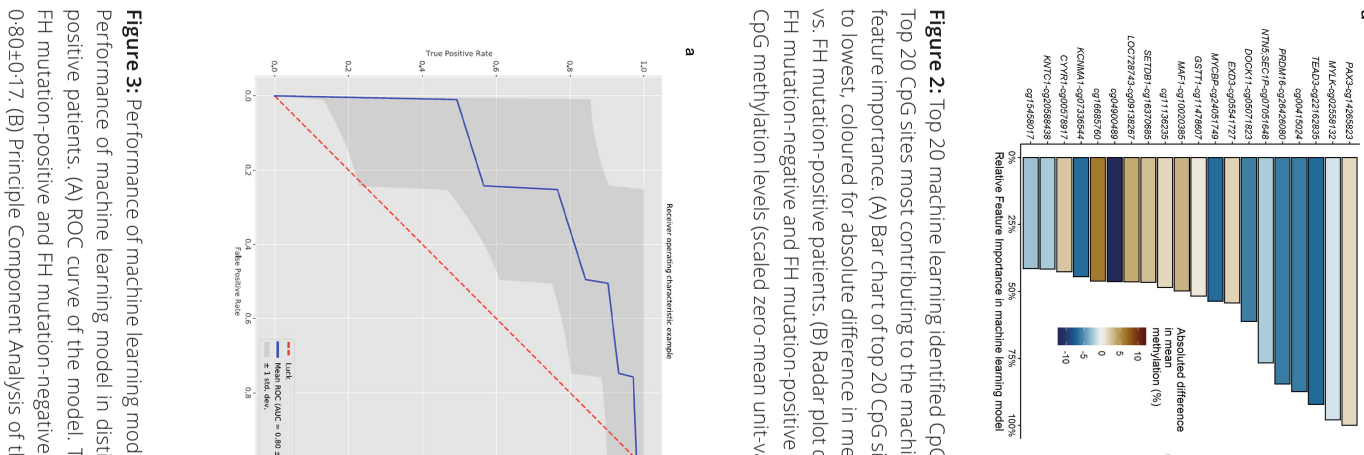

Figure 2: Top 20 machine learning identified CpG sites

Top 20 CpG sites most contributing to the machine learning model performance, selected on relative feature importance. (A) Bar chart of top 20 CpG sites ordered from highest relative feature importance to lowest, coloured for absolute difference in mean methylation (%) in FH mutation-negative patients vs. FH mutation-positive patients. (B) Radar plot displaying top 20 CpG cites that differentiate between FH mutation-negative and FH mutation-positive patients. The axes represent the standardized mean CpG methylation levels (scaled zero-mean unit-variance).

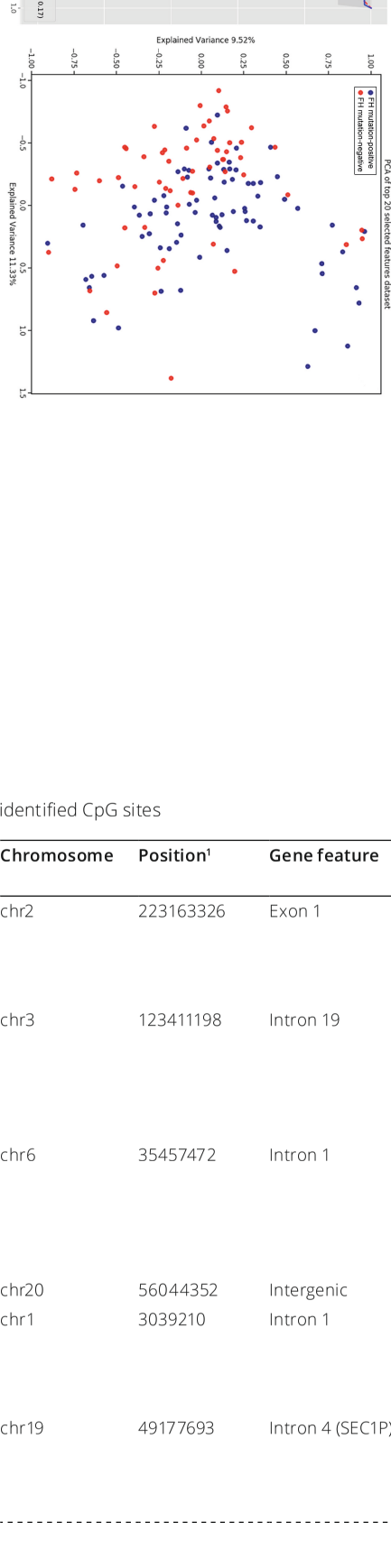

Figure 3: Performance of machine learning model

Performance of machine learning model in distinguishing FH mutation-negative from FH mutation-positive patients. (A) ROC curve of the model. The machine learning model was able to distinguish FH mutation-positive and FH mutation-negative patients with an Area Under the Curve (AUC±SD) of 0·80±0·17. (B) Principle Component Analysis of the top 20 CpG sites with the highest relative feature importance.

Table 2: Top 20 machine learning identified CpG sites

	CpG	Gene	Chromosome	Position[1]	Gene feature	Methylation direction in FH mutation- negative[2]	Relative Feature Importance	Protein function[3]
1	cg14265823	*PAX3*	chr2	223163326	Exon 1	Hyper	100	Paired Box 3; involved in neural development and myogenesis during fetal development.
2	cg02558132	*MYLK*	chr3	123411198	Intron 19	Hypo	97·97	Myosin light chain kinase; involved in smooth muscle contraction via phosphorylation of myosin light chains.
3	cg22162835	*TEAD3*	chr6	35457472	Intron 1	Hypo	92·2	TEA Domain Transcription Factor 3; mainly expressed in placenta and involved in transactivation of chorionic somatomammotropin-B.
4	cg00415024		chr20	56044352	Intergenic	Hypo	87·39	
5	cg26426080	*PRDM16*	chr1	3039210	Intron 1	Hypo	84·61	PR/SET Domain 16; transcriptionfactor involved brown adipose tissue differentiation.
6	cg07051648	*NTN5/SEC1P*	chr19	49177693	Intron 4 (SEC1P)	Hypo	76·65	Netrin 5; plays a role in neurogenesis, prevents motor neuro cell body migration out of the neural tube.

Table 2: (Continued)

	CpG	Gene	Chromosome	Position[1]	Gene feature	Methylation direction in FH mutation- negative[2]	Relative Feature Importance	Protein function[3]
7	cg05071823	*DOCK11*	chrX	117628671	Intergenic	Hypo	61·17	Dedicator Of Cytokinesis 11; involved in megakaryocyte development and platelet production.
8	cg05541727	*EXD3*	chr9	140277740	Intron 2	Hyper	54·31	Exonuclease 3'-5' Domain Containing 3; involved in RNA degradation.
9	cg24051749	*MYCBP*	chr1	39340282	Intron 1	Hypo	53·71	MYC Binding Protein; can bind to oncogenic protein C-MYC and is possibly involved in spermatogenesis
10	cg11478607	*GSTT1*	chr22	24384400	Intergenic	Hyper	51·79	Glutathione S-Transferase Theta 1; conjungates reduced glutathione to exogeneous and endogeneous hydrophobic electrophiles.
11	cg10020385	*MAF1*	chr8	145159706	Exon 1	Hyper	49·8	Repressor of RNA polymerase III transcription MAF1 homolog; involved in repression of RNA polymerase III-mediated transcription.
12	cg11136235		chr10	81077552	Intergenic	Hyper	48·55	

Table 2: (Continued)

	CpG	Gene	Chromosome	Position[1]	Gene feature	Methylation direction in FH mutation- negative[2]	Relative Feature Importance	Protein function[3]
13	cg16370685	*SETDB1*	chr1	150899163	Intron 1	Hyper	46·59	SET Domain Bifurcated 1; regulates histone methylation, potential target for treatment in Huntington Disease
14	cg09138267	*LOC728743*	chr7	150102791	Intron 1	Hyper	46·47	Zinc Finger Protein Pseudogene
15	cg04900489		chr13	31272551	Intergenic	Hypo	46·29	
16	cg16685760		chrX	145701257	Intergenic	Hyper	46·17	
17	cg07336544	*KCNMA1*	chr10	79194347	Intron 1	Hypo	44·54	Potassium Calcium-Activated Channel Subfamily M Alpha 1; encodes alpha subunit of the MaxiK calcium-sensitive potassium channels in smooth muscle cells.
18	cg00578917	*CYYR1*	chr21	27945542	Exon 1	Hyper	42·69	Cysteine And Tyrosine Rich 1
19	cg20588438	*KNTC1*	chr12	123089881	Exon 51	Hypo	41·65	Kinetochore Associated 1; involved in proper chromosome segregation during cell division
20	cg15458017		chr17	9672274	Intergenic	Hyper	41·5	

Top 20 CpG sites sorted by relative feature importance for contribution in the machine learning model distinguishing FH mutation-negative from FH mutation-positive subjects. [1]Genomic positions as provided in human genome build – hg19. [2]Hypo- or hypermethylation in FH mutation negative group compared to FH mutation-positive group, based on direction of difference in median normalized beta's in both groups (see Supplementary Figure 2). [3]Gene names and functions (when known/available) were derived from GeneCards.org(Stelzer *et al.*, 2016)

DISCUSSION

Two findings stand out from our analysis. First, no alterations were observed in the candidate gene analysis, apart from a significant 1·3% decrease in methylation in the *CPT1A* gene in the FH mutation-negative group, suggesting that single gene methylation is not a cause of FH in our cohort. Secondly, gradient boosting machine learning revealed an overall difference in genome-wide DNA methylation between the FH mutation-positive and FH mutation-negative subjects, with a reasonable model performance (AUC 0·80±0·17). This finding underscores that these groups do differ from each other with regards to the epigenetic architecture at a genome-wide scale.

CPT1A was the only locus at which a statistical difference in methylation between the two groups was found. This gene encodes Carnitine palmitoyltransferase (*CPT1A*) and was found to be less methylated in FH mutation-negative patients compared to FH mutation-positive patients. CPT1A is a mitochondrial enzyme that catalyses the transfer of an acyl group from fatty acids to a carnitine molecule, hence controlling mitochondrial uptake and subsequent oxidation of the acyl group, especially in the liver. In line with this role in the regulation of fatty acid metabolism, hypomethylation of cg00574958 in the *CPT1A* gene is associated with plasma triglyceride concentrations.[4,29] However, in previous studies it has been shown that triglycerides affect methylation of *CPT1A* and not vice versa.[30] In fact, the observed lower cg00574958 methylation in the FH mutation-negative patients thus might be explained by the higher triglyceride levels in this group compared to the group of FH patients where a causative variant was identified (Table 1), since triglyceride levels were also found to negatively correlate with cg00574958 methylation in our study. Altogether, our results confirm the earlier described association between methylation in *CPT1A* and triglyceride levels, and a underlying mechanism of its relation to LDL cholesterol is likely not present and cannot be deducted from this study. Moreover, it is uncertain how a small methylation difference of 1.3% in this gene accounts for the severe hypercholesterolemic phenotype observed in the patients.

Next, we set out to incorporate methylation of CpG sites among the whole epigenome in a machine learning model to investigate whether the net effect of multiple small methylation differences could be used to identify specific patterns in FH mutation-negative and FH mutation-positive patients. Indeed, the resulting model performed well in distinguishing FH mutation-negative and FH mutation-positive patients (AUC 0·80±0·17), which emphasizes that the two selected FH groups differ on a genome-wide methylation level. The question arises whether the epigenetic changes in the group are causal or the consequence of environmental influences. For example, it might be that lifestyle factors resulting in triglyceride level differences between the two groups might also cause epigenetic difference, or that resulting triglycerides themselves influence genome wide methylation.

The top 20 CpG sites with a considerable impact on the model comprised two genes that have been linked to cholesterol metabolism in previous studies; *PRDM16* and *GSTT1*. *PRDM16* encodes PR/SET Domain 16, a protein involved in brown adipose tissue differentiation.[31] Common variants in the *PRDM16* locus are associated with plasma LDL-C and triglyceride levels[32], and methylation at CpG site cg26426080 is positively associated with *PRDM16* gene expression (Supplementary Table 3), suggesting that the observed hypomethylation in FH mutation-negative patients also reflects *PRDM16* expression differences in these patients. *GSTT1*, encoding Glutathione S-Transferase Theta 1, is an enzyme involved in the cellular defence against oxidative stress and genetic variants in this gene have been associated with risk for diabetes and atherosclerosis[33], and plasma total cholesterol, LDL-C and apolipoprotein B levels.[34,35] Like *PRDM16*, methylation of the identified CpG site in *GSTT1* (cg11478607) is correlated with expression of *GSTT1* (Supplementary Table 3), suggesting that the differential methylation observed in our study has an effect on *GSTT1* expression. However, the absolute differences in methylation in these two and the other top 20 CpG sites between the two groups is small (Supplementary Figure 2), suggesting that no single CpG methylation site is the causal factor for the phenotype in FH mutation-negative patients, but rather a result of the aggregate of a number of small methylation effects.

Our study has several limitations. Firstly, we measured DNA methylation in peripheral white blood cells, while the liver is known for its central role in LDL

homeostasis. The results we obtained from the analyses in peripheral blood cells may therefore not reflect the deranged hepatic LDL metabolism in our patients. Secondly, the mutation-negative FH patient group comprised patients in whom not only epigenetic factors, but also other unknown genetic phenomena such as intronic variants[36] or polygenic hypercholesterolemia may be the causal factor.[37] Thirdly, as can be appreciated from Supplementary Figure 2, the machine learning model supposedly identified some CpG sites that had two or three distinguishable groups of methylation levels (e.g., *MYCBP*-cg24051749), suggesting the presence of a SNP despite the fact that we rigorously excluded CpG sites near SNPs according to the Illumina manifest using widely accepted pre-processing steps before the analysis. The used gradient boosting model, however, allows for the identification of DNA methylation differences between the two groups despite the presence of skewed distributed methylation data because of a SNP. Further studies should be executed to assess whether the SNP has biological relevant effects in these patients or that they are coincidently identified. Moreover, in our study the group of FH mutation-negative patients were diagnosed with FH by the referring physician based on national guidelines[12,13] and thus potentially is a non-homogenous clinical FH group characterized by some characteristic differences with the FH mutation-positive patients. For example, the FH patients with a *LDLR* pathogenic variant were younger and the LDL-C levels were higher compared to FH variant negative patients (Table 1). Although age and lipoproteins can modulate DNA methylation[30], we estimate this effect to be minimal since we explored methylation only in patients with very high LDL-C levels (above 6 mmol/L and above the 99th percentile in the general population) in both groups. Furthermore, we selected only male participants who were not using statins, since these lipid lowering drugs have been shown to alter DNA methylation through reducing DNA methyltransferase mRNA levels[38], and are associated with less methylation in promotor regions of various genes.[39,40] It is also possible that other confounders, such as obesity, are present in the current study. Additionally, we enrolled a relatively small number of individuals in our study. Our stringent selection criteria to avoid spurious findings did not allow for a larger study group to be analysed. Lastly, in the current model we analysed the data at a group level, and we might therefore have missed specific causal methylation patterns that would explain the FH phenotype at an individual patient level.

Despite extensive sequencing efforts, a causative genetic variant is not found in a large proportion of patients with a clinical FH diagnosis.[1] Hence efforts to find novel factors causing the FH phenotype are deemed of great relevance. The data presented in the current study suggest that monogenic DNA methylation alterations are not a major contributing factor in FH in our cohort and thus are unlikely to be a common contributing factor to the FH phenotype in FH mutation-negative patients. Nevertheless, with the current study we have not excluded the possibility that rare monogenic DNA methylation alterations can cause FH in some individuals. On the other hand, the genome-wide methylation differences observed with advanced machine learning models between FH mutation-negative and FH mutation-positive subjects might suggest that a large number of small DNA methylation effects play a role in high plasma LDL-C. This phenomenon resembles the polygenic score where the inter individual differences in LDL-C levels are not explained by individual genetic variations but rather by the sum of a large number of small effect-size genetic factors. The question whether this is clinically relevant ensues from this finding. In contrast to monogenic FH, family screening for the presence of polygenic hypercholesterolemia, and epigenetic hypercholesterolemia, does not make sense as these do not follow an autosomal dominant inheritance pattern. At this stage, the treatment of these patients will not change either, since FH guidelines recommend the same aggressive lipid lowering with statins and add-on therapeutics, irrespective of the FH cause. Epigenetic hypercholesterolemia may only prove to be clinically relevant in case it has an impact on the efficacy of lipid lowering therapies.

This study was the first of its kind to be conducted in FH patients and tried to control for confounding by differences in lipid levels by the inclusion of two unique FH patient groups: those of interests, FH mutation-negative patients, and a group of FH mutation-positive patients. Although classical candidate gene analysis did, except for *CPT1A*, not reveal major DNA methylation differences in known lipid genes, a machine learning approach showed that FH mutation-negative patients are characterized by a different genome wide DNA methylation pattern compared to FH mutation-positive patients, with important model features for the genes *PRDM16* and *GSTT1*.

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DECLARATION OF INTERESTS

LFR is co-founder of Lipid Tools B.V. MN reports other from kaleido biosciences, other from caelus health, outside the submitted work. GKH has served as consultant and speaker for biotechnology and pharmaceutical companies that develop molecules that influence lipoprotein metabolism, including Regeneron, Aegerion Pfizer, Merck, KOWA, Sanofi, and Amgen; has served as principal investigator for clinical trials conducted with a.o. Amgen, Sanofi, Eli Lilly, Novartis, Kowa, Genzyme, Cerenis, Pfizer, Dezima, and AstraZeneca; has received research grants from ZonMW (Vidi grant [016.156.445]), Klinkerpad fonds, the European Union, Amgen, Sanofi, AstraZeneca, Aegerion, and Synageva; has received honoraria and investigator fees (to the Department of Vascular Medicine) for sponsor-driven studies and lectures for companies with approved lipid-lowering therapy in the Netherlands; and is partly employed by Novo Nordisk AS, Copenhagen, Denmark (0.7FTE) and the Amsterdam UMC, Amsterdam, the Netherlands (0.3FTE).

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SUPPLEMENTAL MATERIALS

Supplementary Table 1: Candidate gene tiers

Tier	Gene	Tier	Gene
1	*LDLR*	3	*KCNK17*
1	*APOB*	3	*HBS1L*
1	*PCSK9*	3	*GPR146*
2	*LDLRAP1*	3	*VIM*
2	*STAP1*	3	*CUBN*
2	*ABCG5*	3	*PHLDB1*
2	*ABCG8*	3	*PHC1*
2	*APOE*	3	*A2ML1*
2	*LIPA*	3	*TOM1*
3	*ANXA9*	3	*EVI5*
3	*CERS2*	3	*RAB3GAP1*
3	*EHBP1*	3	*RAF1*
3	*BRCA2*	3	*C6orf106*
3	*FN1*	3	*SPTY2D1*
3	*APOH*	3	*MAMSTR*
3	*PRKCA*	3	*ERGIC3*
3	*SPTLC3*	3	*PPP1R3B*
3	*SNX5*	3	*APOE*
3	*MTMR3*	3	*CETP*
3	*NYNRIN*	3	*TRIB1*
3	*INSIG2*	3	*FADS1*
3	*LINC01101*	3	*FADS2*
3	*CMTM6*	3	*FADS3*
3	*CSNK1G3*	3	*APOA1*
3	*SOX17*	3	*PIGV*
3	*UGT1A1*	3	*NR0B2*
3	*VLDLR*	3	*ACAD11*
3	*DLG4*	4	*DHCR24* - cg17901584
3	*PRARA*	4	*OXER1* - cg23759710
3	*SORT1*	4	*SQLE* - cg00285394
3	*MYLIP*	4	*NLRC5* - cg00285394
3	*HFE*	4	*SREBF2* - cg09978077
3	*LPA*	4	*TNIP1* - cg22178392
3	*PLEC1*	4	*STAT5A* - cg03001305
3	*ABO*	4	*CPT1A* - cg00574958
3	*ST3GAL4*	4	*PTAFR* - cg20460771
3	*OSBPL7*	4	*IL6R* - cg09257526

Supplementary Table 1: (Continued)

Tier	Gene	Tier	Gene
3	*TOP1*	4	*ACP1* - cg05464506
3	*MARC1*	4	*CIAO1* - cg25522181
3	*IRF2BP2*	4	*FYN* - cg08376209
3	*HMGCR*	4	*ARID3B* - cg02384859
3	*HLA-DRA*	4	*ARHGEF1* - cg12168357
3	*FRK*	4	*DEDD2* - cg13790259
3	*DNAH11*	4	*APOBEC3H* - cg06229674
3	*NPC1L1*	4	*ARHGEF9* - cg00369058
3	*CYP7A1*		
3	*GPAM*		
3	*BRAP*		
3	*HNF1A*		
3	*HPR*		
3	*MAFB*		
3	*ANGPTL3*		
3	*MIR148A*		
3	*LRPAP1*		
3	*TIMD4*		
3	*CILP2*		
3	*ASAP3*		
3	*ABCB11*		
3	*FAM117B*		
3	*PXK*		

Supplementary Table 2: CpG sites with >10% relative feature importance in machine learning model

CpG	Gene	Chromosome	Position	Relative Feature Importance
cg14265823	*PAX3*	chr2	223163326	100
cg02558132	*MYLK*	chr3	123411198	97.97
cg22162835	*TEAD3*	chr6	35457472	92.2
cg00415024		chr20	56044352	87.39
cg26426080	*PRDM16*	chr1	3039210	84.61
cg07051648	*NTN5/SEC1P*	chr19	49177693	76.65
cg05071823	*DOCK11*	chrX	117628671	61.17
cg05541727	*EXD3*	chr9	140277740	54.31
cg24051749	*MYCBP*	chr1	39340282	53.71
cg11478607	*GSTT1*	chr22	24384400	51.79
cg10020385	*MAF1*	chr8	145159706	49.8
cg11136235		chr10	81077552	48.55
cg16370685	*SETDB1*	chr1	150899163	46.59

Supplementary Table 2: (Continued)

CpG	Gene	Chromosome	Position	Relative Feature Importance
cg09138267	*LOC728743*	chr7	150102791	46.47
cg04900489		chr13	31272551	46.29
cg16685760		chrX	145701257	46.17
cg07336544	*KCNMA1*	chr10	79194347	44.54
cg00578917	*CYYR1*	chr21	27945542	42.69
cg20588438	*KNTC1*	chr12	123089881	41.65
cg15458017		chr17	9672274	41.5
cg21166457		chr14	106539756	41.34
cg23350716	*PPIAL4B*	chr1	147956744	40.89
cg17251658	*CBLN1*	chr16	49315754	40.29
cg14705391		chr2	16161202	39.64
cg07052624	*ZNF565*	chr19	36706445	38.29
cg26111308	*TVP23B*	chr17	18699050	38.25
cg09661370	*HOXA11AS*	chr7	27225299	38.02
cg04515200		chr5	135415762	37.1
cg00828689	*PIGZ*	chr3	196697064	36.07
cg09049879	*TEX30*	chr13	103423502	36.03
cg22867893	*ARRB1*	chr11	74988026	34.02
cg12739319	*INSL6*	chr9	5186766	32.06
cg10207850		chr2	26955177	32.04
cg19384241		chr2	55393977	31.58
cg20758756		chr18	9321560	31
cg04497520	*PHF13*	chr1	6673429	30.44
cg07158503		chr5	135415693	29.79
cg14755254	*ERICH1*	chr8	637813	29.18
cg03515999	*PSMD5*	chr9	123606332	28.72
cg18091165	*BAT1*	chr6	31509352	27.93
cg12367543		chr22	48602194	27.92
cg06489993	*PPP5D1*	chr19	47082996	25.88
cg20707527		chr8	105343122	25.85
cg25374269		chr13	44887281	25.71
cg22713356		chr15	30763199	24.55
cg02333792	*RASGRF1*	chr15	79269735	24.14
cg27219185		chr20	11360021	24.1
cg03881738		chr6	170547103	24.08
cg27586797		chr5	13664584	23.66
cg05745656		chr6	22271391	23.34
cg19872188	*LOC285740*	chr6	143884511	23.08
cg22138327	*GTF3A*	chr13	27999177	22.59
cg05194426	*CYP2E1*	chr10	135343193	21.64

Supplementary Table 2: (Continued)

CpG	Gene	Chromosome	Position	Relative Feature Importance
cg16604835	*NRCAM*	chr7	108043396	21.63
cg26999053	*MYH10*	chr17	8414418	20.92
cg14614539	*NOTCH4*	chr6	32170458	20.87
cg01053087	*ERICH1*	chr8	637909	20.78
cg01519350	*ARMC8*	chr3	137906342	20.26
cg04255814	*LINC00222*	chr6	109072980	20.24
cg09125754	*POTEF*	chr2	130886714	19.81
cg14378789		chr2	112483666	19.68
cg07629776	*FRMD4A*	chr10	13972210	19.61
cg08159444	*PNMA5*	chrX	152160229	19.53
cg10667338	*LIN7B*	chr19	49617042	18.28
cg12002139	*SYNJ2*	chr6	158478872	17.73
cg20432732	*UBR4*	chr1	19451037	17.63
cg17662387	*ABLIM1*	chr10	116286880	17.34
cg09506600	*OR2L13*	chr1	248100228	17.3
cg18434912	*AMD1*	chr6	111194786	17.21
cg20227896	*SPTBN4*	chr19	41069670	17.14
cg13984289	*MYH13*	chr17	10220829	17.13
cg11787167	*NPAS3*	chr14	33407370	17.09
cg12718519	*PRKCZ*	chr1	2058417	17.04
cg21754534	*DGKD*	chr2	234294601	16.78
cg20787649	*PADI4*	chr1	17636898	16.67
cg06069504		chr19	10707453	16.44
cg19469447	*CYP2E1*	chr10	135341870	16.41
cg25533845	*MTRF1*	chr13	41838126	16.38
cg21415084		chr12	84218134	16.17
cg08291957	*P4HA1*	chr10	74821035	16.13
cg09723679	*RNF145*	chr5	158634676	16.06
cg07167872	*PM20D1*	chr1	205819463	16.04
cg08164151		chr12	131118432	15.97
cg10980791	*FAM155A*	chr13	108118957	15.94
cg02087342	*TP53*	chr17	7579047	15.86
cg02673002	*RNF169*	chr11	74459317	15.75
cg10154826	*FAM8A1*	chr6	17600994	15.64
cg20347269	*P2RX1*	chr17	3808157	15.64
cg19850149		chr5	180397496	15.45
cg04898932		chr22	35645717	15.36
cg11261447	*NLRP1*	chr17	5465054	15.3
cg09840753	*WDR25*	chr14	100844352	15.18
cg26491092		chr1	23510886	15.04

Supplementary Table 2: (Continued)

CpG	Gene	Chromosome	Position	Relative Feature Importance
cg17190362	*LAMA4*	chr6	112475675	14.86
cg06427702	*PPAPDC1A*	chr10	122228021	14.84
cg14398247	*ARFGAP3*	chr22	43216462	14.83
cg08299859		chr10	13259274	14.73
ch.7.770055F		chr7	31513616	14.68
ch.8.1073630F	*KIAA0146*	chr8	48637817	14.65
cg11878555		chr1	31920316	14.64
cg03485872		chr4	127657778	14.59
cg13033971		chr13	46291973	14.27
cg14331362		chr2	242948396	14.21
cg27629640	*ALOXE3*	chr17	8022337	14.18
cg13023205		chr17	81060243	14.13
cg06344265	*GRIK4*	chr11	120530973	13.95
cg25420767	*ABLIM3*	chr5	148620091	13.56
cg12067536	*ARHGAP42*	chr11	100558822	13.54
cg10096454	*LDB1*	chr10	103880868	13.15
cg06675417		chr18	77292443	13.13
cg18792536	*UPK3B*	chr7	76145562	13.11
cg09296044	*CCNDBP1*	chr15	43477606	13.08
cg06734157		chr3	150066462	13.03
cg16079430		chr5	10856757	12.95
cg27281559		chrX	145701263	12.94
cg21240272	*NXNL2*	chr9	91174472	12.84
cg19090364	*ITCH*	chr20	32972071	12.83
cg01451203	*CYFIP1*	chr15	22955743	12.82
cg02352685	*BTNL8*	chr5	180377617	12.76
cg14554157	*BCL11A*	chr2	60780027	12.75
cg17215819	*LOC101927502*	chr9	84386662	12.71
cg21149944	*ZNF718*	chr4	124344	12.67
cg01780345	*CFAP58*	chr10	106147097	12.56
cg13691793	*OSBPL1A*	chr18	21750891	12.51
cg14353184		chr6	32584893	12.46
cg22730047		chr1	161410551	12.34
cg26725559		chr18	9321178	11.98
cg15098922		chrX	114579157	11.97
cg03966322	*NADK2*	chr5	36242514	11.87
cg04221355	*BRE*	chr2	28114116	11.65
cg25880954	*MGC12982*	chr1	47900630	11.63
cg19347782	*MICAL2*	chr11	12159762	11.63
cg09062922		chr5	131436890	11.6

Supplementary Table 2: (Continued)

CpG	Gene	Chromosome	Position	Relative Feature Importance
cg11186706		chr14	54815745	11.43
cg02331830	*PLEC1*	chr8	145008288	11.41
cg12628883	*PRTFDC1*	chr10	25219837	11.39
cg20517941	*LOC100507140*	chr2	201600636	11.16
cg02484732	*COL21A1*	chr6	56096198	11.06
cg17460047	*ZNRF2*	chr7	30323918	10.97
cg07796016	*LCE1C*	chr1	152779584	10.84
cg16106431	*ABLIM2*	chr4	8034335	10.84
cg10681804	*GGT7*	chr20	33433114	10.82
cg11304899	*LINC01055*	chr13	46267080	10.8
cg07504457	*VPS28*	chr8	145652410	10.78
cg11688874	*WAC*	chr10	28822482	10.77
cg09015484		chr9	96929106	10.73
cg16511841	*C6orf167*	chr6	97730490	10.7
cg21635219	*ACSL4*	chrX	108973840	10.61
cg16145915	*ZFAND2A*	chr7	1198662	10.57
cg00013655	*PIGQ*	chr16	629015	10.49
cg10625758	*WBP2*	chr17	73851514	10.46
cg20792978	*ZDHHC9*	chrX	128977934	10.36
cg01462207	*POLE*	chr12	133202012	10.3
cg19437126		chr12	91720401	10.24
cg05571310	*KIF19*	chr17	72350354	10.1

CpG sites, their respective linked genes and genomic positions, that are most predictive for distinguishing FH mutation-positive and FH mutation-negative patients based on the machine learning model measured as relative feature importance. Only CpG sites with a relative feature importance >10% are reported. Genomic positions as provided in human genome build – hg19.

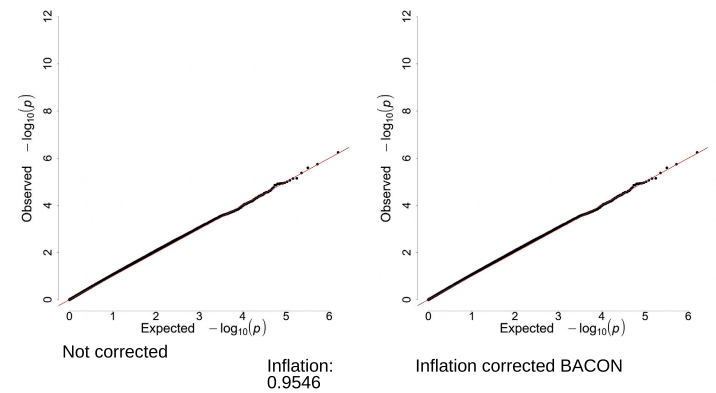
Supplemental Table 3: Association between identified CpG sites of interest methylation and expression in public databases

	SMART		MEXPRESS	
CpG	**R***	**P-value**	**R****	**P-value**
Candidate Gene Analysis				
CPT1A- cg00574958	-0.083	0.093	-0.198	<0.001
Top Features Machine Learning Model				
PAX3-cg14265823	-0.12	0.019	-0.0862	≥0.05
MYLK-cg02558132	0.019	0.71	0.0732	≥0.05
PRDM16-cg26426080	0.38	1.5×10^{-15}	0.496	<0.001
NTN5-cg07051648	0.071	0.15	NA	NA
DOCK11-cg05071823	0.029	0.56	0.0971	≥0.05
MYCBP-cg24051749	NA	NA	NA	NA
GSTT1-cg11478607	NA	NA	-0.876	<0.001
cg11136235	NA	NA	NA	NA
SETDB1-cg16370685	-0.26	2.2×10^{-7}	-0.157	<0.01
LOC728743-cg09138267	NA	NA	NA	NA
cg16685760	NA	NA	NA	NA

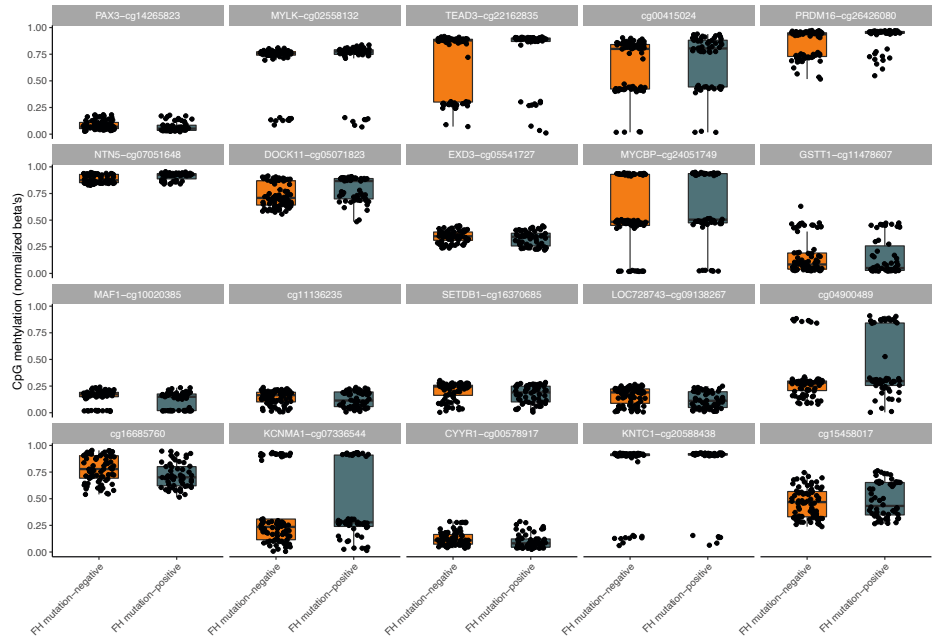
Correlation and P-values for CpG methylation and expression were derived from two publicly accessible databases for tumor specific tissues (SMART [Li 2019] and MEXPRESS [Koch 2015]). The reported correlation was derived from liver hepatocellular carcinoma(LIHC) subset. Since these two databases only contain CpG sites that are available on the 450k Illumina Infinium beachip assay, only those CpG sites are reported in this table. * Spearman rank correlation. ** Pearson correlation.

Supplementary Table 4: Next-generation sequencing gene panel

GENE	
LDLR	*SCARB1*
APOB	*CETP*
PCSK9	*LIPG*
LIPA	*LIPC*
LDLRAP1	*APOC3*
ABCG5	*LPL*
ABCG8	*APOC2*
STAP1	*APOA5*
ANGPTL3	*GPIHBP1*
MTTP	*LMF1*
MYLIP	*APOE*
ABCA1	*SAR1B*
LCAT	*CYP27A1*
APOA1	



Supplementary Figure 1: Inflation correction with BACON package of candidate gene analysis



Supplementary Figure 2: Methylation of the top 20 individual CpG sites derived from the machine learning model

PART II

The role of ANGPTL3 in dyslipidemia

8

The next generation of triglyceride-lowering drugs: will reducing apoC3 or angptl3 reduce CVD?

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ABSTRACT

Purpose of review: ApoC-III and angptl3 have emerged as key regulators of triglyceride metabolism. Based on Mendelian randomisation studies, novel therapeutic strategies inhibiting these proteins using monoclonal antibodies or gene silencing techniques might reduce residual cardiovascular disease (CVD) risk in dyslipidemic patients. This paper aims to review the role of apoC-III and angptl3 in triglyceride metabolism and combine early clinical evidence of CVD reducing potential of these new therapeutic targets.

Recent findings: Angptl3 inhibition by monoclonal antibody or antisense therapy has recently completed phase I and II studies, respectively, and demonstrate robust apoB lowering up to 46%. Volanesorsen is an antisense therapy approved for patients with extremely elevated plasma triglyceride levels where it showed no consistent apoB reduction. However, the GalNAc-conjugated oligonucleotide showed moderate (up to ~30%) apoB reduction in a phase 1/2a dose-finding study.

Summary: Angptl3 and apoC-III are novel targets in lipoprotein metabolism that reduce triglycerides when inhibited. The expected CVD risk reduction may be mediated through reduced triglyceride-rich lipoprotein particle number, reflected by apoB, rather than triglyceride reduction per se. Limited human evidence shows that apoC-III and angptl3 inhibition both potently lower triglycerides, but since angptl3 inhibition reduces apoB more robustly it may be expected to confer more favorable CVD risk reduction.

INTRODUCTION

Within the spectrum of lipid abnormalities, elevated low-density lipoprotein cholesterol (LDL-C) is the most firmly established causal risk factor for CVD.[1,2] Discovery of therapeutic targets and ensuing development of potent LDL-C lowering drugs (i.e. statins, PCSK9 inhibitors) can be counted among the success stories of modern medicine. While cardiovascular risk reduction through LDL-C lowering has been in the limelight in recent decades, it should not be forgotten that multiple large epidemiological as well as genetic studies have firmly established that triglyceride levels are also independently associated with CVD.[3,4]

Following the potential to eradicate LDL-C by applying combination therapies, efforts to reduce the residual CV-burden have shifted towards additional triglyceride lowering strategies. The search for novel therapeutic targets has revealed two promising candidates: apolipoprotein C-III (apoC-III) and angiopoietin like protein 3 (angptl3).

In the current article we review the roles of apoC-III and angptl3 in triglyceride metabolism and their relation to cardiovascular disease. Next, we combine early clinical evidence from novel therapeutic strategies aimed at these targets to speculate whether apoC-III or angptl3 inhibition will indeed reduce CVD.

The role of apoC-III and angptl3 in triglyceride metabolism

Plasma triglycerides routinely measured in clinical laboratories entail triglycerides that are present in all lipoprotein classes, but predominantly in chylomicrons and very-low density lipoproteins (VLDL). Chylomicrons are assembled in the intestine and deliver exogenous (dietary) triglycerides to peripheral tissues. VLDL is excreted into the circulation by the liver and contains endogenous triglycerides derived from lysosomal breakdown of other triglyceride-rich lipoproteins (TRL) or by *de novo* lipogenesis in the liver. Triglycerides in both chylomicrons and VLDLs undergo intravascular lipolysis by Lipoprotein Lipase (LPL), resulting in the release of free fatty acids to be used as fuel for peripheral tissues (e.g. muscles and heart) or stored as fat (i.e., adipose tissue). The left-over particles, so called, remnant lipoproteins (i.e. chylomicron remnants and intermediate-density lipoproteins (IDLs)) can be cleared from the circulation by the

liver. Alternatively, IDLs can be further hydrolyzed resulting in LDL particles. These processes are outlined in Figure 1.

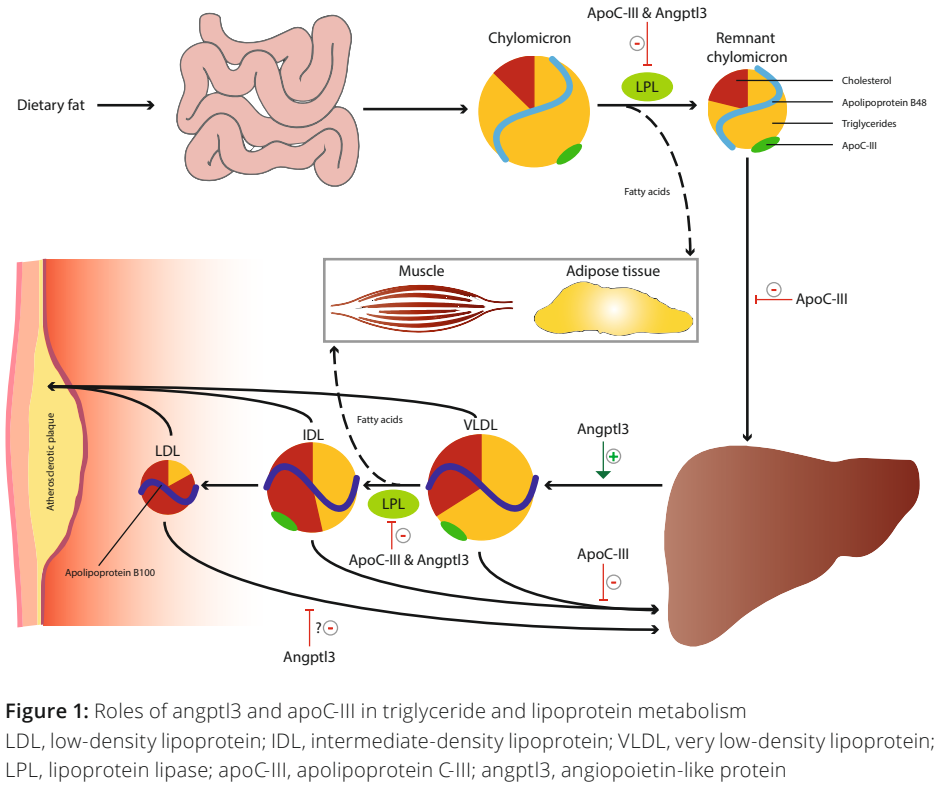

Figure 1: Roles of angptl3 and apoC-III in triglyceride and lipoprotein metabolism
LDL, low-density lipoprotein; IDL, intermediate-density lipoprotein; VLDL, very low-density lipoprotein; LPL, lipoprotein lipase; apoC-III, apolipoprotein C-III; angptl3, angiopoietin-like protein

All processes involved in triglyceride metabolism are highly controlled by multiple proteins. Two pivotal proteins regulating triglyceride metabolism are apoC-III and angptl3.[5,6] ApoC-III is a glycoprotein that is primarily synthesized in the liver and to a lesser extent in the intestine. ApoC-III binds to the surface of almost all types of lipoproteins, predominantly to high-density lipoproteins (HDL), but also to LDL and TRLs such as chylomicrons and VLDL.[7] Two mechanisms by which apoC-III increases triglyceride levels have been proposed: inhibition of LPL-mediated lipolysis of TRLs and/or attenuation of hepatic TRL uptake (See Figure 1). Which mechanism predominates *in vivo* in humans is a matter of continued debate [8] Hepatic TRL uptake appears important since patients with Familial Chylomicronemia Syndrome (i.e. in full absence of residual LPL activity) still displayed a 70% reduction of triglyceride levels following administration of an *APOC3* antisense therapy.[9]

More recently, a stable isotope study compared lipoprotein metabolism in five heterozygous carriers of an *APOC3* null allele with unaffected siblings matched for age and gender. It showed that partial loss of apoC-III synthesis had no significant effect on the uptake of VLDL remnants by the liver, whereas the conversion rate of VLDL to LDL particles was higher.(Reyes-Soffer et al., 2019) Angiopoietin-like protein 3 (angptl3) is solely produced by the liver in humans and also functions as a potent inhibitor of LPL, especially in the presence of angptl8.[6] Its triglyceride raising effects may also originate from increased secretion of VLDL triglycerides from the liver[10] or increased apoB secretion from the liver.[11] Since angptl3 is not associated with increased hepatic apoB production, the exact mechanism leading to increased VLDL excretion remains to be elucidated.[6,10]

Causes of hypertriglyceridemia

VLDL particles harbor the largest part of plasma triglycerides, which is therefore used to reflect plasma VLDL levels. For clinical and epidemiological purposes, hypertriglyceridemia is classified as mild-to-moderate (triglyceride levels ranging from 2.0 to 10 mmol/L (177 - 886 mg/dL)) or severe hypertriglyceridemia (>10 mmol/L (886 mg/dL).[12] In the latter group, pancreatitis is the most predominant complication of hypertriglyceridemia.[13] The rarest and most severe forms of hypertriglyceridemia are caused predominantly by homozygous defects in genes related to triglyceride metabolism (i.e. LPL, APOC2, APOA5, LMF1, GPIHBP1), termed Familial Hyperchylomicronemia Syndrome. With decreasing levels of triglycerides, the likelihood of finding a genetic cause for hypertriglyceridemia decreases from heterozygous large-effect variants in the above mentioned genes, via accumulated common small-effect variants in multiple genes (polygenic hypertriglyceridemia) [14] to multifactorial causes such as lifestyle related factors (high fat or high glycemic diet, alcohol consumption, metabolic syndrome, diabetes mellitus type 2).[12] The pathophysiology and diagnosis of hypertriglyceridemia is extensively described elsewhere.[12]

Triglyceride-rich lipoproteins and CVD risk

Several lines of research link triglyceride levels to cardiovascular disease. Multiple large cohort studies have shown that both fasting and non-fasting triglyceride levels are independently associated with CVD. For example, in the Copenhagen

General Population and Copenhagen City Heart studies non-fasting triglycerides of 6.6 mmol/L were associated with a 5.1 (95% CI: 3.5-7.2) hazard ratio (HR) for myocardial infarction.[15] Similarly, the Emerging Risk Factors Collaboration showed that 1-standard deviation increase in fasting triglycerides was associated with a HR of 1.37 (95% CI: 1.31-1.42) for coronary heart disease.[16] However, this association was no longer significant after adjustment for high-density lipoprotein cholesterol (HDL-C) and non-HDL-C. In fact, since non-HDL-C plasma concentration reflects the plasma cholesterol present in all apolipoprotein B (apoB)-containing particles, the latter finding highlights the importance of apoB as a measure for CVD risk in hypertriglyceridemia.[17,18] Recent genetic data lend further support to this observation. Thus, in the UK biobank, Ference et al. elegantly showed that the achieved CVD risk reduction over a lifetime is proportional to genetically reduced apoB levels using mendelian randomization methods to investigate the effect of genetically lowering triglycerides or cholesterol.[4] Both triglyceride lowering genetic variants in the LPL gene (reflecting triglyceride clearance pathways) as well as genetic variants in the LDL receptor gene (reflecting cholesterol clearance pathway) were associated with an identically lower risk for coronary heart disease per 10 mg/dL (0.1 g/L) lower levels of apoB (odds ratios [OR] of 0.771 and 0.773, respectively).

To date, randomized controlled trials (RCTs) investigating potent triglyceride-lowering drugs (fibrates) have shown inconsistent results concerning VLDL-LDL and apoB-reduction. The vast majority of fibrate RCTs failed to demonstrate a benefit of (marked) TRIGLYCERIDE-lowering on CVD outcome [19]. However, subsequent posthoc analyses demonstrated a significant CVD-benefit in patients characterized by hypertriglyceridemia (> 200 mg/dL) and/or low HDL-C (<40 mg/dL) [19]. As pointed out by Sniderman et al, the fibrate trial results may support the notion that lowering of atherogenic apoB-particles but not the triglyceride reduction itself drives CVD benefit from fibrate therapy.[17] More recently, the impact of fish oils on CVD outcome were also reported. The REDUCE-IT study revealed a modest triglyceride-lowering of approximately 19% with a concomitant marked CVD reduction of -25% major adverse cardiovascular events [20], leading to the hypothesis that the benefits of icosapent ethyl (a highly purified eicosapentaenoic acid (EPA) ethyl ester) are likely due to pleiotropic effects of the fish oil rather than its triglyceride-lowering properties [21]. In contrast, the STRENGTH study (using omega-3 carboxylic acid; a

mixture of EPA and docosahexaenoic acid (DHA)) was recently discontinued due to futility, and its results await presentation and publication[22].

Collectively, these RCTs imply that the benefit on CVD risk of novel triglyceride-lowering drugs may not be proportional to the achieved TRIGLYCERIDE reduction and more likely relate to the total reduction of all atherogenic apoB containing lipoprotein particles [23].

Targeting apolipoprotein C-III (apoC-III)

Ample genetic evidence links apoC-III levels with CVD risk. In 2008, Pollin et al. were the first to show how a rare *APOC3* null-mutation found in the Lancaster county Amish population (n=1033) correlated with reduced triglycerides levels and CVD risk as measured by lower coronary calcium score in mutation-carriers.[24] This finding was later replicated in a multi-ethnic cohort in the US.[25] In 2014, two landmark studies firmly linked *APOC3* loss of function (LOF) mutations with around 40% lower triglycerides and 40% reduction in coronary heart disease.[26,27] In the Framingham heart study, every 1 mg/dL decrease in plasma apoC-III levels was associated with 4% decrease in CVD risk, adjusted for age and sex.[26]

In 2018, a meta-analysis of 137895 individuals showed that in the 776 *APOC3* LOF carriers, remnant cholesterol was 43% lower whereas the mean LDL-C was only non-statistically significantly decreased by 4%.[28] 37 of the observed 41% lower risk of ischemic vascular disease could be explained by reduction in remnant cholesterol. Specifically, apolipoprotein B was 13% lower in heterozygous APOC3 LOF carriers compared to non-carriers. Interestingly, homozygous loss of APOC3 function is not lethal. In the Pakistani kindred in which the homozygous mutations were discovered, individuals were healthy, and post-prandial triglycerides elevation was markedly blunted.[29]

Capelleveen et al. measured plasma lipoproteins (including apoC-III and apoB) and assessed their predictive capacity for coronary artery disease (CAD) in the European Prospective Investigation of Cancer (EPIC)-Norfolk cohort comprising 1879 controls and 832 cases with CAD.[30] The top apoC-III quintile predicted CAD after adjustment for traditional risk factors and lipid-lowering therapy (OR 1.50, 1.13-

1.98), but lost statistical significance after adjusting for other lipoprotein variables. This may strengthen the notion that CVD risk is mediated by apoB containing TRL particles rather than by apoC-III or triglycerides per se.

Results from the first in-human apoC-III inhibiting therapy were published in 2014.[9] This antisense oligonucleotide therapy targets apoC-III expression at mRNA level and showed spectacular decrease in triglyceride levels in patients with familial chylomicronemia syndrome[31,32]. The therapy (volanesorsen) gained market access in several European countries in 2019 but was not approved in the United States, primarily due to concerns of drug-induced side effects comprising platelet count reduction, injection site reactions and flu-like symptoms following antisense administration.[32]

In order to overcome these side effects, the antisense oligonucleotide sequence was coupled to a GalNAc3 moiety to improve specific uptake in the liver and increasing potency by at least 15x.[33] This AKCEA-APOCIII-LRx has recently completed a phase 1/2a dose escalating trial without safety concerns and was able to markedly reduce triglycerides and yielded a more favorable lipid profile (see Table).[33] ApoB reduction was significant (up to ~30% in the highest dosed cohort). To date, no (plans for a) CVD endpoint trial have been announced.

Targeting angiopoietin-like 3 (angptl3)

Multiple studies have shown that rare loss of function variants in the angptl3 coding gene *ANGPTL3* are associated with decreased plasma levels of triglycerides, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, and with decreased risk for coronary heart disease (CHD).[34,35]. These findings were preceded by the discovery of combined hypolipidaemia phenotypes (i.e. low LDL-C, low HDL-C, and low triglyceride levels) in *ANGPTL3* knock-out mice [36] and later in a family with autosomal dominantly inherited hypolipoproteinemia with loss-of-function variants in *ANGPTL3*.[37]

Additionally, angptl3 plasma levels have been shown to be associated with myocardial infarction in the PROMIS cohort (a matched case-control study of Pakinstani patients with myocardial infarction comprising 1493 cases and 3231

matched controls).[35] Patients in the lowest two tertiles of angptl3 plasma levels had a reduced risk of MI compared with the highest tertile with odds ratios of 0.75 (95%-CI: 0.64-0.88) and 0.65 (95%-CI: 0.55-0.77), respectively.

Unsurprisingly, these results have led to the development of two pharmacological compounds that target angptl3. The first one, IONIS-ANGPTL3-L_{Rx}, a GalNAc-conjugated antisense oligonucleotide directed against *ANGPTL3* mRNA, showed dose-dependent and potent reductions in all lipid fractions in a phase 1 dose-finding study in 44 healthy volunteers.[38] Especially triglycerides were lowered up to 63% and LDL-C up to 33%. This coincided with a maximum decrease in apoB of 26% (See Table). No serious adverse events were observed in this trial. The IONIS-ANGPTL3--L_{Rx}, is currently being investigated in subjects with hypertriglyceridemia, type 2 diabetes mellitus, and nonalcoholic fatty liver disease (NCT03371355).

In parallel, a fully human monoclonal antibody directed against angptl3, evinacumab, was developed and investigated for its efficacy in multiple patient categories. A phase 1, double-blind, single ascending dose study in 83 healthy volunteers with triglyceride levels between 1.7-5.1 mmol/L or LDL-C levels above 2.6 mmol/L received different doses of Evinacumab subcutaneously or intravenously, which resulted in 50% and 28% reduction in triglyceride and LDL-C in the highest dosed intravenous treatment arms respectively.[34] The full results of this single ascending dose study were later combined with a multiple ascending dose study in 56 healthy subjects.[39] These results showed an increase in triglyceride- and LDL-C-lowering efficacy at day 15 when subjects received multiple subcutaneously doses (see Table). Evinacumab was subsequently administered to 9 homozygous familial hypercholesterolemia (FH) patients, which resulted in a mean LDL-C reduction of almost 50%.[40] Evinacumab was generally well tolerated and safe. In the three reported trials no serious adverse events were observed, although there were numerically more liver enzyme abnormalities and headaches reported in the groups receiving active treatment.[34,38–40] Evinacumab is now further investigated in patients with homozygous and heterozygous familial hypercholesterolemia in multiple trials (NCT03399786 & NCT03175367), as well as in patients with severe hypertriglyceridemia (NCT03452228).

For both the antisense oligonucleotide and monoclonal antibody therapy, the observed apoB reductions ranged from -14 to -46%. Depending on the baseline apoB levels, these reductions can be expected to result in a proportional risk reduction for CVD, as predicted by mendelian randomization studies.[4]

CONCLUSIONS & FUTURE PERSPECTIVES

Triglyceride-rich particles causally and independently contribute to CVD risk. RCTs evaluating the effect of lowering TRLs have not (yet) provided sufficient evidence to demonstrate a consistent CVD risk reduction. Indirect genetic evidence implies a predominant effect of a reduction in TRL particle number, best reflected by apoB or non-HDL cholesterol. ApoC-III and angptl3 are two novel targets in lipoprotein metabolism that are currently being investigated in clinical trials for reducing triglyceride levels and TRL particle number. The limited data available show marked triglyceride reduction with moderate apoB reduction following apoC-III inhibition, whereas both triglycerides and apoB are reduced following angptl3 inhibition. Combining these RCT data with recent mendelian randomization studies, it can be postulated that the angptl3 inhibition offers the best profile for reducing CVD risk with a marked apoB reduction ranging from -14 to -46% in RCTs. Conversely, both apoC-III and angptl3 genetic data revealed CVD benefit. Further studies using these compounds are eagerly awaited to evaluate whether and to what extent reduction of TRLs will be able to further reduce the residual CV burden in patients.

CONFLICTS OF INTEREST

LFR and TRT declare no conflicts of interest. ESGS reports personal fees from Novartis, personal fees from Amgen, personal fees from Sanofi-Regeneron, personal fees from Mylan, personal fees from Esperion, unrelated to the submitted work. All fees were paid to the institution

Table: Effect of angptl3 or apoC-III inhibition on lipoproteins in humans

Study	Target	Intervention	Study design	Study population	Dose range	Outcome measured at	Main findings*			
							LDL-C	HDL-C	TG	apoB
Graham *et al.* 2017 [38]	angptl3	2nd gen GalNAc ASO IONIS-ANGPTL3-L_{Rx}	Phase 1, double-blind, placebo controlled	Healthy volunteers with TG >1.7 mmol/L, n = 44	Sc: 10-60 mg	Day 43	-33±10%	-27±16%	-63±11%	-26±11%
Dewey *et al.* 2017*** [34]	angptl3	Monoclonal antibody	Phase 1, double-blind, placebo controlled	Healthy volunteers with TG >1.7 & <5.1 mmol/L, and/or LDL-C >2.6 mmol/L, n = 83	Sc: 75-250 mg Iv: 5-20 mg/kg	Day 15	-28±17%	-27±10%	-63 [56, 70]%	NR
Gaudet *et al.* 2017 [40]	angptl3	Monoclonal antibody	Phase 2, open-label, single-arm	HoFH patients, n = 9	250 mg sc on day 1, 15 mg/kg iv on day 15	Week 4	-49±23%	-36±16%	-47 [-38,-57]%	-46±18%
Ahmad *et al.* 2019*** [39*]	angptl3	Monoclonal antibody	Phase 1, double-blind, placebo controlled, single ascending dose study	Healthy volunteers with TG >1.7 & <5.1 mmol/L, and/or LDL-C >2.6 mmol/L, n = 83	Sc: 75-250 mg	Day 15	~-15%**	~-12%**	~-34%**	~-14%**
					Iv: 5-20 mg/kg	Day 15	~-28%**	~-27%**	~-64%**	~-27%**
			Phase 1, double-blind, placebo controlled, multiple ascending dose study	Healthy volunteers with TG >1.7 & <5.1 mmol/L, and/or LDL-C >2.6 mmol/L, n = 56	Sc: 75-250 mg	Day 15	~-20%**	~-13%**	~-72%**	~-19%**
					Iv: 20 mg/kg	Day 15	~-40%**	~-31%**	~-58%**	~-38%**
Graham *et al.* 2013 [41]	apoC-III	2nd gen ASO Volanesorsen	Phase 1 double-blind, placebo-controlled, dose escalation clinical study	Healthy volunteers aged 18-55, normal TG and LDL, n = 33	Sc: 50-400 mg	Day 29	-4%	+8%	-44%	NR

Table: (Continued)

Study	Target	Intervention	Study design	Study population	Dose range	Outcome measured at	Main findings*			
							LDL-C	HDL-C	TG	apoB
Gaudet *et al.* 2014 [8]	apoC-III	2nd gen ASO Volanesorsen	Open label study	FCS patients with TG 15.9-23.5 mmol/L, n = 3	Sc. 300mg weekly	Day 92	+3, +103, +173% (yet all <1 mmol/L)	+50, +163, +121%	-56, -86, -64%	-46, -55, -74% ****
Gaudet *et al.* 2015 [31]	apoC-III	2nd gen ASO Volanesorsen	Randomized, double-blind, placebo-controlled, dose-ranging phase 2 study	Patients with severely elevated TG aged 28-81. TG 6.6±3.3 mmol/L, n = 57	Sc: 100-300 mg weekly	Day 92	+118±177%	+46±24%	-71±14%	+1±36 ****
Gouni-Berrhold *et al.* 2017 (abstract) [42]	apoC-III	2nd gen ASO Volanesorsen	Phase 3, double-blind placebo controlled randomized trial	Fasting TG >5.6 mmol/L, n = 113	Sc: 300 mg weekly	3 months	NR	NR	-72%	NR
Witztum *et al.* 2019 [32*]	apoC-III	2nd gen ASO Volanesorsen	Phase 3, double-blind, placebo controlled randomized trial	FCS patients aged 20-75, median TG 22.4 mmol/L, n = 66	300 mg sc weekly	3 months	+136%	+46%	-77%	+20%
Alexander *et al.* 2019 [33**]	apoC-III	2nd gen GalNAc ASO AKCEA-APOCIII-L_{Rx}	Phase 1 /2a, double-blind, placebo controlled single ascending dose study	Healthy volunteers with TG >1.0 mmol/L, n = 40	Sc: 10-120mg	Day 15	-7%±17	+62±28%	-77±4%	-26±7%
			Multiple ascending dose study	Healthy volunteers with TG >2.3 mmol/L, n = 27	Sc: 10, 30mg weekly dosing Sc: 60mg 4-week dosing cohort	Day 43 Day 92	-17±18% -22±15%	+56±30% +76±50%	-71±10% -65±16%	-26±13% -30±6%

* For studies that tested multiple doses, the maximum mean reduction per lipid class is displayed. ** Numbers are estimated from figures reported in Ahmad et al.[39] *** The data reported in Dewey et al. 2017[34] is the same for the single-arm ascending dose study with Evinacumab iv as reported in Ahmad et al.[39] **** Non-HDL is reported. angptl3, angiopoietin-like protein 3; ASO, antisense oligonucleotide; FCS, familial chylomicronemia syndrome; GalNAc, N-acetylgalactosamine; ow, once weekly; TG, triglyceride; HoFH, homozygous familial hypercholesterolemia; Max, maximum; sc, subcutaneously; iv, intravenously; NR, not reported.

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Evinacumab for Homozygous Familial Hypercholesterolemia

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ABSTRACT

Background: Homozygous familial hypercholesterolemia is characterized by premature cardiovascular disease caused by markedly elevated levels of low-density lipoprotein (LDL) cholesterol. This disorder is associated with genetic variants that result in virtually absent (null–null) or impaired (non-null) LDL-receptor activity. Loss-of-function variants in the gene encoding angiopoietin-like 3 (ANGPTL3) are associated with hypolipidemia and protection against atherosclerotic cardiovascular disease. Evinacumab, a monoclonal antibody against ANGPTL3, has shown potential benefit in patients with homozygous familial hypercholesterolemia.

Methods: In this double-blind, placebo-controlled, phase 3 trial, we randomly assigned in a 2:1 ratio 65 patients with homozygous familial hypercholesterolemia who were receiving stable lipid-lowering therapy to receive an intravenous infusion of evinacumab (at a dose of 15 mg per kilogram of body weight) every 4 weeks or placebo. The primary outcome was the percent change from baseline in the LDL cholesterol level at week 24.

Results: The mean baseline LDL cholesterol level in the two groups was 255.1 mg per deciliter, despite the receipt of maximum doses of background lipid-lowering therapy. At week 24, patients in the evinacumab group had a relative reduction from baseline in the LDL cholesterol level of 47.1%, as compared with an increase of 1.9% in the placebo group, for a between-group least-squares mean difference of –49.0 percentage points (95% confidence interval [CI], –65.0 to –33.1; P<0.001); the between-group least-squares mean absolute difference in the LDL cholesterol level was –132.1 mg per deciliter (95% CI, –175.3 to –88.9; P<0.001). The LDL cholesterol level was lower in the evinacumab group than in the placebo group in patients with null–null variants (–43.4% vs. +16.2%) and in those with non-null variants (–49.1% vs. –3.8%). Adverse events were similar in the two groups.

Conclusions: In patients with homozygous familial hypercholesterolemia receiving maximum doses of lipid-lowering therapy, the reduction from baseline in the LDL cholesterol level in the evinacumab group, as compared with the small increase in the placebo group, resulted in a between-group difference of 49.0 percentage points at 24 weeks.

INTRODUCTION

Homozygous familial hypercholesterolemia is a rare genetic disorder of lipid metabolism affecting approximately 1 in 300,000 persons. The condition is most often caused by the presence of loss-of-function variants in the low-density lipoprotein (LDL) receptor, which leads to low or absent hepatic clearance of LDL cholesterol from the circulation.[1] Genetic alterations that cause a virtually complete absence of LDL-receptor expression (null homozygotes) result in higher LDL cholesterol levels than alterations that partially reduce LDL-receptor activity with either two non-null alleles or one null and one non-null allele (non-null homozygotes).[2]

This disorder is characterized by a markedly elevated plasma LDL cholesterol level from birth, which results in an increased risk of premature atherosclerotic cardiovascular disease.[1] Attempts to lower cholesterol levels often require multiple lipid-lowering drugs and LDL apheresis.[1,3] Despite these therapies, a majority of patients with this disorder do not reach guideline-recommended LDL cholesterol levels.[4] Because traditional lipid-lowering therapies such as statins and proprotein convertase subtilisin–kexin type 9 (PCSK9) inhibitors act by up-regulating LDL-receptor expression, they have little efficacy in these patients and virtually no activity in those with two null alleles.

Angiopoietin-like 3 (ANGPTL3) is an inhibitor of lipoprotein and endothelial lipase and plays a key role in lipid metabolism by increasing the levels of triglycerides and other lipids.[5–7] Loss-of-function variants in ANGPTL3 have been associated with low levels of both LDL cholesterol and triglycerides and with a 41% lower risk of coronary artery disease, despite the presence of low levels of high-density lipoprotein (HDL) cholesterol.[8,9] Both ANGPLT3 loss-of-function variants and ANGPTL3 pharmacologic inhibition reduce LDL cholesterol levels independently of the LDL receptor.[5,10,11]

Evinacumab is a fully human monoclonal antibody that is an inhibitor of ANGPTL3.[10] (A description of the methods used in the development of evinacumab is provided in the Supplementary Appendix) In a phase 2, open-label, proof-of-concept study

involving nine patients with homozygous familial hypercholesterolemia, evinacumab treatment resulted in a mean reduction from baseline of 49% in the LDL cholesterol level.[12] Here, we describe the results of a phase 3, randomized, placebo-controlled, parallel-group trial, the Evinacumab Lipid Studies in Patients with Homozygous Familial Hypercholesterolemia (ELIPSE HoFH) trial, which we conducted to further evaluate the efficacy and safety of evinacumab in patients with nullnull variants and in those with non-null variants.

METHODS

Trial design and oversight

We conducted the trial at 30 sites in 11 countries. The principal investigators and the sponsor (Regeneron Pharmaceuticals) designed the trial protocol (available at NEJM.org) and selected the participating sites. The protocol was approved by the institutional review board or ethics committee at each site.

The trial was conducted in accordance with the principles of the Declaration of Helsinki, consistent with the Good Clinical Practice guidelines of the International Conference on Harmonisation. Monitoring and site supervision were performed by a contract research organization (ICON) with oversight by the sponsor. The sponsor also participated in the collection, analysis, and interpretation of the data and checked information provided in the manuscript. Editorial support for the writing of the manuscript was provided by Prime Global and financed by the sponsor. All the authors had access to the data, contributed to the drafting of an initial version of the manuscript, participated in revisions, and concurred with the decision to submit the manuscript for publication. The authors vouch for the accuracy and completeness of the data and for the fidelity of the trial to the protocol.

Patients

Patients with homozygous familial hypercholesterolemia who were 12 years of age or older were eligible for inclusion. Patients were required to be receiving stable lipid-lowering therapy at the maximum dose that did not cause unacceptable side effects and to have an LDL cholesterol level of 70 mg per deciliter (1.8 mmol per

liter) or more at screening. The full list of inclusion and exclusion criteria is provided in the Supplementary Appendix. Written informed consent was obtained from each patient.

The diagnosis of homozygous familial hypercholesterolemia was based on either genetic or clinical criteria. The genetic diagnosis was defined as a documented variant in two *LDLR* alleles or the presence of homozygous or compound heterozygous variants in apolipoprotein B (*APOB*) or *PCSK9*. Patients who had compound heterozygosity or homozygosity for variants in the gene encoding LDL receptor adaptor protein 1 (*LDLRAP1*) were also eligible. The clinical diagnosis was defined as an untreated total cholesterol level of more than 500 mg per deciliter (12.9 mmol per liter), with either the presence of cutaneous or tendinous xanthomas before the age of 10 years or documentation of an untreated total cholesterol level of more than 250 mg per deciliter (6.5 mmol per liter) in both parents. A patient was considered to be a null–null variant carrier if the LDL-receptor activity was less than 15% according to in vitro assessments of functionality, as reported in the literature.[11] A post hoc analysis of data from patients who had very little or no LDL-receptor activity (based on a definition of less than 2% functional activity) was also conducted.[13]

Procedures

The trial included a run-in period of up to 8 weeks for patients who did not have a diagnosis of homozygous familial hypercholesterolemia and opted to undergo genotyping for confirmation or whose background lipid-lowering therapy or apheresis schedules were not stable before screening. The run-in period was followed by a 2-week screening period to determine trial eligibility.

Eligible patients were randomly assigned in a 2:1 ratio through an interactive voice- or Web-response system to receive an intravenous infusion of either evinacumab (at a dose of 15 mg per kilogram of body weight) every 4 weeks or matching placebo. The randomization was stratified according to whether the patients had received previous apheresis treatment and whether they lived in Japan (to provide data on pharmacodynamic and safety findings in an Asian population). Double-blind treatment continued for 24 weeks. Concomitant lipid-lowering therapies were to

be continued for the duration of the trial. After completion of the double-blind treatment period, patients had the option of entering a 24-week open-label study to receive the same regimen of evinacumab that was used in the randomized trial. Patients who did not choose to enter the open-label study entered a 24-week follow-up period after the last dose of evinacumab or placebo.

Outcomes

The primary outcome was the percent change in the calculated LDL cholesterol level from baseline to week 24 during the double-blind treatment period. The baseline LDL cholesterol level was defined as the last calculated LDL cholesterol value obtained before the administration of the first dose of evinacumab or placebo. For the efficacy analysis, the LDL cholesterol level was obtained within the 24-week window, regardless of adherence to treatment and subsequent therapies. Secondary outcomes are listed in the Supplementary Appendix. The mean total serum level of evinacumab over time was also analyzed.

Statistical analysis

We estimated that a sample size of 57 patients (38 assigned to receive evinacumab and 19 assigned to receive placebo) was required to provide a power of 90% to confirm the primary efficacy hypothesis of a between-group absolute difference in the mean percent change in the LDL cholesterol level of 38 percentage points, according to a two-sample t-test with a two-sided significance level of 0.05. This assumption was based on a common standard deviation of 35% of the percent change from baseline in the two groups, after a 20% adjustment to account for patients who had withdrawn from the trial or could not otherwise be evaluated.

We used a mixed-effects model for repeated measures to analyze the percent change from baseline in the calculated LDL cholesterol level at week 24 in the intention-to-treat population. The model included the fixed categorical effects of trial-group assignment (evinacumab vs. placebo), randomization strata (apheresis [yes vs. no] and geographic region [Japan vs. rest of world]), time point (week 2, 4, 8, 12, 16, 20, or 24), and interactions between strata and time point and between treatment and time point, as well as the continuous fixed covariates of the interaction between baseline levels of calculated LDL cholesterol and time point.

We assessed the continuous secondary outcomes using the same model that was used for the primary outcome, except for variables that were anticipated to have a non-normal distribution, including triglycerides and lipoprotein(a), which we assessed using a robust regression model[14] after applying a multiple-imputation approach (i.e., a log transformation of data before multiple imputation) for handling missing data. In the model, the outcome of interest was the response variable with trial group, randomization strata, and corresponding baseline values as covariates. Binary outcomes were assessed by logistic regression after the application of a multiple-imputation approach, with the trial group and corresponding baseline values as covariates, stratified according to randomization strata. The overall type I error was controlled for primary and key secondary outcomes with a hierarchical inferential approach, as described in the Supplementary Appendix.

The safety analysis population included all the patients who had undergone randomization and had received at least one dose of evinacumab or placebo. The period for the evaluation of adverse events was defined as the interval from the day of administration of the first dose of evinacumab or placebo until week 24. All safety data were assessed descriptively. The percent change from baseline in the HDL cholesterol level was assessed descriptively as a safety outcome because of reductions in this measure that had been observed after evinacumab treatment in previous studies.[12,15]

RESULTS

Patients

Of the 75 patients who were screened, 65 underwent randomization (43 to receive evinacumab and 22 to receive placebo (Fig. S1 in the Supplementary Appendix). The first patient was enrolled on February 15, 2018, and the last on December 18, 2018. The date of the database lock was July 29, 2019. All the patients who had undergone randomization also received at least one dose of evinacumab or placebo. One patient in the placebo group received evinacumab in error at week 20 during the double-blind treatment period and was therefore included in the evinacumab group for all safety analyses.

The demographic and baseline characteristics of the patients were generally well balanced in the two groups (Table 1). One adolescent patient (12 to <18 years of age) was included in each group. The mean baseline LDL cholesterol level was 260 mg per deciliter (6.7 mmol per liter) in the evinacumab group and 247 mg per deciliter (6.4 mmol per liter) in the placebo group. A total of 53 patients (82%) had a genetically confirmed diagnosis of homozygous familial hypercholesterolemia. Genotype data are provided in Table S1.

Null–null LDL-receptor variants (<15% activity) were identified in 15 of 43 patients (35%) in the evinacumab group and in 6 of 22 patients (27%) in the placebo group. At baseline, the mean (±SD) LDL cholesterol level was 312±158 mg per deciliter (8.1±4.1 mmol per liter) in the 21 patients with null–null variants and 228±164 mg per deciliter (5.9±4.2 mmol per liter) in the 44 patients with non-null variants.

The majority of the trial patients (94%) were receiving a statin (a high-intensity statin in 77%). In addition, a PCSK9 inhibitor was being administered in 77% of the patients, ezetimibe in 75%, and lomitapide in 25%; 34% of the patients were undergoing apheresis (Table S2). A total of 63% of the patients were taking at least three lipid-modifying drugs.

Primary efficacy and subgroup outcome analyses

At week 24, patients in the evinacumab group had a 47.1% reduction from baseline in the LDL cholesterol level, as compared with a 1.9% increase in the placebo group, for a between-group least-squares mean difference of –49.0 percentage points (95% confidence interval [CI], –65.0 to –33.1; P<0.001) (Table 2). The reduction in LDL cholesterol levels with evinacumab was observed at the first post-treatment lipid assessment at week 2 and was maintained throughout the 24-week double-blind treatment period (Figure 1). The between-group least-squares mean absolute difference in the LDL cholesterol level was –132 mg per deciliter (–3.4 mmol per liter); 95% CI, –175 to –89 mg per deciliter (–4.5 to –2.3 mmol per liter) (P<0.001) (Table 2). Waterfall plots for the percent and absolute changes in LDL cholesterol levels as grouped according to genotype for each patient are provided in Figure S2.

Table 1: Demographic and Clinical Characteristics of the Patients at Baseline

Characteristic	Evinacumab (N=43)	Placebo (N=22)	Total (N=65)
Age			
Mean — yr	44.3±16.8	36.7±11.5	41.7±15.5
Distribution — no. (%)			
12 to <18 yr	1 (2)	1 (5)	2 (3)
18 to <45 yr	23 (53)	16 (73)	39 (60)
45 to <65yr	11 (26)	5 (23)	16 (25)
≥65 yr	8 (19)	0	8 (12)
Female sex — no. (%)	24 (56)	11 (50)	35 (54)
Race — no. (%)†			
White	31 (72)	17 (77)	48 (74)
Black	2 (5)	0	2 (3)
Asian	6 (14)	4 (18)	10 (15)
Other or not reported	4 (9)	1 (5)	5 (8)
Body-mass index‡	26.1±5.9	24.6±5.7	25.6±5.8
History of coronary heart disease — no. (%)	38 (88)	21 (95)	59 (91)
Method of HoFH diagnosis — no. (%)			
Genotyping	29 (67)	15 (68)	44 (68)
Clinical diagnosis	14 (33)	7 (32)	21 (32)
Activity of LDL-receptor variants — no. (%)			
<2%	8 (19)	2 (9)	10 (15)
<15%	15 (35)	6 (27)	21 (32)
Cholesterol — mg/dl			
Calculated LDL	259.5±172.4	246.5±153.7	255.1±165.2
High-density lipoprotein	43.6±14.9	46.0±16.1	44.4±15.2
Non–high-density lipoprotein	281.9±172.6	269.9±157.8	277.8±166.6
Total cholesterol	325.6±170.8	315.9±150.4	322.3±163.1
Median triglycerides (IQR) — mg/dl	91 (65–145)	104 (59–182)	97 (65–162)
Median lipoprotein(a) (IQR) — nmol/liter	59 (22–173)	53 (32–60)	57 (29–166)
Apolipoprotein B — mg/dl	169.1±82.8	175.9±98.8	171.4±87.8

Plus–minus values are means ±SD. Percentages may not total 100 because of rounding. To convert the values for cholesterol to millimoles per liter, multiply by 0.02586. To convert the values for triglycerides to millimoles per liter, multiply by 0.01129. HoFH denotes homozygous familial hypercholesterolemia, IQR interquartile range, and LDL low-density lipoprotein. † Race was reported by the patients. ‡ The body-mass index is the weight in kilograms divided by the square of the height in meters.

The degree of lowering of cholesterol levels was higher in the evinacumab group than in the placebo group among patients with null–null variants (–43.4% and +16.2%, respectively) and non-null variants (–49.1% and –3.8%, respectively) (Figure 2). The percent changes in LDL cholesterol levels from baseline to week 24 were consistent across the range of background therapies, including statins, ezetimibe, lomitapide, PCSK9 inhibitors, and apheresis (Table S3).

Table 2: Trial Outcomes at 24 Weeks

Outcome	Evinacumab	Placebo	LS Mean (±SE) Difference	Odds Ratio	P Value
	(N=43)	(N=22)	95% CI	95% CI	
Primary outcome					
Percent change from baseline in LDL cholesterol	−47.1±4.6	1.9±6.5	−49.0±8.0 (−65.0 to −33.1)	—	<0.001
Key secondary outcomes					
Percent change from baseline in apolipoprotein B	−41.4±3.3	−4.5±4.8	−36.9±5.9 (−48.6 to −25.2)	—	<0.001
Percent change from baseline in non-HD lipoprotein cholesterol	−49.7±3.8	2.0±5.4	−51.7±6.6 (−64.8 to −38.5)	—	<0.001
Percent change from baseline in total cholesterol	−47.4±3.0	1.0±4.2	−48.4±5.1 (−58.7 to −38.1)	—	<0.001
Patients with ≥30% reduction from baseline in LDL cholesterol — no. (%)†	36 (84)	4 (18)	—	25.2 (5.7 to 110.5)	<0.001‡
Patients with ≥50% reduction from baseline in LDL cholesterol — no. (%)†	24 (56)	1 (5)	—	24.2 (3.0 to 195.6)	0.003‡
Absolute change from baseline in calculated LDL cholesterol — mg/dl	−134.7±12.4	−2.6±17.6	−132.1±21.5 (−175.3 to −88.9)	—	<0.001
Patients who met U.S. apheresis eligibility criteria — no. (%)†§	3 (7)	5 (23)	—	0.1 (0.0 to 1.3)	0.09‡
Patients with LDL cholesterol <100 mg/dl — no. (%)†	20 (47)	5 (23)	—	5.7 (1.3 to 24.9)	NA¶
Patients who met EU apheresis eligibility criteria — no. (%)‖	14 (33)	17 (77)	—	0.1 (0.0 to 0.3)	NA
Other secondary outcomes					
Percent change from baseline in triglycerides	−55.0±3.1	−4.6±7.0	−50.4±7.7 (−65.6 to −35.2)	—	NA
Percent change from baseline in lipoprotein(a)	−5.5±4.0	−3.6±5.8	−1.9±7.1 (−15.7 to 12.0)	—	NA
Percent change from baseline in apolipoprotein C-III	−84.1±3.9	5.8±5.5	−90.0±6.7 (−103.5 to −76.5)	—	NA
Patients with calculated LDL cholesterol <70 mg/dl — no. (%)†	12 (28)	1 (5)	—	20.9 (1.6 to 276.8)	NA

Plus–minus values are means ±SD unless otherwise indicated. The outcome categories are listed in the hierarchical-testing order. The between-group differences and odds ratios are for the value in the evinacumab group, as compared with the placebo group. Details regarding the percent and absolute changes in LDL cholesterol levels according to genotype for each patient are provided in Figure S2 in the Supplementary Appendix. HD denotes high density, LS least squares, and NA not applicable. † In this category, the combined estimate for the number of patients and odds ratio was based on a logistic-regression model that used 100 simulation data sets for imputation of missing data. ‡ P value is based on the odds ratio. § In the United States, the criterion for eligibility to undergo apheresis is an LDL cholesterol level of 300 mg per deciliter or more. ¶ Hierarchical testing was terminated with the previous outcome, since it did not meet the cutoff for statistical significance. ‖ In the European Union (EU), the criterion for eligibility to undergo apheresis is either an LDL cholesterol level of more than 160 mg per deciliter if the patient is being treated for primary prevention of cardiovascular disease or an LDL cholesterol level of more than 120 mg per deciliter if the patient is being treated for secondary prevention of cardiovascular disease.

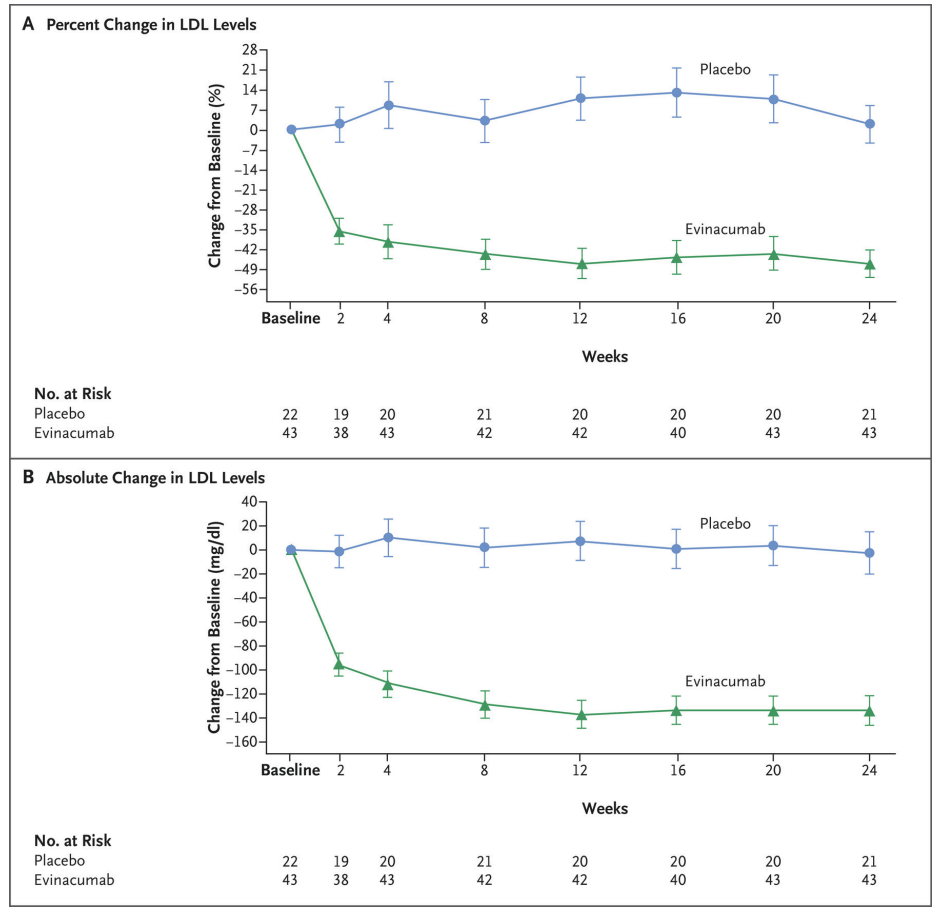

Figure 1: Changes from Baseline in Low-Density Lipoprotein (LDL) Cholesterol Levels at 24 Weeks
Shown is the least-squares mean percent change (Panel A) and absolute change (Panel B) in calculated LDL cholesterol levels from baseline to week 24 in the evinacumab group and the placebo group. The I bars indicate standard errors.

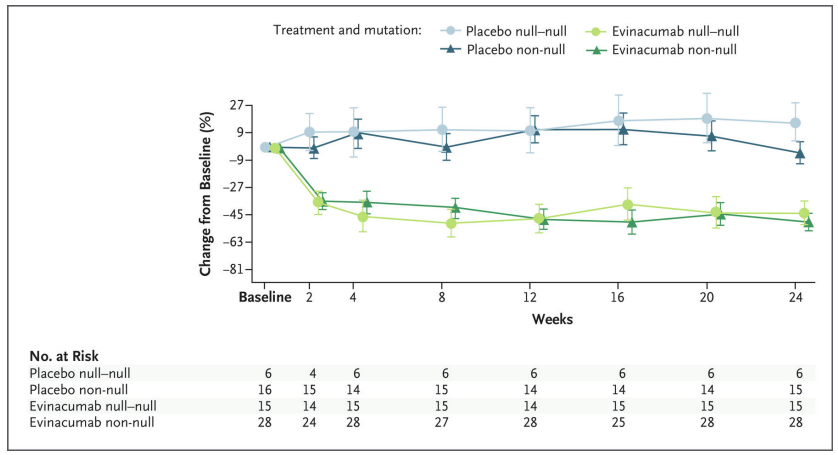

Figure 2: Percent Change in LDL Cholesterol Levels, According to Type of LDL-Receptor Variants
Shown is the least-squares mean percent change from baseline in LDL cholesterol levels in the evinacumab group and the placebo group, according to the type of loss-of-function variant in the LDL receptor. Genetic variants that cause a virtually complete absence of LDL-receptor expression (null–null variants) result in higher LDL cholesterol levels than variants that partially reduce LDL-receptor activity (non-null variants). Patients with null–null variants have LDL-receptor activity of less than 15%.

Secondary and post hoc efficacy outcome analyses

Patients in the evinacumab group had significantly lower levels of apolipoprotein B, non-HDL cholesterol, and total cholesterol from baseline to week 24 than those in the placebo group (P<0.001 for all comparisons) (Table 2). Percent and absolute changes for apolipoprotein B are shown in Figure S3. HDL cholesterol levels (which were assessed as a safety outcome) were reduced from baseline by 29.6% in the evinacumab group, as compared with an increase of 0.8% in the placebo group. A reduction in the LDL cholesterol level of at least 30% was observed in 84% of patients in the evinacumab group and in 18% of those in the placebo group (P<0.001). Results for all other secondary outcomes are provided in Table 2.

Variants with less than 2% functional LDL-receptor activity were identified in 8 patients in the evinacumab group and in 2 patients in the placebo group.13 In a post hoc analysis investigating the effects of evinacumab in these 8 patients, the baseline mean LDL cholesterol level was 261 mg per deciliter (6.7 mmol per liter). At week 24, the change from baseline in the LDL cholesterol level was a decrease of 53.5% in the evinacumab group and an increase of 18.8% in the placebo group

(least-squares mean difference, −72.3 percentage points; 95% CI, −121.8 to −22.8; P=0.005), with an absolute difference in the LDL cholesterol of 245 mg per deciliter (6.3 mmol per liter).

Pharmacokinetics

The mean total levels of evinacumab in serum over time are shown in Figure S4. Overall, serum levels of evinacumab were similar in patients receiving apheresis and in those not receiving apheresis.

Safety

Adverse events during the treatment period occurred in 66% of the patients in the evinacumab group and in 81% of those in the placebo group (Table 3). No patients discontinued either evinacumab or placebo because of an adverse event; there were no deaths. Antidrug antibodies did not develop during the treatment period in any of the patients. Low titers of preexisting antibodies to evinacumab at baseline were reported in 4 patients (3 in the evinacumab group and 1 in the placebo group); the pharmacokinetics of evinacumab were not altered in these patients.

Serious adverse events during the treatment period occurred in 2 patients (5%) in the evinacumab group and were reported as urosepsis and a suicide attempt. Both patients recovered. No cardiovascular events were reported in either group during the double-blind treatment period. An influenza-like illness was reported in 5 of 44 patients (11%) in the evinacumab group and in no patients in the placebo group.

An increase in the level of either alanine or aspartate aminotransferase was reported in 2 of 44 patients (5%) in the evinacumab group and in 2 of 21 patients (10%) in the placebo group, increases that were less than 3 times and 5 times the upper limit of the normal range, respectively. Only elevations in the aspartate aminotransferase level in the placebo group were reported as adverse events during the treatment period. In all cases, elevations were not associated with any symptoms and returned to a normal range while the patients continued to receive either evinacumab or placebo. None of the patients in either trial group met the criteria for drug-induced liver injury, according to Hy's law.[16]

Table 3: Adverse Events during the Treatment Period

Adverse Events	Evinacumab (N=44) no. (%)	Placebo (N=21)
Any adverse event	29 (66)	17 (81)
Nasopharyngitis	7 (16)	5 (24)
Influenza-like illness	5 (11)	0
Headache	4 (9)	5 (24)
Rhinorrhea	3 (7)	0
Gastroenteritis	2 (5)	0
Infusion-site pruritus	2 (5)	0
Pyrexia	2 (5)	1 (5)
Cough	2 (5)	0
Dental caries	2 (5)	0
Diarrhea	2 (5)	1 (5)
Dyspepsia	2 (5)	0
Toothache	2 (5)	2 (10)
Dizziness	2 (5)	0
Urinary tract infection	0	2 (10)
Increased aspartate aminotransferase	0	2 (10)
Myalgia	0	2 (10)
Any serious adverse event	2 (5)	0
Urosepsis	1 (2)	0
Suicide attempt	1 (2)	0

* No adverse event was associated with a discontinuation of evinacumab or placebo. There were no deaths in either group.

DISCUSSION

In this multicenter trial of ANGPTL3 inhibition involving patients with homozygous familial hypercholesterolemia,12 the reduction from baseline in the LDL cholesterol level in the evinacumab group, as compared with the small increase in the placebo group, resulted in a between-group difference of 49.0 percentage points at 24 weeks; the corresponding between-group difference in apolipoprotein B levels was 36.9 percentage points. These reductions were achieved regardless of the use of extensive background lipid-lowering therapies with or without apheresis.

In this patient population, currently available therapies do not typically reduce LDL cholesterol levels to guideline-recommended levels, so new therapies are needed.[17] In such patients, statins and PCSK9 inhibitors, which have a mechanism of action that largely depends on the up-regulation of LDL-receptor function,[17,18] have been shown to reduce LDL cholesterol levels by approximately 20 to 30%, with minimal to no effect among those with null–null homozygosity.[19,20] Although lomitapide and mipomersen act independently of LDL-receptor function, adverse effects limit their wide use.[17,21]

It is well established that LDL cholesterol levels predict cardiovascular risk and that cardiovascular benefit from lipid-lowering therapies is proportional to the absolute reduction in the LDL cholesterol level.[17] In a large study of PCSK9 inhibitors (the ODYSSEY OUTCOMES trial), patients who had a baseline LDL cholesterol level of 100 mg per deciliter (2.6 mmol per liter) or more had the greatest benefit with alirocumab, with a relative 24% lower incidence of major adverse cardiovascular events and a 29% lower incidence of overall mortality than with placebo.[22,23] Our trial was not designed to assess the effect of treatment on a reduction in clinical events, but the absolute reduction in LDL cholesterol levels was substantial. Furthermore, genetic studies of ANGPTL3 loss-of-function variants support the concept that ANGPTL3 inhibition should reduce both LDL cholesterol levels and cardiovascular events, despite a concurrent reduction in HDL cholesterol levels.[8] Studies of PCSK9 loss-of-function variants provide an important precedent by showing that genetic studies can faithfully predict lipid changes as well as cardiovascular outcomes resulting from pharmacologic intervention in the pathway of interest.[8,10,22,24]

Some patients with homozygous familial hypercholesterolemia are treated with apheresis, an invasive therapy that has a considerable effect on health care costs and quality of life. In our trial, evinacumab provided a similar reduction in LDL cholesterol levels regardless of whether patients were being treated with apheresis, and the receipt of apheresis did not meaningfully affect plasma evinacumab levels. With evinacumab treatment, very few patients (7%) met the criteria for undergoing apheresis in the United States. Also, reductions in the levels of LDL cholesterol and apolipoprotein B from baseline to week 24 were observed with evinacumab both in patients with null–null variants and in those with non-null variants. This finding

is important because patients with null–null variants have a higher cardiovascular risk and are less responsive to therapies that depend on LDL-receptor activity than those with non-null variants.[2]

Limitations of this trial include the relatively short duration of treatment and the small number of patients studied. As a consequence, the ability to assess safety, especially long-term safety, is limited. Also, the size and duration of the trial are not sufficient to assess the effect of evinacumab on cardiovascular outcomes in these high-risk patients.

In conclusion, in this phase 3 trial, evinacumab substantially lowered LDL cholesterol levels in patients with homozygous familial hypercholesterolemia, regardless of the degree of their LDL-receptor function.

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We dedicate this article to the memory of Daniel A. Gipe, M.D., for his major contributions and commitment to patients with homozygous familial hypercholesterolemia and other dyslipidemias.

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CONFLICTS OF INTERESTS

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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SUPPLEMENTARY APPENDIX

Inclusion Criteria

A patient must meet the following criteria to be eligible for inclusion in the study:

1. Male or female ≥12 years of age at the time of the screening visit
2. Diagnosis of functional homozygous familial hypercholesterolemia by at least one of the following:
    a. Documented functional mutation or mutations in both low-density lipoprotein (LDL) receptor alleles
    Note: patients who have null receptor mutations on both LDLR alleles, i.e., double null, are eligible
    b. Presence of homozygous or compound heterozygous mutations in apolipoprotein B or proprotein convertase subtilisin/kexin type 9 (PCSK9)
    Note: patients who are double heterozygous, i.e., mutations on different genes (e.g., LDLR/PCSK9), and patients with homozygous LDLRAP1 mutations are eligible
    c. Untreated total cholesterol greater than 500 milligrams per deciliter (12.93 millimoles per liter) and triglycerides less than 300 milligrams per deciliter (3.39 millimoles per liter)
    AND both parents with documented TC > 250 milligrams per deciliter (6.47 millimoles per liter)
    OR cutaneous or tendinous xanthoma before the age of 10 years
3. If undergoing LDL apheresis, must have initiated LDL apheresis at least 3 months prior to screening and must have been on a stable weekly (every 7±1 days) or every other week (every 14±2 days) schedule and stable settings for at least 8 weeks
4. Willing and able to comply with clinic visits and study-related procedures
5. Willing to consistently maintain his/her usual low fat or heart-healthy diet for the duration of the study
6. Provide signed informed consent or assent

Exclusion Criteria

A patient who meets any of the following criteria will be excluded from the study:

1. LDL cholesterol level less than 70 milligrams per deciliter (1.81 millimoles per liter) at the screening visit.
2. Background medical LMT (if applicable) that has not been stable for at least 4 weeks (6 weeks for fibrates, 8 weeks for PCSK9 inhibitor antibodies, 12 weeks for MTD of lomitapide, 24 weeks for mipomersen) before the screening visit
3. Lipid-apheresis schedule (every 7 or 14 days)/apheresis settings (if applicable) that have not been stable for at least 8 weeks before the screening visit or an apheresis schedule that is not anticipated to be stable over the next 48 weeks. Plasma exchange is excluded.
4. Use of nutraceuticals or over-the-counter therapies known to affect lipids, at a dose/amount that has not been stable for at least 4 weeks prior to the screening visit or between the screening and randomization visits
5. Presence of any clinically significant uncontrolled endocrine disease known to influence serum lipids or lipoproteins
    Note: patients on thyroid replacement therapy can be included if the dosage of replacement therapy has been stable for at least 12 weeks prior to screening and the thyroid-stimulating hormone level is within the normal range of the central laboratory at the screening visit.
6. Newly diagnosed (within 3 months prior to randomization visit [week 0/day 1]) diabetes mellitus or poorly controlled (glycated hemoglobin greater than 9%) diabetes
7. Unstable weight (variation greater than 5 kg) within 2 months prior to the screening visit (week -2)
8. Initiation of a new diet or major change to a previous diet within 4 weeks prior to screening
9. Use of systemic corticosteroids, unless used as replacement therapy for pituitary/adrenal disease with a stable regimen for at least 6 weeks prior to screening. Note: topical, intra-articular, nasal, inhaled, and ophthalmic steroid therapies are not considered as 'systemic' and are allowed
10. Use of estrogen or testosterone therapy unless the regimen has been stable for 6 weeks prior to the screening visit and there are no plans to change the regimen during the study

11. Systolic blood pressure greater than 160 millimeters of Mercury or diastolic blood pressure greater than 100 millimeters of Mercury at the screening visit or time of randomization (week 0/day 1)
12. History of a myocardial infarction, unstable angina leading to hospitalization, coronary artery bypass graft surgery, percutaneous coronary intervention, uncontrolled cardiac arrhythmia, carotid surgery or stenting, stroke, transient ischemic attack, valve replacement surgery, carotid revascularization, endovascular procedure, or surgical intervention for peripheral vascular disease within 3 months prior to the screening visit
13. History of New York Heart Association Class IV heart failure within 12 months before screening.
14. Age less than 12 years at the screening visit
15. Tanner stage less than 2 at the screening visit
16. History of cancer within the past 5 years, except for adequately treated basal cell skin cancer, squamous cell skin cancer, or in situ cervical cancer
17. Use of any active investigational drugs (except alirocumab) within 1 month or five half-lives prior to the screening visit, whichever is longer
18. Conditions/situations such as:
    a. Any clinically significant abnormality identified at the time of screening that, in the judgment of the investigator or any sub-investigator, would preclude safe completion of the study or constrain endpoints assessment; e.g., major systemic diseases or patients with short life expectancy
    b. Considered by the investigator or any sub-investigator as inappropriate for this study for any reason, e.g.:
        i. Deemed unable to meet specific protocol requirements, such as scheduled visits
        ii. Investigator or any sub-investigator, pharmacist, study coordinator, other study staff, or relative thereof directly involved in the conduct of the protocol, etc.
        iii. Presence of any other conditions (e.g., geographic or social), either actual or anticipated, which the investigator feels would restrict or limit the patient's participation for the duration of the study

19. Laboratory findings during the screening period (not including randomization labs):
    a. Positive test for hepatitis B surface antigen and/or hepatitis C antibody (associated with a positive hepatitis C virus RNA polymerase chain reaction)
    b. Positive serum beta-human chorionic gonadotropin or urine pregnancy test in women of childbearing potential
    c. Estimated glomerular filtration rate of less than 30 mL/min/1.73 m2 (calculated by central lab)
    d. Alanine aminotransferase or aspartate aminotransferase greater than three times the upper limit of normal (one repeat lab is allowed)
    e. Creatine phosphokinase greater than three times the upper limit of normal (one repeat lab is allowed)
    f. Thyroid-stimulating hormone greater than 1.5 times the upper limit of normal of the central laboratory (one repeat lab is allowed) for patients not on thyroid replacement therapy
20. Known hypersensitivity to monoclonal antibody therapeutics
21. Member of the clinical site study team and/or his/her immediate family
22. Pregnant or breastfeeding women
23. Sexually active women of childbearing potential* who are unwilling to practice a highly effective birth control method prior to the initial dose, during the study, and for 24 weeks after the last dose of study drug. Highly effective contraceptive measures include:
    a. Stable use of combined (estrogen and progestogen containing) hormonal contraception associated with inhibition of ovulation initiated two or more menstrual cycles prior to screening
        1. Oral
        2. Intravaginal
        3. Transdermal
    b. Stable use of progestogen-only hormonal contraception associated with inhibition of ovulation initiated two or more menstrual cycles prior to screening
        1. Oral
        2. injectable
        3. Implantable

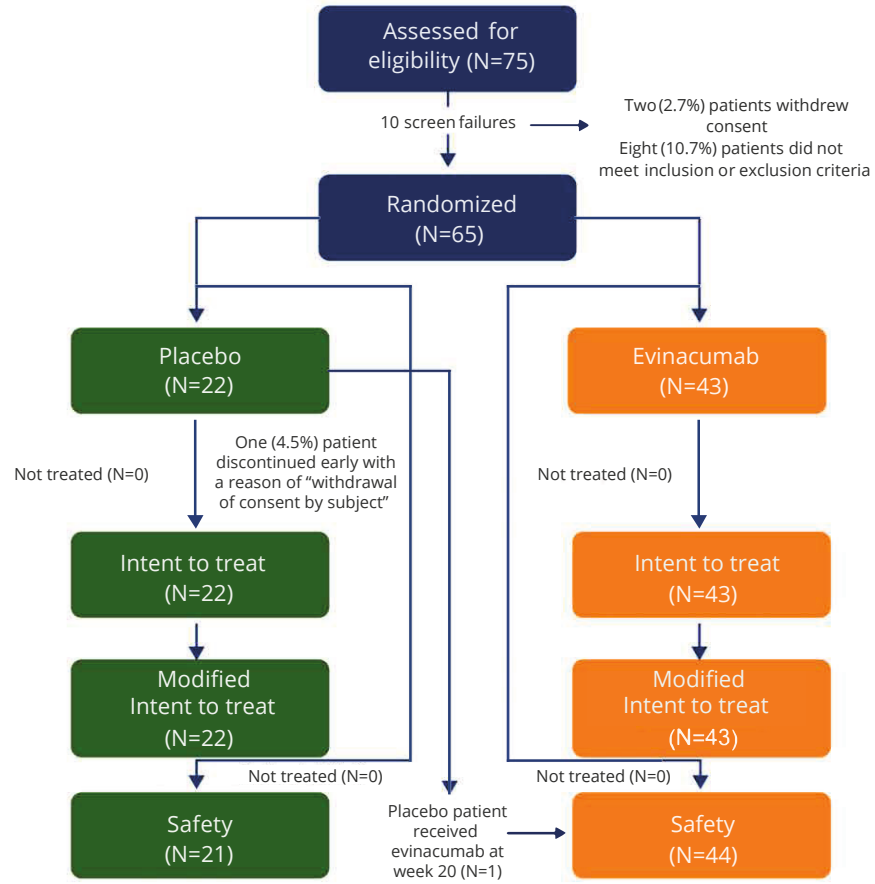
c. Intrauterine device
d. Intrauterine hormone-releasing system
e. Bilateral tubal ligation
f. Vasectomized partner. Note: vasectomized partner is a highly effective birth control method provided that the partner is the sole sexual partner of the female trial participant and that the vasectomized partner has received medical assessment of the surgical success
g. Sexual abstinence. Note: Sexual abstinence is considered a highly effective method only if defined as refraining from heterosexual intercourse during the entire period of risk associated with study treatments. True abstinence: When this is in line with the preferred and usual lifestyle of the patient. Periodic abstinence (calendar, symptothermal, post-ovulation methods), withdrawal (coitus interruptus), spermicides only, and lactational amenorrhea method are not acceptable methods of contraception.

24. Sexually active men who are unwilling to use the following forms of medically acceptable birth control during the study drug treatment period and for 24 weeks after the last dose of study drug: vasectomy with medical assessment of surgical success OR consistent use of a condom. Sperm donation is prohibited during the study and for up to 24 weeks after the last injection of study drug.
25. Housed in an institution on the basis of an administrative or judicial order.
26. Dependent on the sponsor, investigator, or the study site.

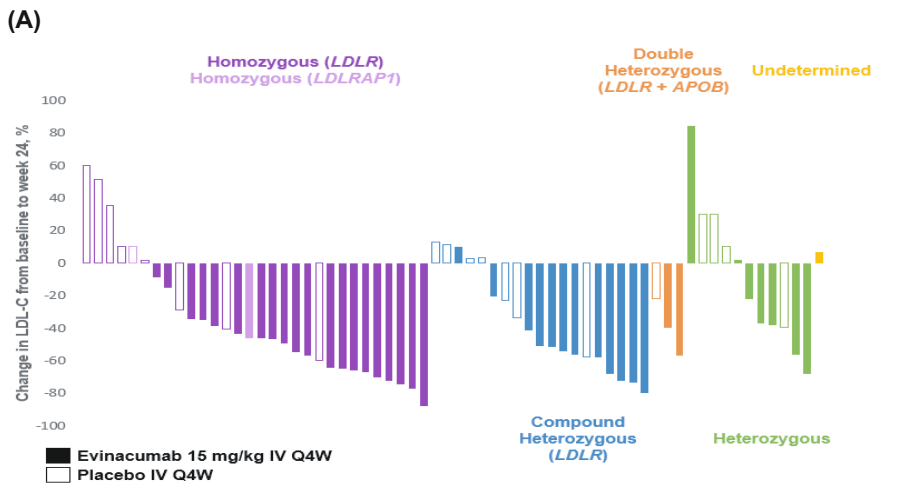
*Postmenopausal women must be amenorrheic for at least 12 months in order not to be considered of childbearing potential. Postmenopausal status will be confirmed by measurement of follicle-stimulating hormone. Pregnancy testing and contraception are not required for women with documented hysterectomy and/or oophorectomy.

VelocImmune Technology

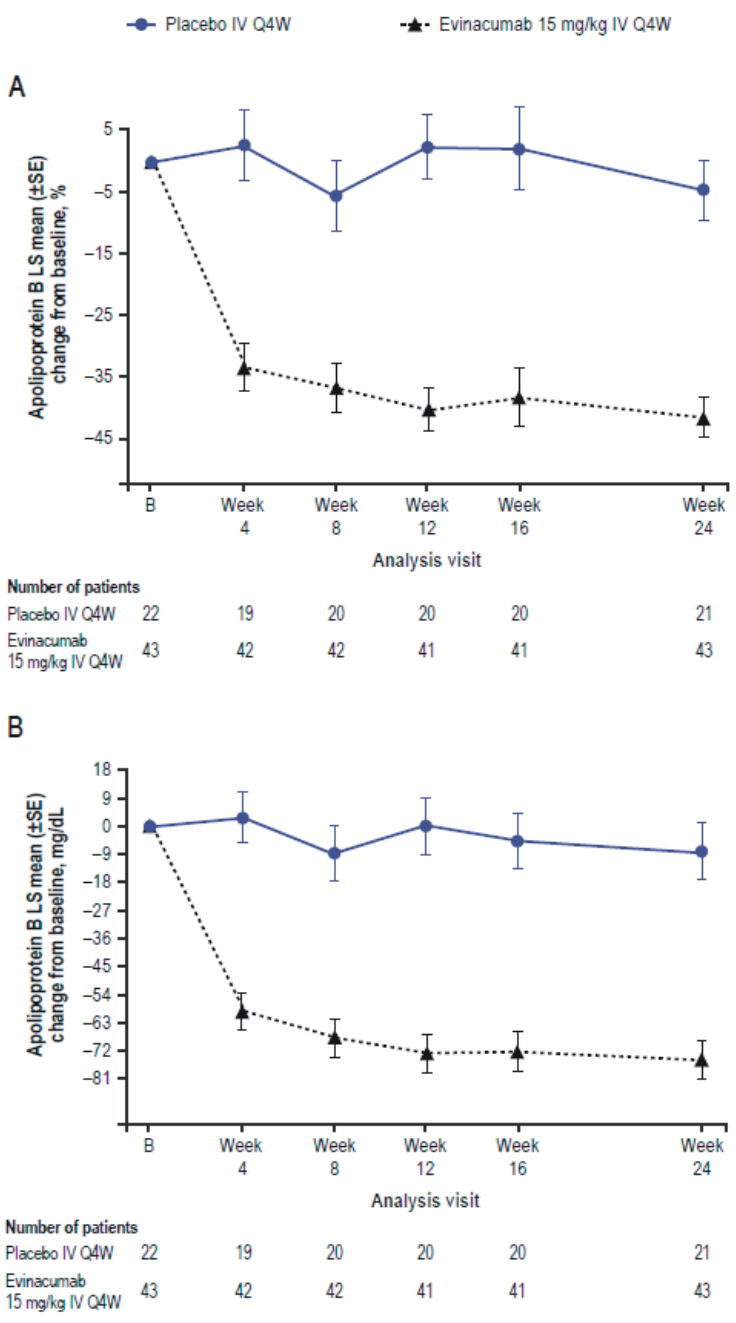
Evinacumab was invented using Regeneron's VelocImmune technology, which utilizes a proprietary genetically-engineered mouse endowed with a genetically-humanized immune system in order to produce fully-human antibodies. VelocImmune creates antibody drug candidates directly from immunized mice. This approach creates fully human antibodies that tightly bind to therapeutic targets and avoid potential immune responses that may occur in patients receiving antibodies that contain nonhuman (typically mouse) components.



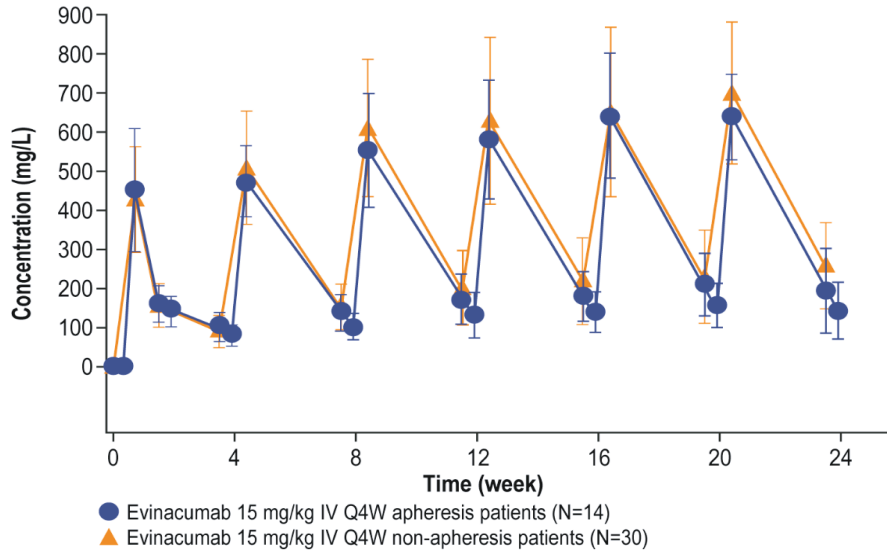
Supplementary Figure S1: Patient flow throughout the study.



Supplementary Figure S2: Waterfall plots for individual patient (A) percent and (B) absolute changes in LDL cholesterol
IV, intravenous; LDL-C, low-density lipoprotein cholesterol; Q4W, every 4 weeks



Supplementary Figure S3: Least squares mean (±SE) (A) percent change and (B) absolute change in apolipoprotein B from baseline to week 24
IV, intravenous; LS, least squares; Q4W, every 4 weeks



Supplementary Figure S4: Mean (+SD) concentrations of total evinacumab in serum by time and apheresis status in patients with homozygous familial hypercholesterolemia
Pre-dose and end-of-infusion concentrations are presented for non-apheresis patients. Pre-apheresis, post-apheresis and end-of-infusion concentrations are presented for apheresis patients. IV, intravenous; Q4W, every 4 weeks; SD, standard deviation.

Supplementary Table S1: Patients by grouped by genotype

Genotype	Evinacumab 15 mg/kg IV Q4W (N=43)	Placebo IV Q4W (N=22)	Total (N=65)
Homozygous *(LDLR)*	21	7	28
Homozygous *(LDLRAP1)*	1	1	2
Compound Heterozygous *(LDLR)*	12	8	20
Double Heterozygous (*LDLR* and *APOB*)	2	1	3
Heterozygous	7	4	11
Undetermined	0	1	1

IV, intravenous; Q4W, every 4 weeks

Supplementary Table S2: Lipid-lowering therapy at baseline

Lipid-lowering therapy, n (%)	Evinacumab 15 mg/kg IV Q4W (N=43)	Placebo IV Q4W (N=22)	Total (N=65)
Statin	41 (95.3)	20 (90.9)	61 (93.8)
Ezetimibe	33 (76.7)	16 (72.7)	49 (75.4)
PCSK9 inhibitor	34 (79.1)	16 (72.7)	50 (76.9)
Lomitapide	11 (25.6)	3 (13.6)	14 (21.5)
Apheresis	14 (32.6)	8 (36.4)	22 (33.8)
Lipid-lowering therapy combinations, n (%)			
Ezetimibe + PCSK9 inhibitor + statin	21 (48.8)	8 (36.4)	29 (44.1)
Ezetimibe + lomitapide + PCSK9 inhibitor + statin	4 (9.3)	3 (13.6)	7 (10.8)
At least three lipid- lowering therapies	30 (69.8)	11 (50.0)	41 (63.1)

Supplementary Table S3: Percentage Change in Calculated LDL Cholesterol for Patients with Background Lipid-Lowering Therapy from Baseline to Week 24

Background Therapy at Baseline, mean (SD)*	Background Therapy at Baseline		No Background Therapy at Baseline	
	Placebo IV Q4W	Evinacumab 15 mg/kg IV Q4W	Placebo IV Q4W	Evinacumab 15mg/kg IV Q4W
Statin	N=61		N=4	
	2.2 (32.3)	–47.3 (30.6)	–5.7 (22.7)	–46.2 (11.0)
Ezetimibe	N=49		N=16	
	–2.0 (30.6)	–53.1 (21.0)	12.2 (34.1)	–28.0 (45.5)
Lomitapide	N=14		N=51	
	–17.2 (47.6)	–49.6 (22.5)	4.5 (28.4)	–46.4 (32.3)
PCSK9 inhibitor	N=50		N=15	
	1.7 (30.3)	–49.5 (31.9)	0.7 (36.2)	–38.9 (20.1)
Apheresis	N=22		N=43	
	–7.3 (34.3)	–46.2 (18.1)	6.8 (29.2)	–47.8 (34.4)

Patients were taking these medications with or without other medications. IV, intravenous; LDL, low-density lipoprotein; PCSK9, proprotein convertase subtilisin/kexin type 9; Q4W, every 4 weeks; SD, standard deviation.

10

Marked plaque regression in homozygous familial hypercholesterolemia

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Submitted

ABSTRACT

Background: Both plasma low-density lipoprotein (LDL) cholesterol levels and risk for premature cardiovascular disease are extremely elevated in patients with homozygous familial hypercholesterolemia (HoFH), despite the use of multiple cholesterol lowering treatments. Given its inborn nature, atherosclerotic plaques are commonly observed in young HoFH patients. Whether aggressive lipid lowering strategies result in plaque regression in adolescent patients is unknown.

Methods: Two HoFH patients with null/null *LDLR* variants who participated in the R1500-CL-1629 randomized clinical trial (NCT03399786), evaluating the LDL cholesterol lowering effect of evinacumab (a human antibody directed against ANGPTL3; 15mg/kg intravenously once monthly), were included in this study. Patients underwent coronary computed tomography angiography (CCTA) before randomization and after 6 months of treatment.

Results: Both patient A (aged 12) and B (aged 16) were treated with a statin, ezetimibe and weekly apheresis. Evinacumab decreased mean pre-apheresis LDL cholesterol levels form 213±29 and 196±56 mg/dL to 96±12 and 85±5 mg/dL and post-apheresis LDL levels from 56±10 and 53±15 mg/dL to 31±6 and 30±5 mg/dL in patient A and B, respectively. Total plaque volumes were reduced by 76% and 85% after 6 months of evinacumab treatment in patient A and B, respectively.

Conclusions: We describe two severely affected young HoFH patients in whom profound plaque reduction is observed with CCTA after aggressive lipid lowering therapy with statins, ezetimibe, LDL apheresis, and evinacumab. This shows that atherosclerotic plaques possess the ability to regress at young age, even in HoFH patients.

INTRODUCTION

Homozygous familial hypercholesterolemia (HoFH) is a rare inherited condition characterized by extremely elevated low-density lipoprotein (LDL) cholesterol levels with untreated plasma concentrations reaching levels over 500mg/dL (>13 mmol/L).[1] Patients with HoFH are at severely increased risk for myocardial infarction (MI) due to massive cholesterol accumulation in the coronary arterial walls, a process that starts at young age. Despite treatment with statins, the majority of HoFH children between 3 and 16 years already show coronary lesions, which typically develop to severe plaques in the second decade of life.[2,3]

Aggressive LDL cholesterol lowering is advocated in current clinical guidelines to slow down plaque progression and lower the risk of premature cardiovascular events.[4] The effect of combining multiple lipid lowering therapies such as statins, ezetimibe, pro-protein convertase subtilisin/kexin type 9 (PCSK9) inhibitors and lomitapide is limited in severe HoFH patients with null/null pathogenic variants in *LDLR,* the gene encoding the LDL receptor. These patients typically rely on weekly lipoprotein apheresis to achieve acceptable LDL cholesterol levels.

In patients at risk for cardiovascular disease it has proven challenging to induce regression of atherosclerotic plaques. In the GLAGOV study it was shown that aggressive LDL cholesterol lowering to mean levels of 30 mg/dL resulted in only very modest (~1%) plaque regression visualized by intravascular ultrasound in patients in the post-acute coronary syndrome (ACS) period.[5] Based on indirect evidence, it has been suggested that intensive LDL cholesterol lowering may offer a larger benefit earlier in the course of atherogenesis, when there is a lower burden of fibrotic and calcified plaque.[6]

In the present study, we report two adolescent HoFH patients in whom highly aggressive LDL cholesterol lowering resulted in reversal of atherosclerotic plaque burden on CCTA imaging. To this end, a combination of established LDL cholesterol lowering drugs, LDL apheresis, and evinacumab, a fully human monoclonal antibody targeting angiopoetin-like protein 3 (ANGPTL3) was used.[7]

METHODS

Patient characteristics

The clinical characteristics of both patients are described in Table 1. Patient A was 12 years old (weight 45 kg, height 163 cm) during the initial CCTA imaging. At 4 years of age, she was diagnosed with HoFH caused by a bi-allelic null/null c.313G>A, p.(?) pathogenic variant resulting in an LDL cholesterol of 805 mg/dL (20.8 mmol/L). At physical examination, multiple tuberous and tendon xanthomas were noted, located between the hand digits, on the Achilles tendons and extensor tendons of the foot and between the buttocks. Upon diagnosis, statin and ezetimibe were started and LDL apheresis (Kaneka LA-15, Osaka, Japan) was initiated at the age of 6 years. At initial CCTA imaging, Patient A was treated with rosuvastatin 20 mg, ezetimibe 10 mg, and weekly LDL apheresis.

Patient B was 16 years old, weighed 65 kg and was 165 cm tall at initial CCTA imaging. She was diagnosed with HoFH at the age of 9, upon the identification of bi-allelic c.(1586+1_1587-1)_(1845+1_1846-1)dup, p(?) pathogenic variants in *LDLR*. At diagnosis, LDL cholesterol levels were 598 mg/dL (15.5 mmol/L), and the patient had multiple tuberous xanthomas at the elbows and between the buttocks, as well as bilateral corneal arcus. LDL apheresis was started at the age of 11 years, in addition to statin and ezetimibe, which were started at diagnosis. At initial CCTA imaging, Patient B was treated with rosuvastatin 20 mg, ezetimibe 10 mg, and weekly LDL apheresis.

Study medication

The two patients participated in the R1500-CL-1629 randomized clinical trial (NCT03399786), which evaluated the LDL cholesterol lowering effect of evinacumab (REGN1500, 15mg/kg intravenously once monthly) in HoFH patients.[7] The most important inclusion criteria were, next to the diagnosis of HoFH, undergoing stable lipid lowering therapies (including LDL apheresis) and a persistent LDL cholesterol level above 70mg/dL (1.81 mmol/L). The trial consisted of a 24-week blinded treatment period (2:1 ratio evinacumab:placebo) followed by a 24-week open label extension period. Both patients were included in the study in November 2018 and received placebo during the blinded treatment period. Patient A received

her first dose of evinacumab 10 months after the initial CCTA. After 6 monthly doses of Evinacumab the CCTA was repeated. Patient B received her first dose of evinacumab 25 months after the first CCTA and the follow up CCTA was performed 5 months after the initiation of evinacumab.

LDL cholesterol levels over time

LDL cholesterol levels were measured directly before the start of apheresis (pre-apheresis) and directly after completion of apheresis (post-apheresis). LDL cholesterol levels during the blinded treatment period were obtained after unblinding of the original trial through the sponsor of the trial (Regeneron) and only consist of pre-apheresis values. We calculated the mean LDL cholesterol during each treatment period (before and during Evinacumab treatment) separately for pre- and post-apheresis values. A linear regression model with LDL cholesterol as dependent and time as independent variable was used to display LDL cholesterol levels before and during evinacumab treatment.

Coronary computed tomography angiography (CCTA)

CCTA imaging was performed to assess atherosclerotic soft plaque progression after intensified LDL cholesterol lowering treatment with evinacumab and to clinically evaluate whether LDL apheresis frequency could be decreased these patients. CCTA imaging was done independently from the evinacumab study protocol and was ordered by the treating physician. A third-generation dual source 2x192 slice CT-scanner (Siemens Somatom Force, Germany) was used for CCTA imaging. A prospective ECG-triggered sequential step and shoot sequence protocol was applied in all scans. The lowest feasible tube voltage was chosen using care-kV (range 70–120 kV). Iodine-based contrast (Ultravist 300 mg/ml, Bayer Healthcare Pharmaceuticals, Berlin, Germany) was administered intravenously and the dose was adjusted to the patient's body weight and the kV setting. All four scans were analyzed by four experienced assessors. First, they were clinically assessed by one clinical assessor (R.N.P) for the presence of plaque and stenosis severity. Next, the CCTA scans were quantified by a second assessor (M.B.). To confirm their findings, scans were assessed by two additional assessors who were blinded for the order of the scans and were uninformed for any clinical information of the two patients (R.S.D. and P.A.D.). Furthermore, coronary lesions were analyzed for the presence

of qualitative atherosclerotic adverse plaque characteristics, i.e. low-attenuation plaque (LAP) and positive remodeling (PR).[8] LAP was defined as a plaque containing any voxel <30 Hounsfield Units (HU).[8] The remodeling index was computed as the ratio of vessel area at the site of the maximal lesion to that of a proximal reference point, with an index >1.1 representing PR.[8] Additionally, atherosclerotic plaque burden was quantified by assessing total plaque volumes automatically within manually designated regions. In a consensus meeting between the two blinded assessors (R.S.D. and P.A.D.), one discrepancy in plaque volume quantification was resolved (Table S1).

RESULTS

Effect of LDL cholesterol lowering treatment

In the three years before participation in the R1500-CL-1629 trial, while treated with a statin, ezetimibe and weekly LDL-apheresis, patient A and B had mean LDL cholesterol levels of 213±29 and 196±56 mg/dL pre-apheresis and 56±10 and 53±15 mg/dL post-apheresis, respectively (Figure 1; Table 1). Treatment with evinacumab lowered mean pre-apheresis LDL cholesterol levels to 96±12 and 85±5 mg/dL and post-apheresis LDL cholesterol levels to 31±6 and 30±5 mg/dL in patient A and B respectively (Figure 1; Table 1). Other lipid values closest to the day of CCTA imaging are shown in Table 2. Both patients did not experience any serious adverse events during treatment with evinacumab or placebo.

Table 1: Patient characteristics and lipid profile

	Patient A				**Patient B**			
Sex	Female				Female			
Genotype	c.313G>A, p.(?) c.313G>A, p.(?)				c.(1586+1_1587-1)_ (1845+1_1846-1)dup, p.(?) c.(1586+1_1587-1)_ (1845+1_1846-1)dup, p.(?)			
Age of diagnosis (years)	4				9			
LDL cholesterol at diagnosis (mg/dL)	804				599			
FH stigmata at diagnosis	Multiple xanthomas				Multiple xanthomas & bilateral corneal arcus			
Age start statin + ezetimibe (years)	4				9			
Age start LDL apheresis (years)	7				11			
Average LDL per treatment period								
Treatment	**Before evinacumab**		**During evinacumab**		**Before evinacumab**		**During evinacumab**	
Timepoint (pre/post-apheresis)	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**post**
LDL cholesterol in mg/dL (mean±SD)	213±29	56±10	96±12	31±6	196±56	53±15	85±5	30±5

LDL, low-density lipoprotein; FH, Familial Hypercholesterolemia

Table 2: Lipid values of both patients on the dates closest to CCTA imaging

	Patient A				**Patient B**			
Treatment	**Before evinacumab**		**During evinacumab**		**Before evinacumab**		**During evinacumab**	
Date CCTA	07-2017		11-2019		04-2017		12-2019	
Date lipid assessment	07-2018		10-2019		04-2017		01-2020	
Time point (pre/post-apheresis)	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**
Total cholesterol (mg/dL)	249	102	105	56	228	91	129	65
LDL cholesterol (mg/dL)	197	59	77	33	179	49	92	36
HDL cholesterol (mg/dL)	39	36	24	23	43	39	32	28
Triglycerides (mg/dL)	69	35	17	9	31	11	23	9
Apo B (g/L)	1.42	-	0.70	-	1.20	-	1.00	-
Apo A1 (g/L)	1.06	-	0.62	-	1.07	-	0.74	-
Lp(a) (mg/L)	60	-	62	-	115	-	76	-

CCTA, coronary computed tomography angiography; LDL, low-density lipoprotein; HDL, high-density lipoprotein; Apo B, apolipoprotein B; Apo A1, apolipoprotein A1; Lp(a), lipoprotein(a).

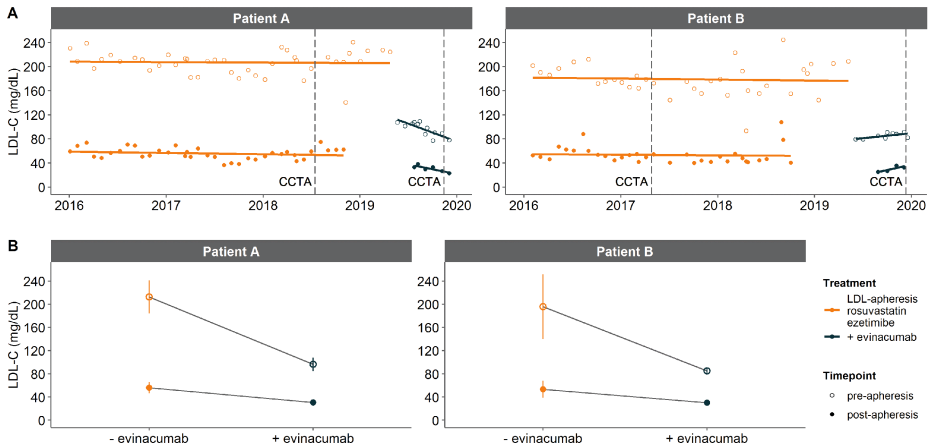

Figure 1: LDL cholesterol levels of the two patients
(A) LDL cholesterol over time, CCTA imaging time points, and evinacumab exposure for both patients, respectively. (B) Average LDL cholesterol per treatment period (before and during evinacumab) for both patients. LDL, low-density lipoprotein; CCTA, coronary computed tomography angiography

Coronary plaque quantification with CCTA

In patient A, baseline CCTA imaging showed a left-dominant coronary circulation and a calcified aortic root. One non-calcified lesion with positive remodeling was observed in the proximal left anterior descending (LAD) coronary artery, resulting in a total plaque volume (TPV) of 12.6±8.1 mm3 (Figure 2; Table S1). Treatment with evinacumab lowered mean pre-apheresis LDL cholesterol levels from 213±29 to 96±12 mg/dL and post-apheresis LDL cholesterol levels from 56±10 to 31±6 mg/dL (Table 1). At follow-up CCTA, which was performed 16 months after the initial CCTA, patient A had undergone a minimum of 6 months of evinacumab treatment. The follow-up CCTA showed large regression of the plaque burden to 3.0±1.5 mm3, which corresponds to a 76% reduction of TPV (Figure 1; Table S1).

In Patient B, baseline imaging showed a right-dominant coronary circulation with a TPV of 233.5±36.0 mm3 (Figure 1; Table S1), based on two non-calcified lesions in the LAD and one larger lesion in the right coronary artery (RCA), which showed signs of positive remodeling. Treatment with evinacumab lowered mean pre-apheresis LDL cholesterol levels from 196±56 to 53±15 mg/dL and post-apheresis LDL cholesterol levels from 85±5 to 30±5 mg/dL (Table 1). At follow-up CCTA, after 31.5 months, including 5 months of evinacumab treatment, no lesions were found

in the RCA, and the LAD lesions had markedly decreased in volume. TPV was shown to be decreased to 34.8±19.3 mm3, which corresponds to a 85% reduction of TPV (Figure 1; Table S1).

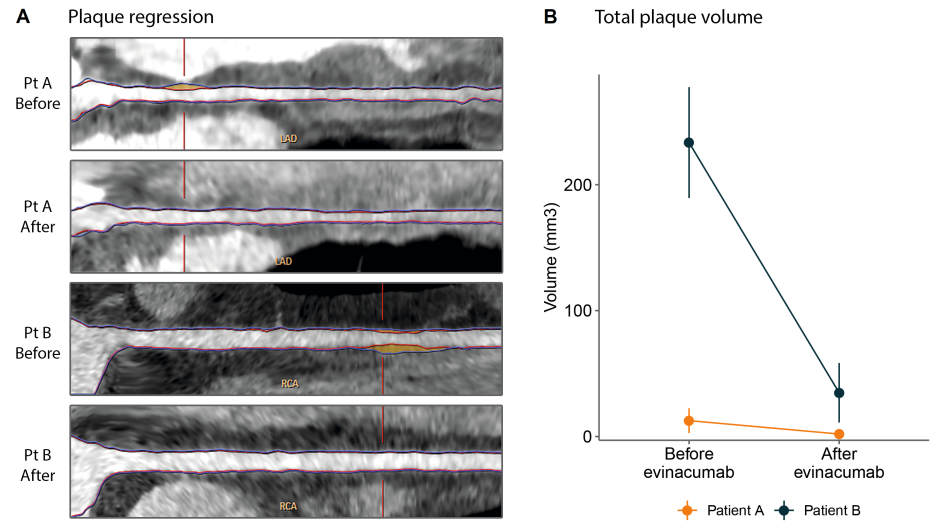

Figure 2: (A) (B) Stretched multi-planar reconstruction of coronary computed tomography angiography for patients A (LAD) and B (RCA) before and after treatment with evinacumab. Plaques are marked yellow. (C) Decrease of total plaque volume in both patients.

DISCUSSION

We describe two severely affected young HoFH patients in whom profound plaque reduction is observed with CCTA imaging after highly aggressive lipid lowering therapy with statins, ezetimibe, LDL apheresis, and evinacumab, a fully human monoclonal antibody against ANGPTL3. To our knowledge these are the first described HoFH patients reaching low enough LDL cholesterol levels where virtually complete atherosclerotic plaque regression was observed.

In contrast to the minor effect on plaque reduction (~1%) observed in older non-HoFH patients following aggressive lipid lowering therapy,[5] we observe a virtually complete plaque regression in our pediatric HoFH patients upon aggressive LDL cholesterol lowering. This observation complements the data showing the impact of early lipid lowering therapy on coronary plaque formation and even shows that

aggressive LDL cholesterol lowering can revert atherosclerosis when initiated at a young age.[6,9,10] The notion that "young plaques" are more susceptible to regression[11] is further strengthened by the fact that we observe this regression in adolescent patients who have had a cumulative LDL cholesterol exposure comparable with the average exposure of a forty year old male[10], while aggressive lipid lowering in these older subjects does only result in a minimally reduced plaque burden.[5] In fact, our data are in line with the recent observation that the risk of cardiovascular events is minimized in heterozygous FH patients, who started lipid lowering therapy around the age of 13 years.[12]

The potent plaque regression in our HoFH patients may have implications for the decision- making process when it comes to the length and aggressiveness of lipid lowering therapy in these rare patients. Our data may imply that following an 'induction' period with very aggressive lipid lowering therapy, a less intensive "maintenance therapy" may be considered in patients with nearly complete plaque regression. Accordingly, complete resolution of plaque in Patient A prompted us to lower the frequency of her weekly LDL apheresis, which has a considerable impact on the quality of life of this patient. Future studies are needed to evaluate the validity of this CCTA guided, personalized medicine approach.

Our study has several limitations. First and foremost, due to the limited number of HoFH patients included in this study, results cannot be readily extrapolated to all HoFH patients, but should be interpreted as a promising signal for early and aggressive CVD risk attenuation in this highly susceptible patient group. Furthermore, we described radiological plaque features, which are known to correlate with CVD risk in the general population.[9] However, validation with plaque features *in vivo* is lacking, nor do we have data to support that these changes will abolish the CVD risk in this specific type of patients.

In conclusion, we observed reversal of plaques on using CCTA imaging in young HoFH patients upon 5 to 6 months of aggressive reduction of LDL cholesterol to pre-apheresis levels below 100 mg/dL and post-apheresis levels below 35 mg/dL. This finding supports the hypothesis that young plaques can regress completely when aggressive LDL cholesterol lowering therapies are started at a (very) young age.

ACKNOWLEDGEMENTS

We would like to thank both patients for their participation in the Evinacumab trial and their consent for sharing the here described results with the rest of the medical and academic world.

DISCLOSURES

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SUPPLEMENTARY APPENDIX

Table S1: Total plaque volume quantifications

Patient	**A**		**B**	
Time point	**Before evinacumab**	**During evinacumab**	**Before evinacumab**	**During evinacumab**
Total plaque volume (mm^3) *Assessor 1 (unblinded)*	6.2	0	194.2	41.5
Total plaque volume (mm^3) *Assessor 2 (blinded)*	7.7	77.7*	281.1	8.5
Total plaque volume (mm^3) *Assessor 3 (blinded)*	24.0	6.0	225.1	54.3
Mean±SD total plaque volume (mm^3)	12.6±8.1	3.0±1.5*	233.5±36.0	34.8±19.3

Quantification of total plaque volumes in patient A and B by three independent assessors. *After discussion of the results during a consensus meeting between the two blinded assessors, the plaque volume based on the lesion in the LAD measured by assessor 2 in patient A was considered to be pathophysiological impossible and to result from a scan artefact. Therefore this volume was not taken into account in the calculated mean total plaque volume.

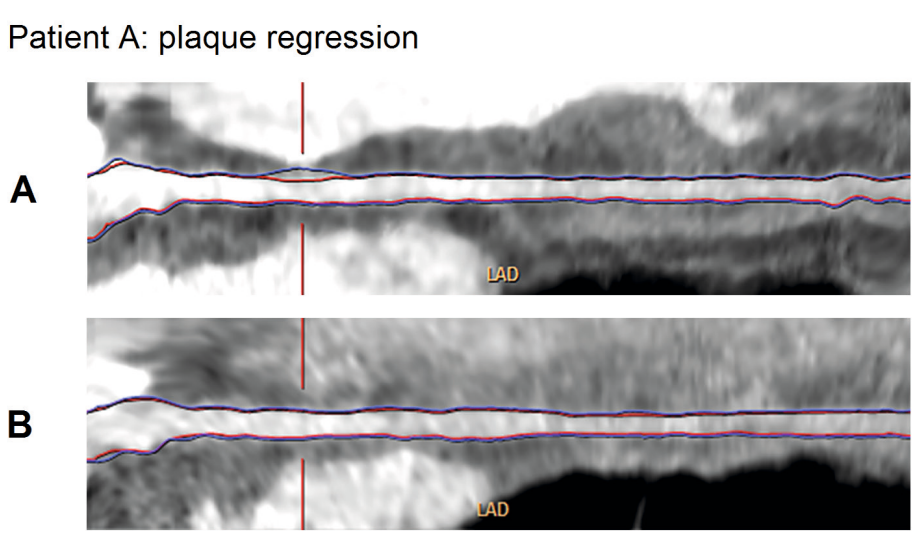

Figure S1: Plaque regression Patient A after evinacumab treatment
Original stretched multi-planar reconstruction of coronary computed tomography angiography for patient A (LAD) before (A) and after (B) treatment with evinacumab.

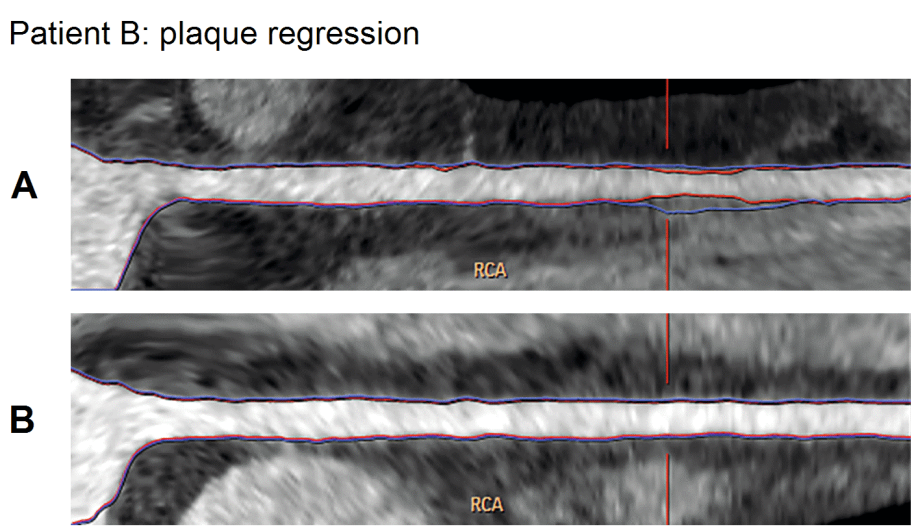

Figure S2: Plaque regression Patient B after evinacumab treatment
Original stretched multi-planar reconstruction of coronary computed tomography angiography for patient B (RCA) before (A) and after (B) treatment with evinacumab.

11

ANGPTL3 inhibition with evinacumab results in faster clearance of IDL and LDL apolipoprotein B in homozygous familial hypercholesterolemia patients

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ABSTRACT

Objective: The mechanism by which evinacumab, a fully human monoclonal antibody directed against Angiopoietin-like 3 protein (ANGPTL3) lowers plasma LDL cholesterol (LDL-C) levels in patients with Homozygous Familial Hypercholesterolemia (hoFH) is unknown. We investigated apolipoprotein B (apoB) containing lipoprotein kinetic parameters in hoFH patients, before and after treatment with evinacumab.

Approach and Results: Four hoFH patients underwent apoB kinetic analyses in two centers as part of a substudy of a trial evaluating the efficacy and safety of evinacumab in hoFH patients. The enrichment of apoB with the stable isotope (5,5,5-$^{2}H_{3}$)-Leucine was measured in VLDL, IDL, and LDL at different time points before and after intravenous administration of 15 mg/kg evinacumab. Evinacumab lowered LDL-C by 59±2% and increased IDL apoB and LDL apoB fractional catabolic rate (FCR) in all four hoFH subjects, by 616±504% and 113±14%, respectively. VLDL-apoB production rate decreased in 2 of the four subjects.

Conclusions: In this small study, ANGPTL3 inhibition with evinacumab is associated with an increase in the FCR of IDL apoB and LDL apoB, suggesting that evinacumab lowers LDL-C predominantly by increasing apoB-containing lipoprotein clearance from the circulation. Additional studies are needed to unravel which factors are determinants in this biological pathway. This study is registered on ClinicalTrials.gov (NCT04722068).

INTRODUCTION

Angiopoietin-like 3 protein (ANGPTL3) is a regulator of lipoprotein metabolism and has recently emerged as a novel therapeutic target to treat patients with dyslipidemia. Carriers of loss-of-function (LOF) variants in *ANGTPL3* present with low triglyceride, low low-density lipoprotein cholesterol (LDL-C), and low high-density lipoprotein cholesterol (HDL-C) plasma levels[1], as well as a decreased risk for cardiovascular disease (CVD) compared to non-carriers[2,3]

ANGPTL3 is mainly expressed in the liver and has been shown to reduce the activity of lipoprotein lipase (LPL) [4] and endothelial lipase (EL) *in vitro* [5], which is widely considered to explain the observed low triglyceride and HDL-C levels, respectively, in carriers of LOF variants in *ANGPTL3.* Hitherto, it is not fully elucidated which mechanism underlies the effect of ANGPTL3 on LDL-C metabolism. Animal models and *in vitro* studies have shown that ANGPTL3 has an effect both on production as well as clearance of apoB containing lipoproteins [6,7]. Interestingly, the LDL-C lowering effects of ANGPTL3 inhibition with either monoclonal antibodies or antisense oligonucleotides seems to be LDL receptor (LDLR) independent since marked LDL-C lowering is observed in both *Ldlr* knock-out (KO) mice [6,8] and homozygous familial hypercholesterolemic (hoFH) patients [9]. Recent reports of studies in mice suggest that endothelial lipase is required for ANGPTL3 inhibition to reduce LDL-C levels in absence of a functional LDLR [10,11].

To further elucidate the physiological effect of ANGPTL3 inhibition on LDL-C lowering, we investigated apoB-containing lipoprotein kinetics in four homozygous FH patients before and after treatment with evinacumab, a fully human monoclonal antibody against ANGPTL3.

MATERIALS AND METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Study population and clinical protocol

We invited hoFH patients who were enrolled in an open-label, single-arm study assessing the efficacy and safety of evinacumab in hoFH patients (NCT02265952)[9] to also participate in a sub-study to evaluate the production and catabolic rates of apoB-containing lipoproteins before and after receiving evinacumab (NCT04722068). In brief, patients ≥18 years old with genetically confirmed hoFH were eligible for inclusion if they had LDL-C levels above 70 mg/dL while on stable lipid lowering therapy for at least 4 weeks for statins and ezetimibe, 8 weeks for PCSK9 inhibition, and 12 weeks for lomitapide. Four subjects (two at Amsterdam UMC [AUMC] and two at University of Pennsylvania [UPENN]) underwent apoB kinetic measurements before the first dose of study drug was given (baseline) and 1 week (subject AUMC_1) or 6 weeks (all other subjects) after receiving one i.v. dose of evinacumab (treatment). Evinacumab was administered intravenously at a dose of 15 mg/kg. All subjects gave informed consent for participation in the sub-study and the parent study. All studies were approved by the medical ethic committees of the two research institutes (Amsterdam UMC and University of Pennsylvania).

Study protocol

Subjects fasted overnight (>10h) prior to the study. (5,5,5-$^{2}H_3$)-Leucine was administered via a venous catheter as 7 mg/kg bolus (AUMC) or as primed (1.34 mg/kg) continuous 12-h infusion (1.34 mg/kg/hr; UPENN). Blood samples were drawn at multiple timepoints for 24-48 hours for the determination of (5,5,5-$^{2}H_3$)-leucine enrichment of apoB in VLDL, IDL, and LDL fractions. The two subjects who were enrolled at the AUMC received a standardized meal (whole wheat bread with light cheese) two hours after bolus infusion and dinner ad libitum in the evening. After a 10 hours admission, patients went home and the blood sample at t=24 h was collected at the patients' home by a trained trial nurse. The two subjects enrolled at UPENN remained in the research unit overnight and were maintained in a constant fed condition for the first 20 hours of the study by receiving their total daily caloric

intake in the form of 10 identical small meals every other hour starting 1 h prior the start of the infusion. They were discharged after the 24-hour time point and returned at the research unit for a 48-hr blood draw.

Laboratory methods

We measured the incorporation of (5,5,5-$^{2}H_3$)-leucine in the apoB moiety in VLDL, IDL and LDL. At the AUMC site, VLDL, IDL and LDL fractions were isolated from plasma by a 1-step gradient ultracentrifugation using a SW41 rotor (Beckman). In short, the density of 3.5 ml of plasma was adjusted to 1.25 g/ml with 2.695 g KBr. 3.0 ml plasma (d=1.25 g/ml) was transferred to an ultra-clear Beckman SW41 tube. The gradient was formed by layering the following salt solutions on top of the plasma: 1) 2 ml d=1.225 g/ml; 2) 4 ml d=1.100 g/ml; 3) 3 ml d=1.006 g/ml. The different fractions were then isolated by centrifugation in a Beckman ultracentrifuge 29,000 rpm, 10^{0}C, 19h and termination without brake. Fractions were frozen and stored at -80 °C for further analysis. For leucine enrichment analysis of apoB VLDL, IDL and LDL fractions were precipitated with isopropanol, delipidated with ethanol-diethyl ether, dried and hydrolyzed with 6M HCl at 110 °C for 24h[12]. The samples were then prepared for analysis of leucine enrichment as described using norleucine as internal standard. Enrichments were determined by GC-MS GC-MSD5975c (Agilent Technologies, Amstelveen, The Netherlands) equipped with a VF17 ms column operated in SIM mode. For the correction and calculation of obtained isotope enrichments, the average values of the m/z 161:158 ratio were determined using a calibration curve with known quantities of labelled and unlabeled leucine[13]. The resulting m/z 161:158 and expressed as molar percentage ratio (MPE).[13]

At the UPENN site, the enrichment of VLDL, IDL and LDL apoB was determined as previously described [14]. Briefly, lipoprotein fractions were isolated from plasma by sequential ultracentrifugation. ApoB100 was isolated from VLDL, IDL, and LDL by SDS-PAGE. ApoB100 bands were hydrolyzed using 6N HCl followed by derivatization of amino acids to their heptafluorobutyryl isobutyl esters. Leucine isotope enrichments were determined in the IDOM Metabolic Tracer Resource at UPENN using GC-MS.[15]

ApoB kinetic modelling and parameter estimation

Fractional transfer and catabolic rates for apoB were determined by fitting the tracer data to a previously described multi-compartmental model[15] (shown in Supplemental Figure I) using the WinSAAM modeling program. The precursor plasma D3-leucine enrichment data were modeled as a forcing function for newly secreted apoB. Clearance from the VLDL and IDL remnants and LDL pools were fit using Bayesian estimation to improve parameter identifiability. On-treatment values for these parameters were set to the same values obtained during the baseline treatment period and were multiplied by another parameter that allowed them to increase, if necessary. VLDL, IDL, and LDL apoB concentrations were either measured directly (patients UPENN_1, UPENN_2) or calculated as a percentage of the total plasma apoB concentration (patients AUMC_1, AUMC_2). Pool sizes were determined by multiplying the apoB concentration in each fraction (mg/dL) by the estimated plasma volume (body weight in kg * 0.45 dL/kg). Production rates were calculated by multiplying fractional transfer and catabolic rates by the corresponding pool size and expressed relative to body weight.

Statistical methods

This is a descriptive study with a small sample size. Therefore, no formal statistical testing was performed. Results obtained from each subject are reported individually and summarized as mean ± standard deviation (SD), if normally distributed, or as median [inter quartile range], if not normally distributed. All analyses were performed in R version 3.6.1 (The R Foundation, Vienna, Austria).

RESULTS

Subject characteristics

The characteristics of the four adult hoFH patients are depicted in Table 1. The two subjects enrolled at the AUMC were compound heterozygous for *LDLR* defective variants (Table 1) and presented with less severe hypercholesterolemic phenotypes compared to the two UPENN subjects who were shown to carry two *null* variants in *LDLR* (Table 1). Both UPENN subjects stopped lipoprotein apheresis at least four weeks before the baseline study. Background lipid lowering therapy consisted of a statin, ezetimibe, and a PCSK9 inhibitor in three subjects and of lomitapide in the fourth subject (UPENN_2) and had been stable as required per protocol, in all participants.

Treatment effect of evinacumab on lipid profile

Exposure to one infusion with the ANGPTL3 antibody evinacumab (15mg/kg) resulted in pronounced decreases (mean percent change ±SD) in plasma levels of total cholesterol (-53±5%), LDL-C (-59±2%), HDL-C (-30±26%), triglycerides (-36±16%), apoB (-47±5%), and apoA-1 (-35±15%) (Table 1). No serious adverse events occurred during this kinetic substudy.

Treatment effect on apoB production and catabolic rates

In order to estimate the effects of evinacumab treatment on apoB turnover, we analyzed the rates at which the stable isotope leucine was incorporated into and removed from apoB in the VLDL, IDL, and LDL fractions. Upon evinacumab treatment apoB concentrations decreased in VLDL, IDL and LDL by 41±38%, 81±11%, and 40±7%, respectively (Supplementary Table I). The effect of evinacumab on the production rate (PR) of apoB in the VLDL, IDL and LDL fractions was variable among the participants (see Figure 1 and Table 1, Supplementary Figures II and IIIA-D for individual results), and showed mean percent changes of -25±50%, 13±20%, and 27±8%, for VLDL, IDL, and LDL, respectively. The effect on fractional catabolic rate (FCR) was more pronounced and consistent. ApoB FCR increased by 16±24%, 616±504%, and 113±14% in VLDL, IDL and LDL fractions respectively (see Figure 1 and Table 1, Supplementary Figures II and IIIA-D for individual results).

Table 1: Characteristics and kinetic parameters for all subjects at baseline and after treatment with evinacumab

Subject ID	AUMC_1		AUMC_2		UPENN_1		UPENN_2		
Age (years)	42		42		38		31		
Sex	Male		Female		Male		Female		
BMI (kg/m²)	25.9		23.8		27.6		29.2		
FH mutations	Compound heterozygote *LDLR:* p.Gly207_Cys231del *LDLR:* p.Gly335Val		Compound heterozygote *LDLR:* p.Gly207_Cys231del *LDLR:* p.Ser306Leu		True homozygote *LDLR:* p.Ser177Leu		Compound heterozygote *LDLR:* p.Ala627Profs*38 *LDLR:* c.314-?_940+?del		
Mutational category	Defective/Defective		Defective/Defective		Null/Null		Null/Null		
Background Lipid Lowering Therapies	Rosuvastatin 20mg Ezetimibe 10mg Evolocumab 140mg Q2W		Rosuvastatin 20mg Ezetimibe 10mg Evolocumab 140mg Q2W		Atorvastatin 80mg Ezetimibe 10mg Evolocumab 140 Q2W		Lomitapide 20mg		
Timepoint	**Baseline**	**Treatment**	**Baseline**	**Treatment**	**Baseline**	**Treatment**	**Baseline**	**Treatment**	**Mean Percent Change (±SD)**
Body weight (kg)	92.7	90.2	66.0	68.0	79.9	81.4	70.2	68.5	0 (3)
Lipid profile (mg/dL)									
Total cholesterol	159	77	167	88	407	177	776	325	-53 (5)
LDL cholesterol	100	39	101	44	339	140	708	286	-59 (2)
HDL cholesterol	41	25	44	30	46	20	17	18	-30 (26)
Triglycerides	41	29	48	32	48	38	114	47	-36 (16)
ApoB	77	44	81	45	187	102	399	182	-47 (5)
ApoA-I	117	76	117	83	119	53	60	47	-35 (15)
Production rate (PR) per lipoprotein sub fraction (mg/kg/day)									
VLDL apoB	1.99	2.36	6.54	7.65	13.24	4.74	45.85	12.23	-25 (50)
IDL apoB	2.02	2.46	4.36	5.42	8.36	6.94	9.91	12.25	13 (20)
LDL apoB	6.35	8.29	12.17	15.93	20.34	26.9	26.31	30.37	27 (8)
Fractional catabolic rate (FCR) per lipoprotein sub fraction (pools/day)									
VLDL apoB	0.79	0.92	9.24	13.74	45.12	44.01	14.08	14.1	16 (24)
IDL apoB	0.38	5.32	0.91	6.12	30.9	57.92	4.15	25.12	616 (504)
LDL apoB	0.23	0.51	0.44	0.86	0.24	0.5	0.16	0.36	113 (14)

Characteristics and kinetic parameters for all subjects at baseline and after treatment with evinacumab. BMI, body-mass index; SD, standard deviation; LDL, low-density lipoprotein; HDL, high-density lipoprotein; apoB, apolipoprotein B100; apoA-I, apolipoprotein A-I; VLDL, very-low density lipoprotein; IDL, intermediate-density lipoprotein; LDLR, LDL receptor.

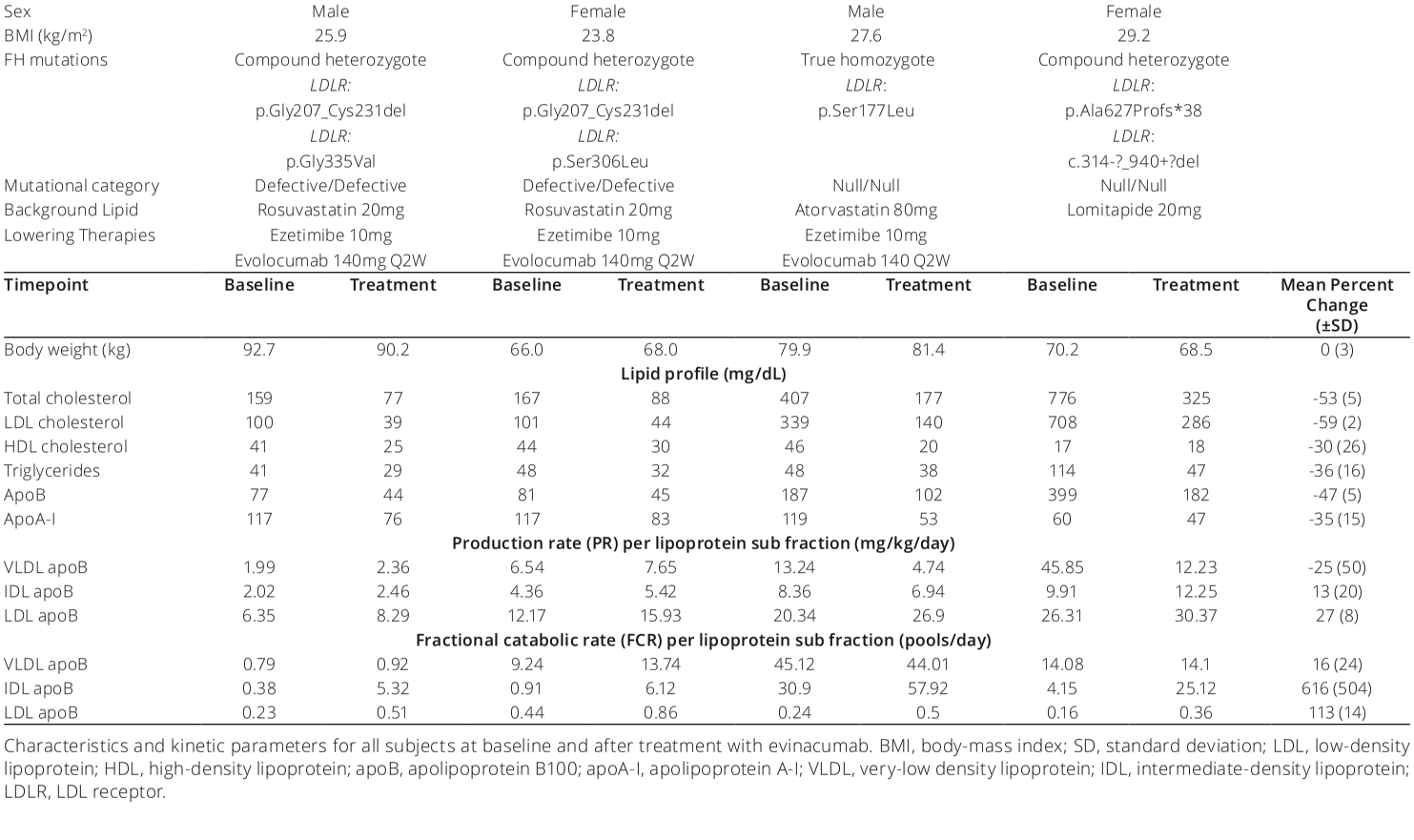

Figure 1: Percentage change between treatment and baseline apoB production and fractional catabolic rates for lipoprotein sub fractions

PR, production rate; FCR, fractional catabolic rate; apoB, apolipoprotein B100; VLDL, very-low density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein.

DISCUSSION

This is the first study to investigate the effects of ANGPTL3 inhibition with a fully human monoclonal antibody on apoB kinetics in humans. In the four hoFH patients evaluated, inhibition of ANGPTL3 by evinacumab resulted in marked increases in fractional catabolic rates of IDL and LDL apoB. The effect on the apoB production rates of the lipoprotein sub-fractions was less clear, with two of the four participants showing a decrease in the production rate of VLDL apoB, and a small increase of the production rate of IDL- and LDL apoB noted in all four subjects. Although we acknowledge the very small number of subjects studied, the heterogeneity of their phenotypic and genotypic characteristics, and the difference in the kinetic study protocol , these data suggest that the decrease in LDL-C plasma concentrations observed following evinacumab administration is mainly due to an increased catabolism of the IDL and LDL fractions.

Our results are in line with an earlier published apoB kinetic study in a family with familial hypobetalipoproteinemia [16], which was later reclassified as familial combined hypolipidemia caused by *ANGPTL3* LOF variants [1]. The affected family

members presented with lower VLDL apoB production rates, and increased IDL and LDL apoB fractional catabolic rates compared to unaffected family controls [16], with a clear gene dose effect [1].

Possible mechanisms underlying the effect of ANGPTL3 inhibition on LDL-C could be related to an enhanced clearance and/or reduced production of LDL precursors. Given the known inhibitory effects of ANGPTL3 on LPL [4], it is possible that the reduced levels of LDL-C are at least in part due to increased lipolytic activity of LPL and consequent accelerated clearance of apoB containing particles from the circulation via LPL and non-LPL-mediated pathways. We observed a substantial increase in IDL apoB FCR in all 4 subjects with only a slight increase in LDL apoB PR, indicating that IDL is mostly cleared from the circulation. This is consistent with the minimal effects in LDL apoB production rate in the family carrying *ANGPTL3* loss-of-function variants [1]. The known effect of ANGPTL3 on LPL cannot, however, by itself explain the observed increase in IDL and LDL apoB FCR.

ANGPTL3 is also known to inhibit endothelial lipase (EL) [5]. EL is well known to affect HDL metabolism and the lowering effect on HDL-C by ANGPTL3 inhibition is an EL-dependent mechanism [17]. Additionally, EL can affect apoB-containing lipoprotein metabolism. EL overexpression was associated with a decrease in total and non-HDL-C levels in several mouse models, including *ldlr* KO mice [18]. Interestingly, overexpression of EL was also associated with faster LDL particle clearance in *ldlr* KO mice [18], suggesting that the increase in LDL apoB catabolism observed during treatment with evinacumab in the four hoFH patients could be mediated, at least in part, by an increase in EL activity. Indeed, two recent reports identified the critical role of EL in mediating LDL-C lowering by an LDLR independent pathway [10,11]. These studies in mice lacking both LDLR and EL support the importance of the ANGPTL3/EL pathway in mediating VLDL remnant particles clearance and LDL-C lowering [10]. The marked increase in IDL apoB FCR observed in our hoFH patients after treatment with evinacumab are in line with those results. We also observed a small but consistent increase in LDL apoB PR and a more substantial increase in LDL apoB FCR, suggesting that ANGPTL3 inhibition may also directly affect LDL apoB metabolism in hoFH patients. Further research is needed to fully elucidate the mechanism of evinacumab induced LDL-C lowering in humans.

Although the existence of an LDLR-independent pathway is supported by studies in mice [8,10] as well as by the remarkable reduction in LDL-C observed in hoFH patients treated with evinacumab [19,20] and the increase in LDL apoB FCR in carriers of either *LDLR* null/null or defective variants observed in this study, it is not yet clear what receptor(s) are responsible for apoB-containing lipoproteins uptake from the circulation. Extensive studies in animal models suggest that the LDL-C lowering effect of evinacumab is not dependent on a number of other receptors or ligands, such as apolipoprotein E, LDLR related protein 1, and syndecan 1, which are known to affect LDL or its precursors [6], and SR-BI [10].

Alternative to an enhanced clearance, a reduction in LDL-C levels could theoretically be caused by a decreased production in LDL precursors (i.e. VLDL and/or IDL). Although the mechanism(s) underlying a decrease in VLDL secretion are not immediately apparent, kinetic studies in fasting carriers of *ANGPTL3* LOF mutations showed a significant decrease in VLDL apoB production rate [1]. A similar finding was observed in hepatocarcinoma cell lines treated with ANGPTL3 siRNA [7]. A decrease in VLDL-TG secretion, but not in VLDL apoB secretion or VLDL clearance, was observed in mouse models treated with a monoclonal antibody [6] or antisense oligonucleotides [8] against ANGPTL3. In our study, we observed a reduction in VLDL apoB secretion in the two subjects carrying two *LDLR* null variants, but not in the two subjects carrying *LDLR* defective variants. Thus, the effect on VLDL production may differ based on the mechanism by which ANGPTL3 is inhibited (i.e., via intrahepatic RNA inhibition or a monoclonal antibody), the genetic background (*ANGPTL3* LOF variant vs. *ANGPTL3* wildtype with pharmacological ANGPTL3 inhibition; *LDLR* null vs. *LDLR* defective variants) and metabolic state (fasting vs. non-fasting) of the studied population. Larger studies are needed to investigate these hypotheses.

The diverse clinical and genetic characteristics in the four subjects warrant further discussion. Although a genetic defect was identified in all patients, the subjects from UPENN carried *LDLR null/null* variants, resulting in total loss of LDLR function [19]. The baseline LDL-C levels were therefore higher compared to the levels in two patients from the AUMC, who carried *LDLR* defective variants [19] and had LDL-C levels that were lower with the concomitant medications. It is of particular interest that, contrary to other lipid lowering drugs, such as PCSK9 inhibitors [21,22], the lipid

lowering effect of ANGPTL3 inhibition by evinacumab seems to be independent of the presence of residual LDLR activity, supporting the data obtained in animal models [6,10], as well as in a pilot clinical trial [9]. These findings are confirmed in the recently published ELIPSE trial (Efficacy and Safety of Evinacumab in Patients With Homozygous Familial Hypercholesterolemia; NCT03399786), that showed a similar LDL-C lowering effect of Evinacumab in hoFH patients with *null/null* and non-*null/null* variants [20].

We acknowledge the several limitations of our study. First and foremost, the sample size of four hoFH patients is small. Due to the inter-patient variability in kinetic parameters we would need a greater number of patients to conduct formal statistical testing on these parameters. For this reason, the study is only descriptive. Furthermore, all four subjects carried *LDLR* variants in the *LDLR* gene, and kinetic results may differ in hoFH carrying variants in other FH-causing genes, namely *APOB*, *PCSK9* and *LDLRAP1*. Secondly, different infusion and lipoprotein isolation protocols were used at the two participating centers and may have contributed to some variability in the fit of the compartmental model and, ultimately, to some variability in the kinetic results. Lastly, while three subjects underwent the on-treatment kinetic study six weeks after the evinacumab infusion, one of the AUMC subjects (AUMC_1) underwent the on-treatment kinetic study one week after receiving the infusion. Based on the data collected during the parent clinical trials and other studies, LDL-C levels typically reach nadir after 4 weeks of receiving evinacumab infusion and remain generally stable at week 6 [9]. Therefore, it is possible that subject AUMC_1 was not yet in a steady state when he underwent the second kinetic study, which may have contributed to some of the observed heterogeneity in the results. An early (non steady state) effect of evinacumab could have resulted in the observed magnitude of effect on IDL-apoB FCR of 1300% in this particular subject (See Figure 1). These limitations, taken together, have likely contributed to some of the variability observed in the kinetic parameters in this study, and may affect the generalizability of the results.

In conclusion, ANGPTL3 inhibition with evinacumab markedly increases IDL and LDL apoB catabolic rates in this small study of four hoFH patients, suggesting that evinacumab lowers LDL-C predominantly by increasing apoB-containing

lipoprotein clearance from the circulation. Additional studies with a larger sample size are needed to confirm our findings as well as to identify the biological pathways involved in this process.

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DISCLOSURES

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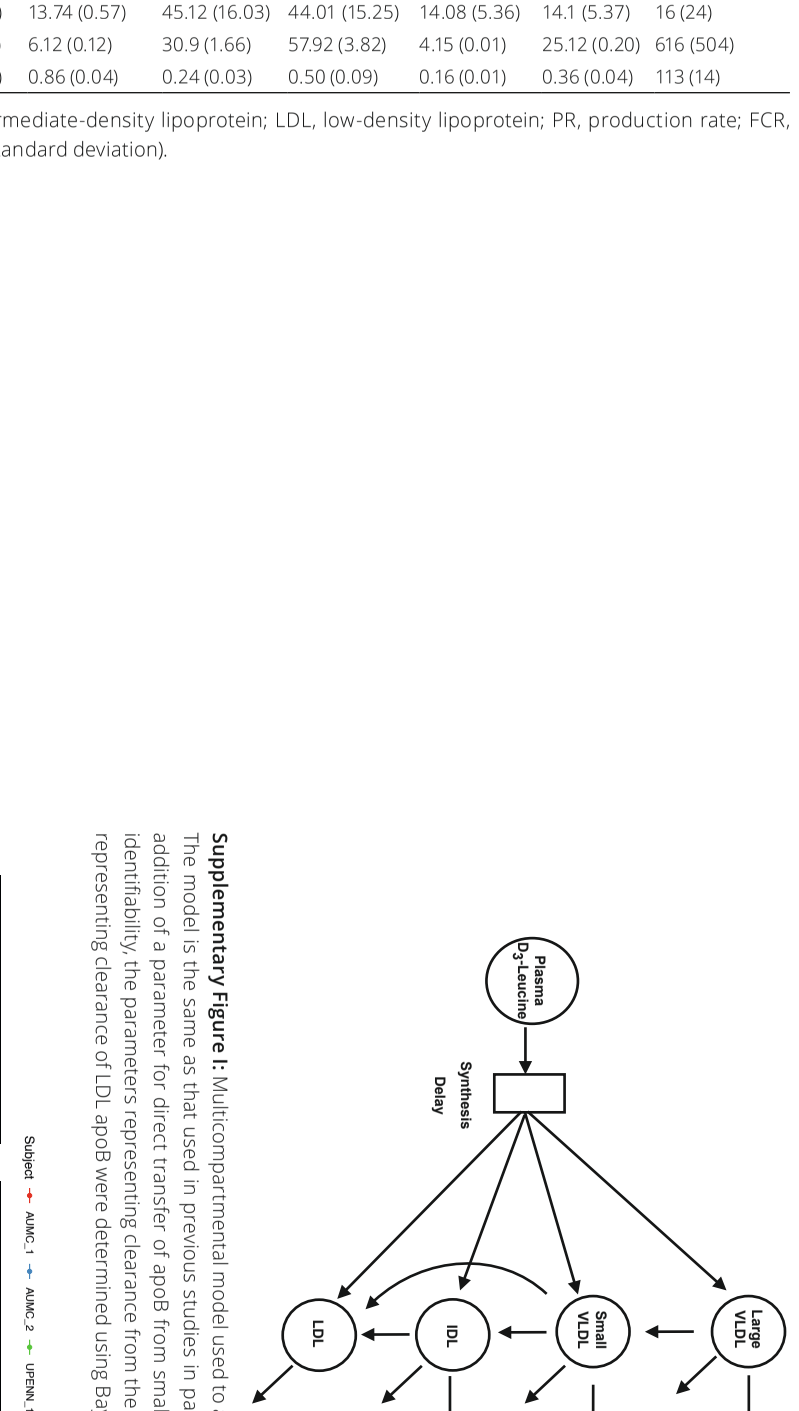
SUPPLEMENTAL MATERIALS

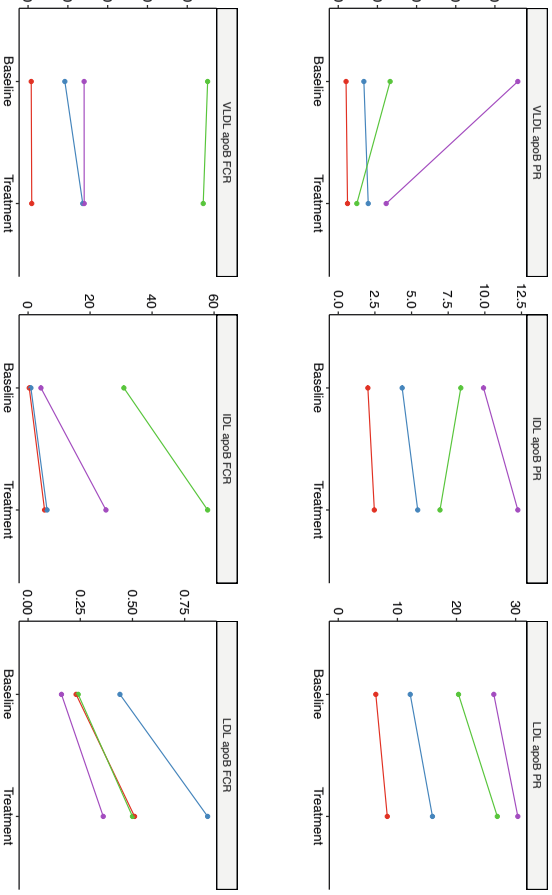
Supplementary Table I: Complete list of apoB kinetic parameters before and after evinacumab

Subject	AUMC_1		AUMC_2		PENN_1		PENN_2		Mean Percent Change (±SD)
Time point	**Baseline**	**Treatment**	**Baseline**	**Treatment**	**Baseline**	**Treatment**	**Baseline**	**Treatment**	
Body weight (kg)	92.7	90.2	66	68	79.9	81.4	70.2	68.5	0 (3)
Plasma apoB	78.83	42.54	73.65	44.57	190.2	119.8	381.8	192.4	-43 (6)
VLDL apoB concentration (mg/dl)	5.61	5.71	1.57	1.24	0.7	0.23	4.5	0.97	-41 (38)
IDL apoB concentration (mg/dl)	11.8	1.03	10.61	1.97	0.62	0.21	4.76	0.66	-81 (11)
LDL apoB concentration (mg/dl)	61.42	35.81	61.46	41.36	188.9	119.4	372.5	190.8	-40 (7)
VLDL apoB PR (mg/kg/d)	1.99 (0.38)	2.36 (0.55)	6.54 (0.18)	7.65 (0.32)	13.24 (4.70)	4.74 (1.64)	45.85 (17.45)	12.23 (4.66)	-25 (50)
IDL apoB PR (mg/kg/d)	2.02 (0.18)	2.46 (0.31)	4.36 (0.06)	5.42 (0.10)	8.36 (0.45)	6.94 (0.46)	9.91 (0.03)	12.25 (0.10)	13 (20)
LDL apoB PR (mg/kg/d)	6.35 (0.65)	8.29 (1.11)	12.17 (0.36)	15.93 (0.68)	20.34 (2.47)	26.9 (4.80)	26.31 (1.88)	30.37 (3.75)	27 (8)
VLDL apoB FCR (pools/d)	0.79 (0.15)	0.92 (0.21)	9.24 (0.26)	13.74 (0.57)	45.12 (16.03)	44.01 (15.25)	14.08 (5.36)	14.1 (5.37)	16 (24)
IDL apoB FCR (pools/d)	0.38 (0.03)	5.32 (0.67)	0.91 (0.01)	6.12 (0.12)	30.9 (1.66)	57.92 (3.82)	4.15 (0.01)	25.12 (0.20)	616 (504)
LDL apoB FCR (pools/d)	0.23 (0.02)	0.51 (0.07)	0.44 (0.01)	0.86 (0.04)	0.24 (0.03)	0.50 (0.09)	0.16 (0.01)	0.36 (0.04)	113 (14)

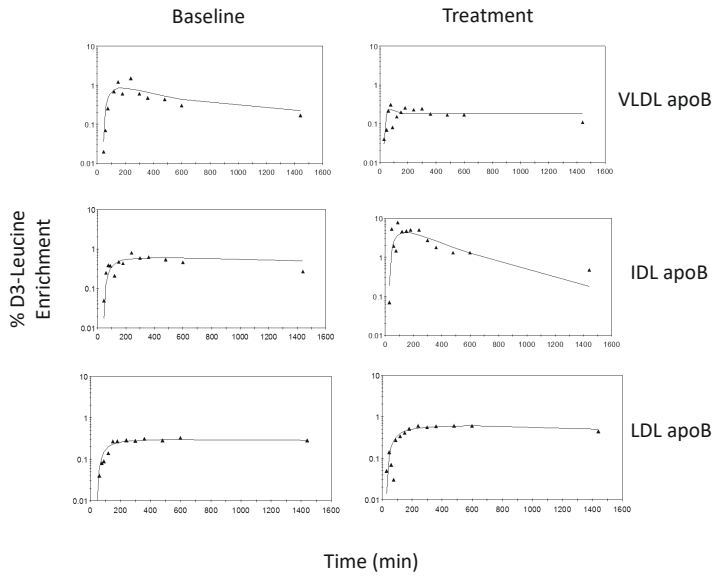
apoB, apolipoprotein B100; VLDL, very low-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; PR, production rate; FCR, fractional catabolic rate. PR and FCR are shown as parameter estimates (standard deviation).



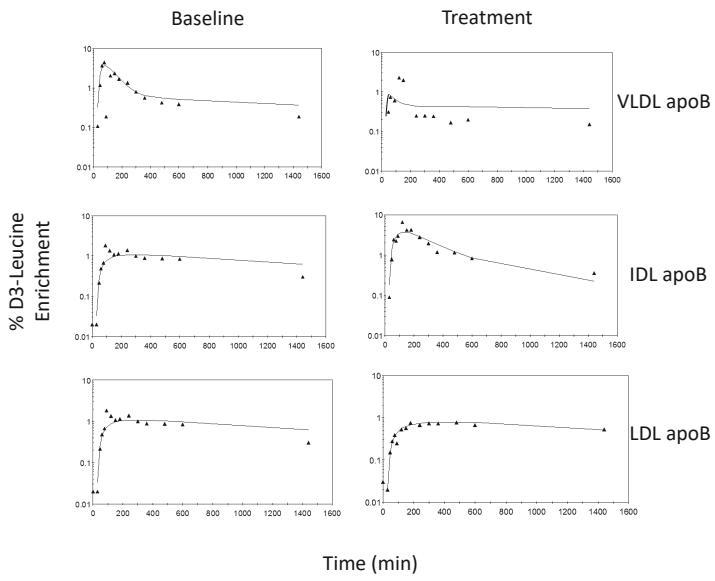
Supplementary Figure I: Multicompartmental model used to analyze tracer kinetic data
The model is the same as that used in previous studies in patients with homozygous FH1 with the addition of a parameter for direct transfer of apoB from small VLDL to LDL. To improve parameter identifiability, the parameters representing clearance from the VLDL and IDL remnant pools and that representing clearance of LDL apoB were determined using Bayesian estimation.



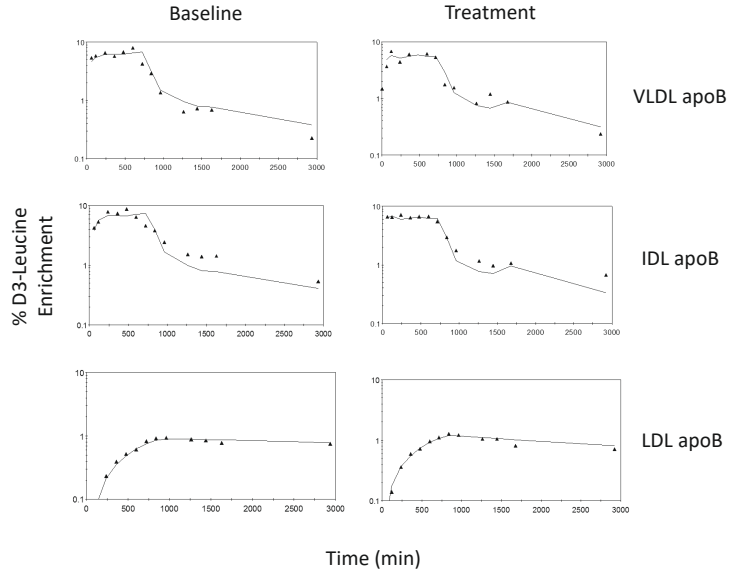
Supplementary Figure II: Individual apolipoprotein B100 production rates and fractional catabolic rates.
Values of apolipoprotein B100 (apoB) production rates (PR) and fractional catabolic rates (FCR) for individual subjects before and after treatment with evinacumab. VLDL, very-low density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein.



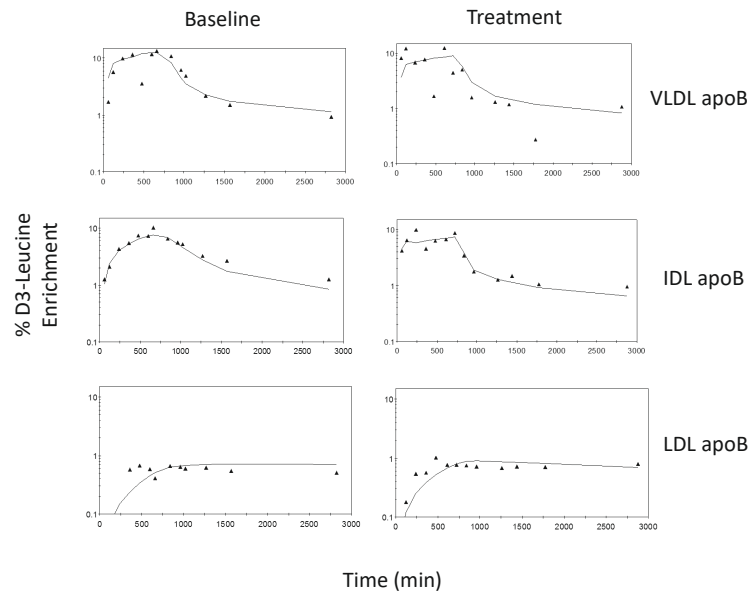
Supplementary Figure IIIA: Model fit (line) to tracer data (triangles) for AUMC_1



Supplementary Figure IIIB: Model fit (line) to tracer data (triangles) for AUMC_2



Supplementary Figure IIIC: Model fit (line) to tracer data (triangles) for UPENN_1



Supplementary Figure IIID: Model fit (line) to tracer data (triangles) for UPENN_2

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Statin therapy reduces plasma angiopoietin-like 3 (ANGPTL3) concentrations in hypercholesterolemic patients via reduced liver X receptor (LXR) activation

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ABSTRACT

Background and aims: Statins suppress hepatic mRNA expression of *ANGPTL3* encoding angiopoietin-like 3 in healthy subjects, but it is unknown if plasma ANGPTL3 concentrations are affected by statins prescribed to hypercholesterolemic patients in clinical practice. We therefore investigated the effect of statin treatment on plasma ANGPTL3 concentrations in hypercholesterolemic patients. In addition, we explored the underlying mechanism by which statins regulate ANGPTL3 *in vitro*.

Methods: Plasma ANGPTL3 concentrations were measured in 93 genetically confirmed familial hypercholesterolemia (FH) patients who were using statin therapy and 61 statin naïve FH patients. Moreover, levels were measured in 14 hypercholesterolemic patients who discontinued their statin treatment for 4 weeks. *In vitro* studies were performed with Huh7 human hepatoma cells.

Results: Plasma ANGPTL3 concentrations were 15% lower in statin treated FH patients compared to statin naïve FH patients (145 (120-193) vs. 167 (135-220) ng/ml, p=0.012). Statin discontinuation resulted in a 21% (p<0.001) increase of plasma ANGPTL3 concentrations. Simvastatin reduced *ANGPTL3* mRNA expression and ANGPTL3 secretion of Huh7 cells. Liver X receptor (LXR) activation with T0901317 increased *ANGPTL3* mRNA expression and ANGPTL3 secretion by 6- and 3-fold, respectively. Adding simvastatin did not mitigate this effect but adding the LXR antagonist GSK2230 to simvastatin incubated Huh7 cells diminished simvastatin-induced reductions in *ANGPTL3* mRNA expression and ANGPTL3 secretion. Simvastatin reduced intracellular oxysterol concentrations. Oxysterols are endogenous LXR ligands, implying that simvastatin suppresses ANGPTL3 secretion via reduced oxysterol-mediated LXR activation.

Conclusions: Statins lower plasma ANGPTL3 concentrations in hypercholesterolemic patients, likely due to decreased oxysterol-mediated LXR activation.

INTRODUCTION

Angiopoietin-like 3 (ANGPTL3) is a member of a family of eight angiopoietin-like proteins (1,2) and is primarily expressed and secreted by the liver (3). ANGPTL3 recently emerged as a key player in lipid metabolism following the observation that loss-of-function (LOF) mutations in *ANGPTL3* result in low plasma triglyceride (TG), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol concentrations compared to controls (1,4-9). ANGPTL3 is considered a potential drug target to treat patients with dyslipidemia (10). Indeed, ANGPTL3 inhibition by a monoclonal antibody or an antisense oligonucleotide reduced plasma LDL cholesterol concentrations by approximately 30% and 50% in healthy volunteers (11,12) and in patients with homozygous familial hypercholesterolemia (FH) (13), respectively.

ANGPTL3 inhibition is most likely to be prescribed to patients with the most severe forms of dyslipidemia, e.g. FH patients, who are, in general, already on statin therapy (14). A study by Pramfalk *et al.* showed that statins suppress hepatic *ANGPTL3* mRNA expression in healthy volunteers (15) which would imply that statin therapy could negatively impact the efficacy of ANGPTL3 inhibition. However, whether statins have an effect on hepatic *ANGPTL3* mRNA expression and hence plasma ANGPTL3 concentrations in hypercholesterolemic patients is unknown. Moreover, the mechanisms via which statins modulate *ANGPTL3* mRNA expression are also not known.

Activation of the liver X receptor (LXR), a nuclear transcription factor, has been shown to increase *ANGPTL3* mRNA expression (16). LXR is activated by oxysterols (17) which are cholesterol metabolites that have been shown to be lower upon statin treatment in macrophages (18) and plasma (19). We therefore hypothesized that statins reduce *ANGPTL3* mRNA expression via decreased oxysterol-mediated LXR activation.

The aim of the current study was to determine the effect of statin therapy on plasma ANGPTL3 concentrations in hypercholesterolemic patients and to gain mechanistic insight into statin's effect on ANGPTL3 expression *in vitro*.

MATERIALS AND METHODS

Study cohorts

We investigated the association between statin treatment and plasma ANGPTL3 concentrations in two cohorts of hypercholesterolemic patients. The first cohort comprised patients with molecularly proven FH who participated in a single center cross-sectional study, described in detail elsewhere (20). These patients were aged between 18 and 55 years at the moment of inclusion and were not using lipid lowering therapy. For the current analysis we selected those FH patients with a pathogenic genetic variant in *LDLR* or *APOB* with plasma LDL cholesterol concentrations above the age- and sex-specific 90th percentile. Some patients started using statins between inclusion and the first study visit, allowing us to determine the association of statin therapy and plasma ANGPTL3 concentrations.

The second cohort comprised severely hypercholesterolemic patients who tested negative for FH-causing variants in *LDLR*, *APOB* and *PCSK9* (SeqCap easy choice, Roche NimbleGen Inc., Pleasanton, USA). All patients were free from cardiovascular disease and on a stable regimen of statin therapy. Blood samples were taken before and after stopping statin therapy for four weeks.

Both studies were approved by the local institutional review board and all participants signed written informed consent before participation.

Plasma lipid profiling of human patients

Blood was obtained in EDTA containing vacutainers after an overnight fast. Plasma was isolated as described (21) and concentrations of total cholesterol, HDL cholesterol and TG 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). Plasma LDL cholesterol concentrations were calculated by the Friedewald formula (22). Plasma ANGPTL3 concentrations were determined using the Bio human ANGPTL3 DuoSet ELISA (R&D Systems, Minneapolis, MI).

Cell culture

The human hepatoma cell-line Huh7 was maintained in DMEM with 4.5 g/l glucose and pyruvate (Gibco-Invitrogen, Breda, Netherlands) supplemented with 10% fetal bovine serum (FBS; Gibco-Invitrogen), 100 IU/mL penicillin (Gibco-Invitrogen) and 100 µg/mL streptomycin (Gibco-Invitrogen) under standard culture conditions at 37 °C with 5% CO_2.

Depending on the experiment, the medium was replaced by the same medium with 10% FBS or lipoprotein depleted human serum (LPDS). Five hours later, the medium was replaced by the same FBS or LPDS containing medium supplemented with the indicated concentrations of simvastatin (Sigma-Aldrich, Zwijndrecht, The Netherlands), the LXR agonist T0901317 (Cayman Chemical, Ann Arbor, MI), the squalene synthase inhibitor TAK475 (Sigma-Aldrich) and/or the LXR antagonist GSK2033 (Tocris, Abingdon, UK) and/or DMSO vehicle control. After 24 hours of incubation, either 1) cells were collected for gene expression analysis; 2) medium and cells were collected to measure ANGPTL3 in the medium and cellular protein concentrations; or 3) cells were collected for oxysterol quantification.

Gene expression analysis

Total RNA was isolated using Tripure Isolation Reagent (Roche Applied Sciences, Almere, The Netherlands) 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). Primer sequences are listed in Supplemental Table 1. The expression of each gene was reported in arbitrary units after normalization to the average expression level of the housekeeping genes 18S ribosomal RNA (*RN18S*) and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) using the $2^{-\Delta\Delta Ct}$ method (23).

ANGPTL3 ELISA and cellular protein concentrations

The amount of ANGPTL3 in the medium was quantified using the Bio human ANGPTL3 DuoSet ELISA (R&D Systems, Minneapolis, MI). The concentrations of ANGPTL3 in the medium were corrected for the cellular protein concentration.

Cells were washed three times with ice-cold PBS 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 and the protein concentration of the supernatant was determined using a BCA protein assay kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

Oxysterol concentrations

For cellular oxysterol concentration, cells were detached with trypsin (Gibco), centrifuged at 300 g for 5 minutes at room temperature and resuspended in PBS. Part of this cellular suspension was used to measure the protein content according to the aforementioned method while the remainder was used for oxysterol quantification. This latter suspension was centrifuged at 300 g for 5 minutes at room temperature, the supernatant was discarded and 100 µl of internal standards (1 µmol/l of both 24-hydroxycholesterol-d7 and 27-hydroxycholesterol-d7 (Medical isotopes inc., Pelham, NH)) and 500 µl methanol was added. Next, the suspensions were centrifuged at 13,523 g for 10 minutes at room temperature and the supernatant was transferred to a new tube and evaporated under a stream of nitrogen at 60°C. The dried extract was derivatized in a 50µl mix of pyridine and chlorosulfonic acid (10/1 v/v) for 30 min at 60°C after which 100 µl of MilliQ water was added to the samples.

The samples were analyzed by an ultra-high pressure liquid chromatography system (Waters, Milford, Massachusetts, USA) with a Acquity C18, 100 x 2.1 mm, 1.7µm particle size column (Waters) coupled to a Premier XE mass spectrometer (Waters). Mass spectrometry was performed in the negative ionization mode using an electron spray ionization source. Data was acquired using the Masslynx software (v4.2 SCN977, 2017, Waters).

The mobile phase was composed of solvent A (25 mM NH4form/MeOH/MilliQ (150/250/600 (v/v))) and solvent B (Acetonitrile/MilliQ (9/1 (v/v))). The flow rate was 0.4 ml/min. The step gradient used was as follows: after 20 µl sample injection the initial conditions was 15% solvent B, 15% to 40% solvent B in 4.0 min, 40% to 100% solvent B in 5.0 min, 100% to 15% solvent B in 0.01 min and re-equilibrate at 15% solvent B for 2.99 min. The total run-time was 12.0 min. The autosampler wash solvent was 1% tetrafluoroethylenein 2-propanol. Calibration curves were prepared by spiking 24-hydroxycholesterol and 27-hydroxycholesterol and calculated using calibration lines (in ethanol) within the appropriate concentration range.

Statistics

Normally distributed values are reported as mean values ± SD and compared using paired or non-paired t-tests when appropriate. Non-normally distributed values are reported as median [interquartile range] and compared using Wilcoxon signed rank test or Mann Whitney U test for paired and non-paired analyses, respectively. Categorical data was compared between groups using the Fisher Exact test. An linear regression analysis was performed to investigate statin treatment effect on log-transformed ANGPTL3 levels with covariates for age sex, and BMI in the first cohort. Statistical analyses were performed with GraphPad Prism (Version 8, GraphPad Software, Inc.) using the indicated tests. P<0.05 was considered statistically significant.

RESULTS

Lower plasma ANGPTL3 concentrations in hypercholesterolemic patients treated with statin compared to non-statin treated patients

We measured plasma ANGPTL3 concentrations in 154 FH patients in whom a variant in *LDLR* or *APOB* was identified. Their characteristics are shown in Table 1. Despite clear inter-subject variability, plasma ANGPTL3 concentrations were significantly lower in 93 FH patients receiving statin therapy compared to 61 FH patients who were not using a statin (167 (135-220) ng/ml *vs.* 145 (120-193) ng/ml in controls *vs.* statin users; p=0.012; Figure 1A).

We further investigated the relationship between statin use and plasma ANGPTL3 concentrations in a linear regression model (Table 2). Statin use remained significantly associated with 14% lower plasma ANGPTL3 concentrations when controlling for age, sex and BMI (β 0.86, 95%-CI: 0.77-0.95, p=0.004). Furthermore, age was positively associated with plasma ANGPTL3 concentrations, which

increased by 1% per year when controlling for statin use, sex and BMI (β 1.01, 95%-CI: 1.01-1.02, p<0.001). Plasma ANGPTL3 concentrations were not different among patients using different statins (Supplemental Table 2).

As the results of the study might have been confounded due to its observational nature, we also measured plasma ANGPTL3 concentrations in 14 patients with severe hypercholesterolemia who temporarily discontinued their statin therapy for four weeks. Patient characteristics are shown in Table 1 and the details on statin treatment are reported in Supplemental Table 3. Upon cessation of statin therapy, plasma LDL cholesterol concentrations increased by 92 ± 54% (p<0.001; Table 1) and plasma ANGPTL3 concentrations increased from 328 (274-351) ng/ml to 354 (343-376) ng/m (p=0.003; Figure 1B). The type and dose of statin did not influence its potential to lower plasma ANGPTL3 concentrations.

Simvastatin reduces ANGPTL3 mRNA expression in vitro which is attenuated by LXR stimulation

Hepatoma Huh7 cells were incubated with 1 μM LXR agonist T0901317 and/or various simvastatin concentrations to explore mechanisms underlying the effect of statins on ANGPTL3. Incubation for 24 hours with simvastatin resulted in a dose-dependent reduction of *ANGPTL3* mRNA expression in cells cultured in medium supplemented with either FBS or LPDS (Figures 2A and 2D). The simvastatin-mediated reduction in *ANGPTL3* mRNA expression was more pronounced in cells cultured in medium supplemented with LPDS than those in medium with FBS. The lowest simvastatin concentration (312 nM) reduced *ANGPTL3* mRNA expression by 50% in cells cultured in LPDS but had no statistically significant effect on *ANGPTL3* mRNA expression in cells cultured with FBS. Incubation with the LXR agonist T0901317 enhanced the *ANGPTL3* mRNA expression more than 6-fold in both culture conditions and prevented the simvastatin-mediated reductions of *ANGPTL3* mRNA expression. Simvastatin also increased the expression of LDLR, as expected (Figures 2B and 2E).

A similar pattern as for ANGPTL3 was found for the validated LXR target gene *ABCA1* (24) that encodes for ATP binding cassette A1, but only in cells kept in medium with LPDS (Figures 2C and 2F). As with the *ANGPTL3* mRNA expression, LXR stimulation

with T0901317 prevented simvastatin-mediated reductions in *ABCA1* mRNA expression of cells cultured in medium with LPDS.

Table 1: Patient characteristics and plasma lipid concentrations.

	Cohort 1. FH patients			Cohort 2. Hypercholesterolemic patients		
	On statin	**Not on statin**	**p-value**	**On statin**	**Off statin**	**p-value**
N	91	63	-	14	-	-
Male (N (%))	38 (42%)	24 (38%)	0.7387	7 (50%)	-	-
Age (years)	36.3 ± 8.6	33.8 ± 8.6	0.0836	58.0 ± 10.9	-	-
BMI (kg/m²)	25.5 ± 4.2	24.2 ± 4.7	0.0157	25.2 ± 3.9	-	-
Total cholesterol (mmol/l)	5.2 ± 1.0	7.1 ± 1.2	<0.0001	5.5 ± 0.8	8.5 ± 1.4	0.0002
LDL cholesterol (mmol/l)	3.4 ± 1.0	5.2 ± 1.1	<0.0001	3.4 ± 0.8	6.2 ± 1.4	0.0004
HDL cholesterol (mmol/l)	1.5 ± 0.4	1.5 ± 0.4	0.9394	1.4 ± 0.3	1.3 ± 0.2	0.0811
Triglycerides (mmol/l)	0.7 (0.5 – 1.0)	0.7 (0.5 – 1.6)	0.8626	1.3 (0.9 – 1.7)	2.1 (1.6 – 2.4)	0.0212

Plasma LDL cholesterol concentrations were calculated by the Friedewald formula (22). Values are mean ± SD or median with interquartile range (Triglycerides). For cohort 1: p-value from Mann-Whitney U test or Fisher exact test (Male); for cohort 2: p-value from Wilcoxon matched-pairs signed rank test.

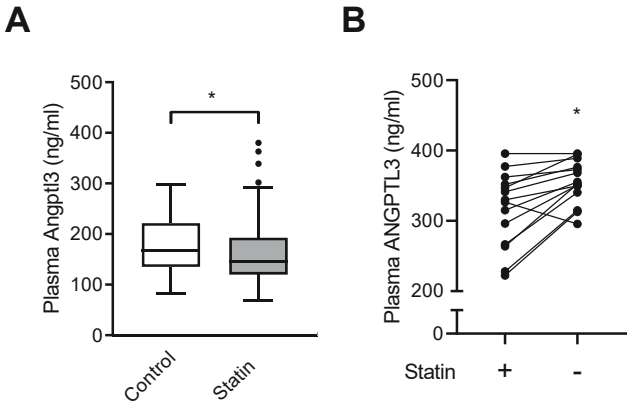

Figure 1: Statin therapy results in reduced plasma ANGPTL3 concentrations in hypercholesterolemic patients
(A) Plasma ANGPTL3 concentrations of familial hypercholesterolemia patients who were treatment naïve or on statin therapy. Data are shown as boxplots; *, p<0.05 (Mann-Whitney U test). (B) Plasma ANGPTL3 concentrations of hypercholesterolemic patients before and after four weeks cessation of statin therapy. Shown are individual values; *, p<0.05 vs. zero (one-sample t-test). ANGPTL3, angiopoietin-like 3.

Table 2: Linear regression model with log-transformed plasma ANGPTL3 concentrations.

Variable	Estimate	p-value	95% confidence interval
(Intercept)	116.52	<0.001	82.91 – 163.74
Statin use = yes	0.86	0.004	0.77 – 0.95
Age (years)	1.01	<0.001	1.01 – 1.02
Sex = male	0.98	0.644	0.88 – 1.08
BMI (kg/m^2)	1.00	0.840	0.99 – 1.01

Liniair regression model with log-transformed plasma ANGPTL3 concentrations as outcome variable and statin use, age, sex and BMI as predictor variables. Estimates are exponiated ($e^{original\_estimate}$) and can be interpretated as the % change in ANGPTL3 plasma levels by (1-estimate)*100. BMI, body-mass index.

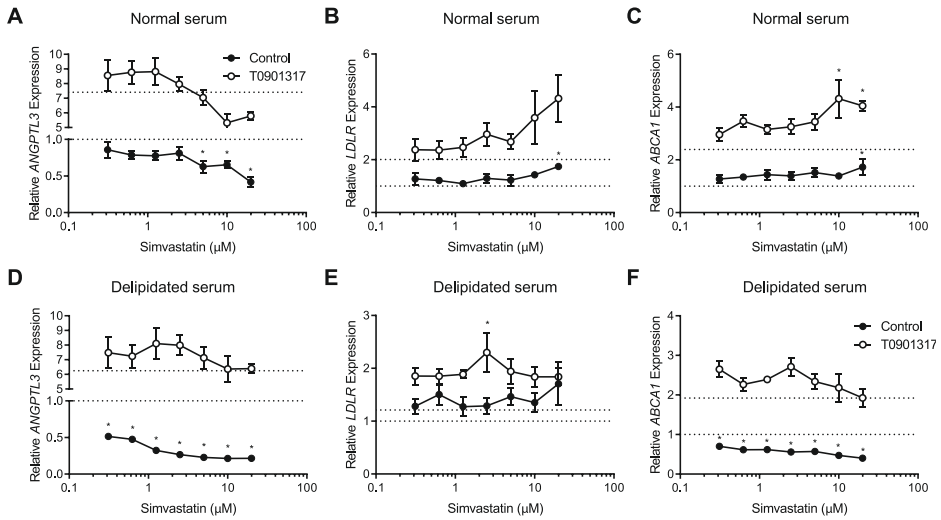

Figure 2: Simvastatin decreases ANGPTL3 mRNA expression in a dose dependent manner
ANGPTL3 (A and D), LDLR (B and E) and ABCA1 (C and F) mRNA expression in Huh7 cells incubated with the indicated concentrations of simvastatin and/or 1 μM LXR agonist T0901317 for 24 hours in medium supplemented with 10% FBS (A, B and C) or 10% LPDS (D, E and F). The dotted line represents the data from cells not incubated with simvastatin, either with (upper line) or without (lower line) 1 μM T0901317. Relative mRNA expression was normalized to housekeeping genes RN18S and HPRT1 with data from cells without simvastatin and T0901317 defined as '1'. Values are averages ± SD; n=4; *, p<0.05 compared to 0 μM simvastatin, same T0901317 concentration (Mann-Whitney U test). ANGPTL3, angiopoietin-like 3; LXR, liver X receptor; FBS, fetal bovine serum; LPDS, lipoprotein depleted serum.

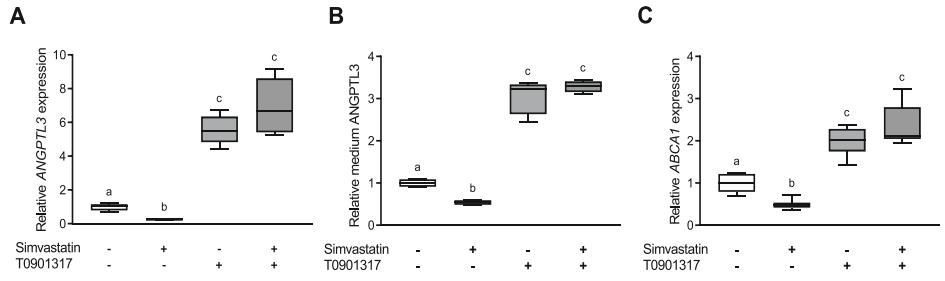

Figure 3: Simvastatin reduces both ANGPTL3 mRNA expression and ANGPTL3 secretion and these effects are negated by LXR stimulation
ANGPTL3 (A) and ABCA1 (C) mRNA expression and ANGPTL3 concentrations in the medium (B) in Huh7 cells incubated with 2.5 μM simvastatin and/or 1 μM LXR agonist T0901317 for 24 hours in medium supplemented with 10% LPDS. Data are shown as boxplots with results from 2 independent experiments; n=6-8. Plots without a similar symbol are significantly different (p<0.05, Mann-Whitney U test). Relative mRNA expression was normalized to housekeeping genes RN18S and HPRT1 with data from cells without simvastatin and T0901317 defined as '1'. ANGPTL3, angiopoietin-like 3; LXR, liver X receptor.

Simvastatin reduces ANGPTL3 secretion in vitro by interfering with LXR signaling

We next studied the effects of a fixed dose of 2.5 μM simvastatin and/or 1 μM T0901317 on *ANGPTL3* mRNA expression and ANGPTL3 secretion of Huh7 cells cultured in medium supplemented with LPDS, a medium completely devoid of exogenous lipids. As a result of this depletion, cells entirely depend on endogenous intracellular cholesterol production. Under these conditions, simvastatin incubation resulted in a 74% reduction of *ANGPTL3* mRNA expression and a 46% lower ANGPTL3 concentration in the medium (Figure 3A and 3B). These reductions were not observed when the cells were co-incubated with T0901317. A similar pattern was seen for the *ABCA1* mRNA expression (Figure 3C). Next, we incubated the Huh7 cells with the LXR antagonist GSK2033 (25). As expected, this prevented the upregulation of the LXR target gene *ABCA1* as wells as *ANGPTL3* upon T0901317 incubation (Figures 4A and 4B). Moreover, LXR antagonism with GSK2033 reduced both *ANGPTL3* and *ABCA1* mRNA expression and ANGPTL3 secretion. These reductions were not exacerbated by simvastatin (Figures 4C-E), underscoring the role of LXR in simvastatin-mediated reductions in ANGPTL3.

Subsequently, we investigated the effect of simvastatin on the intracellular oxysterol content since these oxidized metabolites of cholesterol are known endogenous

LXR ligands (26). 24-hydroxycholesterol and 27-hydroxycholesterol concentrations were reduced by approximately 60% and 85%, respectively, after 24-h incubation with 2.5 µM simvastatin (Figure 5).

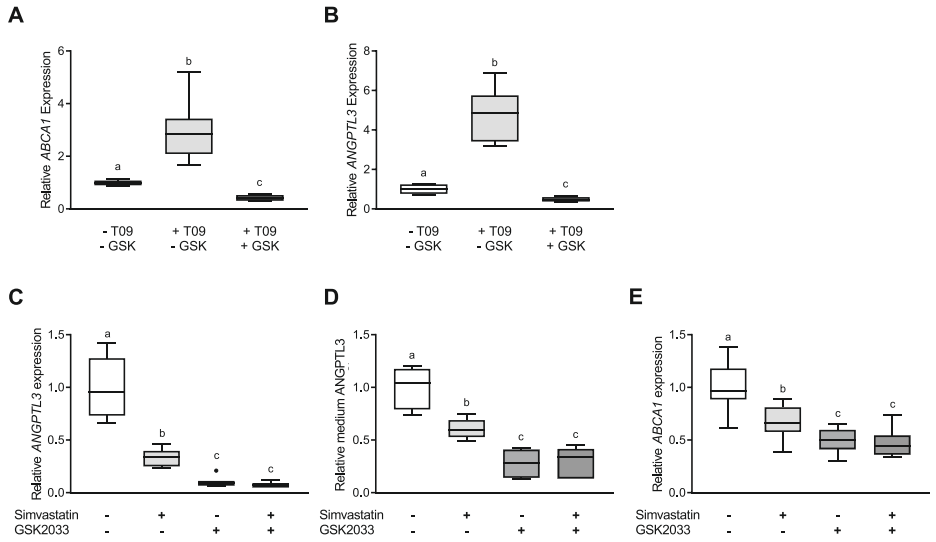

Figure 4: LXR antagonism hampers simvastatin-mediated ANGPTL3 reduction
ANGPTL3 (A) and ABCA1 (B) mRNA expression in Huh7 cells incubated with 1 µM LXR agonist T0901317 (T09) with/or without 10 µM LXR antagonist GSK2033 (GSK) for 24 hours in medium supplemented with 10% LPDS. ANGPTL3 (C) and ABCA1 (E) mRNA expression and ANGPTL3 concentrations in the medium (D) of Huh7 cells incubated with 2.5 µM simvastatin and/or 10 µM GSK2033 for 24 hours in medium supplemented with 10% LPDS. Data are shown as boxplots with results from 2 independent experiments; n=6-8. Plots without a similar symbol are significantly different ($p<0.05$, Mann-Whitney U test). Relative mRNA expression was normalized to housekeeping genes RN18S and HPRT1 with data from cells without T0901317 and GSK2033 defined as '1'. ANGPTL3, angiopoietin-like 3; LXR, liver X receptor.

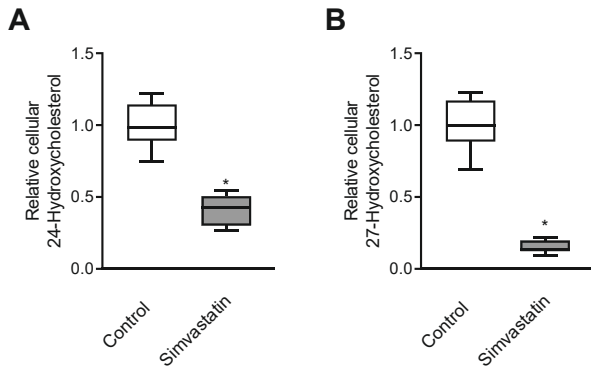

Figure 5: Simvastatin reduces intracellular oxysterol concentrations
Relative 24-Hydroxycholesterol (A) and 27-Hydroxycholesterol (B) content in Huh7 cells incubated with or without 2.5 µM simvastatin for 24 hours in medium supplemented with 10% LPDS. Values are averages ± SD from 2 independent experiments with data from cells without simvastatin defined as '1'; n=8; *, $p<0.05$ (Mann-Whitney U test).

Cholesterol synthesis inhibition reduces both oxysterol content and ANGPTL3 secretion in vitro

We repeated the experiments, this time replacing simvastatin with the squalene synthase inhibitor TAK475 (27) in order to assess whether a downstream regulator of *de novo* cholesterol synthesis has a similar effect on ANGPTL3 and intracellular oxysterols concentrations. Incubation with TAK475 dose-dependently reduced *ANGPTL3* mRNA expression but had no effect on *ABCA1* mRNA expression (Figures 6A and 6B). In line with the reduced *ANGPTL3* mRNA expression, addition of a low concentration of 0.5 µM TAK475 reduced ANGPTL3 secretion by approximately 50% (Figure 6C). The role of LXR was once again underscored by the finding that TAK475 did not result in a reduction of ANGPTL3 secretion when the cells were co-incubated with LXR agonist T0901317. The finding that TAK475 also reduced the cellular oxysterol concentrations (Figures 6D and 6E) reiterates the role of the oxysterol-mediated effects of LXR on ANGPTL3.

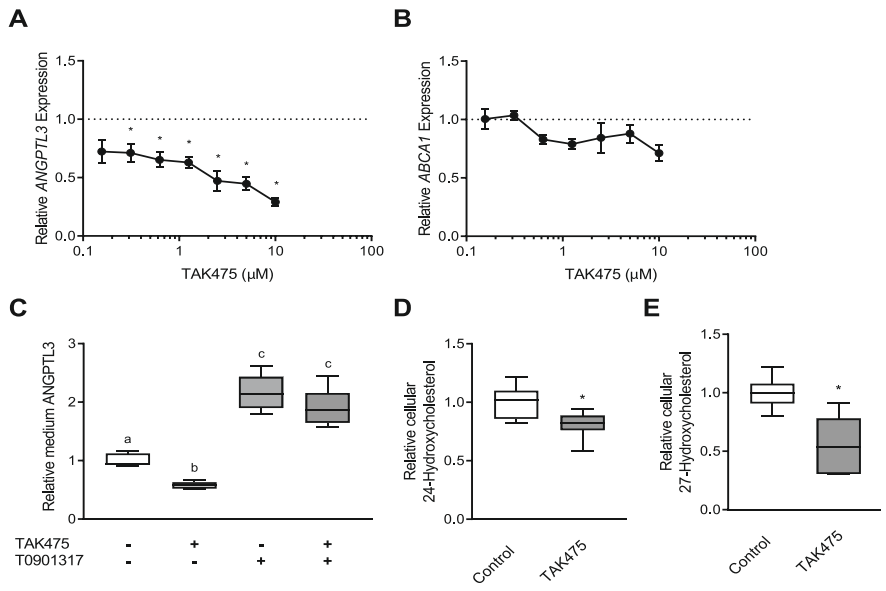

Figure 6: Squalene synthase inhibition reduces intracellular oxysterol concentrations resulting in lower ANGPTL3 mRNA expression and ANGPTL3 secretion.

ANGPTL3 (A) and ABCA1 (B) mRNA expression in Huh7 cells treated with the indicated concentrations of the squalene synthase inhibitor TAK475 for 24 hours in medium supplemented with 10% LPDS. The dotted line represents the data from cells not incubated with TAK475. Relative mRNA expression was normalized to housekeeping genes RN18S and HPRT1 with data from cells without TAK475 defined as '1'. Values are averages ± SD; n=4; *, p<0.05 compared to 0 μM TAK475 (Mann-Whitney U test). ANGPTL3 concentrations in the medium (C) of Huh7 cells treated with 0.5 μM TAK475 and/or 1 μM LXR agonist T0901317 for 24 hours in medium supplemented with 10% LPDS. Data are shown as boxplots with results from 2 independent experiments; n=6. Plots without a similar symbol are significantly different (p<0.05, Mann-Whitney U test). Relative 25-Hydroxycholesterol (D) and 27-Hydroxycholesterol (E) content of Huh7 cells treated with or without 0.5 μM TAK475 for 24 hours in medium supplemented with 10% LPDS. Values are averages ± SD from 2 independent experiments with data from cells without treatment defined as '1'; n=8; *, p<0.05 (Mann-Whitney U test). ANGPTL3, angiopoietin-like 3; LXR, liver X receptor.

DISCUSSION

In the present study we show that plasma ANGPTL3 concentrations are lower in FH patients treated with a statin compared to FH patients not using statins. Moreover, plasma ANGPTL3 concentrations increased in hypercholesterolemic patients who temporarily discontinued statin therapy for four weeks. Additional *in vitro* studies strongly suggest that the statin-mediated reductions in ANGPTL3 are the result of a reduced oxysterol-mediated LXR activation.

The effect of statins on plasma ANGPTL3 concentrations is relatively underinvestigated. Only Pramfalk and colleagues (15) investigated the effect of statins on ANGPTL3. In their study, they found that 10 healthy subjects who used atorvastatin (80 mg per day) for 4 weeks had lower hepatic ANGPTL3 mRNA expression than 9 healthy subjects who received a placebo. However, the study was relatively small scaled and conducted in healthy volunteers. To the best of our knowledge, the effects of statins on ANGPTL3 has not been addressed in hypercholesterolemic patients who are normally treated with statins. As we anticipate ANGPTL3 lowering therapy to be used in hypercholesterolemic patients we deem studying the effects of statins on ANGPTL3 in hypercholesterolemic patients to be of clinical importance. Moreover, plasma ANGPTL3 concentrations were not measured by Pramfalk and colleagues and these may be relevant as neutralizing antibodies against ANGPTL3 administered per intravenous injection (13,28) are likely to only lower ANGPTL3 concentration in the circulation. Our present study now clearly shows that statin therapy reduces plasma ANGPTL3 concentrations in hypercholesterolemic patients.

Since low plasma ANGPTL3 concentrations are associated with low plasma TG and cholesterol concentrations (1,4-9), we hypothesize that the lipid lowering effects of statins may partly depend on its effect on ANGPTL3. This also implies that ANGPTL3 inhibition may be less effective in statin treated compared to statin-naïve patients, since statins reduce the availability of ANGPTL3 protein as a target. Statin therapy is often started after myocardial infarction and a recent case-control study showed that patients with low plasma ANGPTL3 concentrations were relatively protected against myocardial infarction (29). It will be of interest to investigate whether the beneficial effects of statins on myocardial infarction are partly mediated by reducing plasma ANGPTL3 concentrations.

The number of studies addressing the impact on ANGPTL3 of interventions, other than statin therapy, is limited. Apart from our current observation that statins lower plasma ANGPTL3 concentrations, insulin has also been shown to decrease plasma ANGPTL3 concentrations. In fasted healthy volunteers, hyperinsulinemia decreased plasma ANGPTL3 concentrations by 26 to 38% (30), which is comparable to the reductions with statins observed in our study.

In order to unravel the mechanism how statins reduce plasma ANGPTL3 concentrations, we performed *in vitro* experiments with the human hepatoma Huh7 cell line and showed that incubation with simvastatin reduced the oxysterol content of these cells. Oxysterols are endogenous LXR ligands (26) and therefore statins could potentially also regulate other LXR target genes. Indeed, we found that simvastatin reduces *ABCA1* mRNA expression in Huh7 cells. However, these results appear inconsistent with experiments that have used other types of statins. Pravastatin has been shown to induce *LXR* mRNA expression as wells as its target genes *ABCG5*, *ABCG8*, and *CYP7A1* in Hep3B cells (31) while atorvastatin and rosuvastatin did not affect LXR target genes in human primary hepatocytes (32). These contrasting results may be explained by the fact that we cultured our cells in LPDS, making them entirely dependent on intracellular cholesterol production. Since statins suppress endogenous cholesterol synthesis, a reduction of oxysterols and thus LXR target gene expression will only become evident once cells are cultured in medium devoid of lipids. Indeed, when the Huh7 cells were cultured in lipid-containing medium, the suppressive effect of statin treatment on *ANGPTL3* and *ABCA1* mRNA expression was less pronounced or even absent. Moreover, although limited by a small sample size, the statin type had no effect on plasma ANGPTL3 concentration in our FH cohort.

Our studies have certain limitations. First of all, the data from the clinical studies were obtained from cross sectional observational studies. Although we acknowledge that a randomized study is of larger scientific value, we do not think the observational nature of our studies invalidates our results. Secondly, cohort 2 contained only 14 hypercholesterolemic subjects. LDL lowering is the cornerstone in the treatment of hypercholesterolemic patients and we therefore did not deem it to be justified to stop statin therapy in subjects that were using statins in a secondary prevention setting. Thirdly, we only evaluated the effects of statins on ANGPTL3 since they are the most widely prescribed lipid lowering drugs. It is not known whether other lipid lowering drugs such as PCSK9 inhibitors and ezetimibe also affect ANGPTL3. Finally, for feasibility our in vitro studies were only performed with simvastatin. Although the type of statin did not influence its potential to lower plasma ANGPTL3 concentrations in the two studied patient cohorts, it cannot be

ruled out that more potent statins like rosuvastatin and atorvastatin have a bigger effects on ANGPTL3.

A number of ANGPTL3 lowering therapies are emerging, among which the ANGPTL3 antibody evinacumab (13,33). Our data support a partial overlap in the underlying LDL cholesterol lowering mechanisms between statins and ANGPTL3 inhibition. Hence, future studies need to closely address the efficacy of ANGPTL3 inhibition in presence or absence of background statin therapy. This may bear direct clinical consequences for future choices of combined LDL cholesterol lowering regimens in patients with (refractory) hypercholesterolemia.

For future studies, it would be of interest to establish whether baseline plasma ANGPTL3 concentrations are a key determinant of the observed lowering of plasma LDL-cholesterol concentrations upon ANGPTL3 inhibition therapy. Such studies should also include subjects with and without statin therapy in order to uncover whether ANGPTL3 inhibition on top of statin therapy is more efficient in lowering LDL than ANGPTL3 alone.

In conclusion, we show that statins reduce plasma ANGPTL3 concentrations in hypercholesterolemic patients, and in *in vitro* studies we show that this is likely caused by reduced *ANGPTL3* mRNA expression, caused by statin induced suppression of LXR activation. Whether these findings mean that the lipid lowering effect of ANGPTL3 lowering is larger in statin naïve patients compared to patients who already use a statin is a clinical relevant question that should be addressed in intervention studies.

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SUPPLEMENTAL MATERIALS

Supplemental Table 1: Sequences of the primers used for realtime-PCR

Gene	Forward Primer	Reverse Primer
ABCA1	ATGAGGACAACAACTACAAAGCC	GGGAAAGAGGACTAGACTCCAAA
ANGPTL3	ATCTTCCAAGCCAAGAGCACC	AGCAGGAATGCCATCATGTTTT
HPRT1	TGACCTTGATTTATTTTGCATACC	CGAGCAAGACGTTCAGTCCT
RN18S	GAGGGAGCCTGAGAAACGG	GTCGGGAGTGGGTAATTTGC

Supplemental Table 2: Plasma ANGPTL3 concentrations of hypercholesterolemic patients in cohort 1 depending on statin therapy

Statin	No. of FH patients	Plasma ANGPTL3 (ng/ml)
No	61	167 (135 – 220)
Atorvastatin	19	141 (117 – 200)
Fluvastatin	3	120 (116 – 126)
Pravastatin	1	339
Rosuvastatin	20	140 (127 – 183)
Simvastatin	50	154 (123 – 189)

Values are median with interquartile range.

Supplemental Table 3: Treatment characteristics of patients in cohort 2

Subject	Sex	Age	Treatment
1	Male	63	Atorvastatin 10 mg
2	Female	57	Atorvastatin 40 mg
3	Male	52	Simvastatin 40 mg
4	Female	58	Atorvastatin 40 mg
5	Female	57	Atorvastatin 40 mg
6	Male	34	Simvastatin 40 mg
7	Female	71	Simvastatin 40 mg
8	Male	64	Atorvastatin 80 mg
9	Male	69	Atorvastatin 10 mg
10	Female	64	Rosuvastatin 20 mg
11	Female	56	Rosuvastatin 5 mg
12	Male	53	Simvastatin 40 mg
13	Male	41	Atorvastatin 40 mg
14	Female	73	Pravastatin 20 mg

PART III

Novel therapeutic options for hypercholesterolemia

13

Safety and efficacy of mipomersen in patients with heterozygous familial hypercholesterolemia

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ABSTRACT

Background and aims: Heterozygous familial hypercholesterolemia (HeFH) is a common genetic disorder characterized by elevated low-density lipoprotein cholesterol (LDL-C) and increased cardiovascular disease risk. Despite multiple LDL-C–lowering therapies, many HeFH patient do not reach LDL-C targets. Mipomersen, an antisense oligonucleotide against apolipoprotein B (apoB) might further lower LDL-C in HeFH patients. We assessed the efficacy and safety of two mipomersen dosing regimens in HeFH patients and explored whether thrice-weekly dosing improves the benefit-risk profile.

Methods: In this double-blind trial, HeFH patients (LDL-C >160 mg/dl) on maximal tolerated LDL-lowering therapy were randomized to mipomersen 200 mg once weekly (n=104), mipomersen 70 mg thrice weekly (n=102), or placebo in matching frequency (n=103) for 60 weeks. Main outcomes were LDL-C, apoB, and lipoprotein(a) levels after 60 weeks of treatment.

Results: Mipomersen 200 mg once weekly and mipomersen 70 mg thrice weekly significantly lowered LDL-C compared with placebo by 21.0% and 18.8%, respectively, and apoB by 22.1% and 21.7% (all p <0.001). Lipoprotein(a) was significantly lowered by 27.7% (p <0.001) with thrice-weekly dosing. Injection-site reactions and flu-like symptoms led to discontinuation in 21.2% (200 mg), 17.6% (70 mg), and 5.8% (placebo) of participants. Alanine transaminase was elevated (≥3x upper limit of normal at least once) in 21.2%, 21.6%, and 1.0% of subjects, respectively.

Conclusions: Mipomersen 200 mg once weekly and 70 mg thrice weekly are effective in lowering apoB-containing lipoproteins in HeFH patients. This is counterbalanced by limited tolerability and increased hepatic transaminase levels in about 21% of patients. The thrice-weekly dosing regimen was associated with lower frequency of flu-like symptoms, which might help avert discontinuation in some patients, but otherwise had no major benefits.

INTRODUCTION

Familial hypercholesterolemia (FH) is an autosomal dominant disorder characterized by elevated plasma levels of low-density lipoprotein cholesterol (LDL-C), consequently leading to an increased risk for premature cardiovascular disease (CVD)[1]. Heterozygous FH (HeFH) has a prevalence of 1/200–1/500,[1] and typically leads to CVD in men before the age of 55 and in women before the age of 60.[2,3] Most commonly FH is caused by mutations in the LDL receptor gene (*LDLR*); however, a much smaller proportion of FH patients have mutations in the apolipoprotein B (*APOB*) or proprotein convertase subtilisin/kexin type 9 (*PCSK9*) gene, leading to impaired LDL-C uptake or increased LDL receptor degradation, respectively.[4]

Current treatment of HeFH patients consists of high doses of high-intensity statins, often in combination with ezetimibe, with LDL-C goals of <100 mg/dL in primary prevention or <70 mg/dL after a CVD event.[5] However, those goals are often not reached with this therapeutic regimen,[6] underlining the need of additional LDL-lowering options. One of these is PCSK9 inhibition, with promising results of a robust additional LDL-C reduction and relevant reduction in major adverse cardiac events (MACE) even in individuals with FH.[7,8]

Another novel lipid-lowering strategy is mipomersen; an antisense oligonucleotide directed against apolipoprotein B100 (apoB100) mRNA in the liver, ultimately resulting in decreased levels of apoB100-containing lipoproteins such as LDL and lipoprotein(a) [Lp(a)].[9] Mipomersen has showed LDL-C–lowering efficacy in patients with homozygous FH (HoFH), severe HeFH, severe hypercholesterolemia at high CVD risk, and statin intolerance.[10–14] Since mipomersen exerts its lipid-modifying effect independently from the function of the LDL receptor, it is considered an additional or alternative treatment option to traditional cholesterol-lowering drugs targeting the LDL receptor pathway. Mipomersen was approved in 2013 by the U.S. Food and Drug Administration (FDA) for HoFH patients in the United States. However, its use is associated with some frequently occurring side effects: injection-site reactions, flu-like symptoms, and, inherent to its pharmacological mechanism, hepatic fat accumulation and transaminase elevation.[15] A study of different doses

and frequencies of mipomersen has shown a potential beneficial side effect profile regarding injection site reactions for a thrice weekly dosing regimen compared to once weekly dosing.[16]

Therefore, the aim of this long-term double-blind study was to assess the efficacy and safety of 2 different dosing regimens of mipomersen (200 mg subcutaneously [SQ] once weekly or 70 mg SQ thrice weekly) in a large population of patients with HeFH on stable maximally tolerated lipid-lowering regimens, and to explore whether the thrice-weekly dosing regimen improved the benefit–risk profile compared to the FDA-approved weekly dosing regimen.

PATIENTS AND METHODS

Study subjects

The study cohort comprised two populations of very high risk adult HeFH patients,18 years and older, with persistent severe hypercholesterolemia (LDL-C >160 mg/dL) on maximally tolerated LDL-lowering therapy. Cohort 1 (severe HeFH) consisted of subjects with fasting LDL-C at screening ³300 mg/dL (7.77 mmol/L) or LDL-C ³200 mg/dL (5.18 mmol/L) plus documented coronary heart disease (CHD)/ CHD risk equivalents. Cohort 2 (high-risk HeFH) consisted of HeFH subjects with LDL-C levels between 160 mg/dL (4.14 mmol/L) and 200 mg/dL (5.18 mmol/L) plus documented CHD/CHD risk equivalents. A diagnoses of HeFH in cohort 2 had to be made with the Simon Broom, US MedPED, or Dutch lipid Clinic Network criteria. Patients had to be on a stable, maximally tolerated, lipid-lowering regimen for at least 12 weeks prior to screening. Furthermore, a body mass index (BMI) £40 kg/m^2 with stable weight and triglyceride (TG) levels below 350 mg/dL (3.95 mmol/L) were required. Females could not be pregnant and required contraceptive regimens up to 24 weeks after the last mipomersen dose. Males had to be either surgically sterile or they and their partners had to be willing to use effective contraceptive methods.

Exclusion criteria included CVD events within 24 weeks prior to screening, apheresis within 3 months prior to screening or expected apheresis during study treatment,

insulin-dependent diabetes mellitus (type 1) or a glycated haemoglobin >8%, New York Heart Association class III and IV heart failure, uncontrolled hypertension (i.e., systolic and diastolic blood pressures ³160/95 mmHg), a positive test for human immunodeficiency virus or hepatitis B/C/E, uncontrolled hypothyroidism, history of malignancy (other than skin cancer), hepatic disease (e.g., non-alcoholic steatohepatitis), and renal disease. Patients were excluded when their laboratory values at screening showed elevated creatine kinase (³3 × upper limit of normal [ULN]), alanine transaminase (ALT; ³1.5 × ULN) or aspartate transaminase (AST; ³1.5 × ULN), creatinine (>8.8 µmol/L above ULN in women; >17.7 mmol/L above ULN in men), proteinuria (>1000 mg protein/g creatinine), or total bilirubin >1.5 × ULN.

Study design

A multicenter, randomized, double-blind, placebo-controlled, parallel-group study was performed at 131 sites in 30 countries to assess the efficacy and safety of mipomersen in the selected populations. Patients with severe HeFH (cohort 1) or high-risk HeFH (cohort 2) were randomized and stratified by geographic region at a 1:1 ratio to a regimen of mipomersen 200 mg SQ or placebo once weekly (n=155) or a regimen of mipomersen 70 mg SQ or placebo thrice weekly (n=154). See the consort diagram (Figure 1) for a study overview. Patients were then stratified by gender and use of statins and randomized at a 2:1 ratio to receive mipomersen or placebo. The total study duration consisted of a 60-week blinded treatment phase followed by a 24-week post-treatment safety follow-up period. Patients could choose to enter a 26-week open-label continuation period before entering the post-treatment safety follow-up period. During the open-label extension, participants received mipomersen according to their assigned dose regimen, either continuing previously blinded treatment with mipomersen or changing from placebo to mipomersen at the same dosing regimen. Patients, investigators, and study staff were blinded to treatment allocation and lipid data of the patients during the entire blinded treatment period. During the first 8 weeks of the study, patients received 50% of the assigned dose of investigational product, consisting of dosing once every other week for mipomersen 200 mg (or placebo) and thrice every other week for mipomersen 70 mg (or placebo). From week 9 and onwards the dose frequency was increased to weekly. The study was approved by all local Institutional Review Boards of the participating sites and performed in compliance

with the Declaration of Helsinki (revised edition, Washington 2002). All participants gave informed consent prior to participation in this trial. An independent Data Monitoring Committee was established to provide an expert review of unblinded safety, efficacy, and tolerability data and assure safety of study subjects. An independent Cardiovascular Adjudication Committee was established to apply uniform criteria for the adjudication of cardiovascular events in a blinded, uniform manner.

Safety monitoring

The safety of mipomersen was assessed by recording the incidence of (serious) adverse events ([S]AE), physical examinations, 12-lead ECGs, measurement of routine laboratory analytes (chemistry, coagulation, hematology, and urinalysis), inflammatory markers, anti-mipomersen antibodies, and serial hepatic magnetic resonance imaging to assess the percent hepatic fat fraction. ALT and AST levels were measured regularly, and if elevated, dosing adjustment or temporary discontinuation of mipomersen was considered based on predetermined "liver chemistry stopping rules" (Supplementary Table 1).

Lipid assessments

Fasting blood samples were analyzed for LDL-C, very low density lipoprotein cholesterol (VLDL-C), TG, high-density lipoprotein cholesterol (HDL-C), total cholesterol (TC), Lp(a), apoB100, and apolipoprotein A1 (apoA1). TC, HDL-C, and TG were measured with enzymatic colorimetry, apoB100 and apoA1 with rate nephelometry, and Lp(a) with a standardized isoform-independent assay. LDL-C was calculated with the Friedewald formula, or measured through ultracentrifugation if TG levels exceeded 400mg/dL.

Outcome parameters

The primary outcome parameter was the percent change in LDL-C levels from baseline for both dose regimens in cohort 1 compared with placebo. Secondary outcome parameters were percent change in apoB100 and Lp(a) levels in both cohorts, and LDL-C change in cohort 2. Furthermore, other lipid parameters and differences in the safety and tolerability profiles of the two dosing regimens were determined in both cohorts of HeFH patients.

Statistical analysis

For the sample size determination, the standard deviation for the percent change in LDL-C was estimated at 22%. With a 2:1 mipomersen to placebo randomization, the inclusion of 60 patients per regimen (40 mipomersen, 20 placebo) yielded a 90% power to detect a 20% difference between treatment groups using a 2-sided alpha of 5%. The efficacy analysis for primary and secondary outcome parameters was executed by an intention to treat analysis and per protocol analysis. A mixed model for repeated measures with terms for baseline lipid value, geographic region, gender, statin use, treatment group, study visit, and study visit–by–treatment group interaction was used to assess the mean treatment difference at week 61 for the primary and secondary outcome parameters. Statistical significance was defined as p£0.05. Sequential testing was used to control for multiplicity associated with testing the primary and secondary efficacy outcome parameters.

RESULTS

Study subjects

A total of 309 subjects were enrolled in this study and randomized among 4 different treatment arms within severe HeFH and high-risk HeFH patients; 104 subjects received mipomersen 200 mg once weekly, 51 received placebo once weekly, 102 subjects received mipomersen 70 mg thrice weekly, and 52 subjects received placebo thrice weekly (see Figure 1 for consort diagram). Table 1 summarizes demographic and baseline characteristics of the enrolled patients by treatment group. A breakdown per cohort and treatment arm can be found in Supplementary Table 2). Rates of prior CHD were similar between mipomersen regimen groups and placebo; 77.7% of subjects in the pooled placebo group had a history of CHD compared to 73.5% and 77.9% in the mipomersen 200 mg once weekly and 70 mg thrice weekly groups, respectively. In the severe HeFH group (cohort 1), mean baseline LDL-C levels were 255 mg/dL and 262 mg/dL in the placebo and mipomersen 200 mg once weekly groups, respectively, and 263 mg/dL and 274 mg/dL in the placebo and mipomersen 70 mg thrice weekly groups, respectively. In the high-risk HeFH group (cohort 2), mean baseline LDL-C levels were 179 mg/dL and 177 mg/dL in the once-weekly placebo and mipomersen 200

mg groups, respectively, and 169 mg/dL and 178 mg/dL in the thrice-weekly placebo and mipomersen 70 mg groups, respectively.

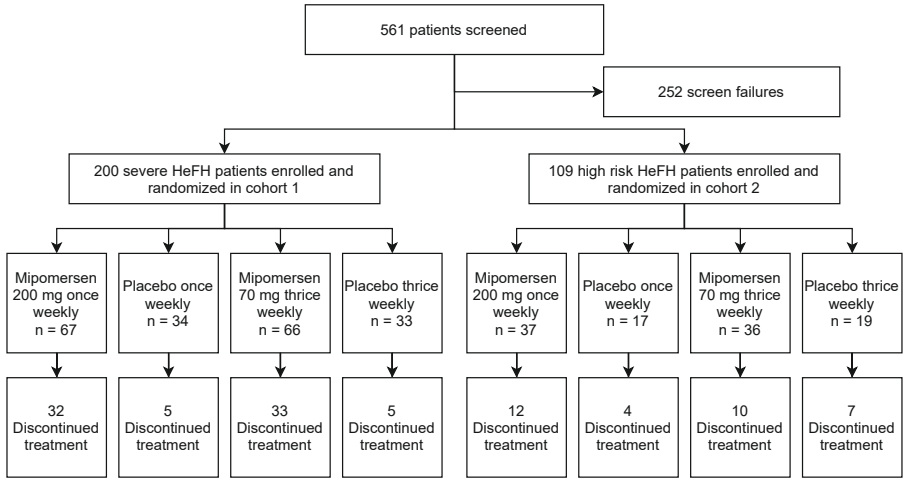

Figure 1: Consort diagram

A total of 201 patients (65.0%) completed the 60-week blinded treatment phase of the study. Most discontinuations were due to AEs. After 60 weeks of treatment, 60 subjects (57.5%) allocated to mipomersen 200 mg and 59 subjects (57.8%) allocated to mipomersen 70 mg were still on active treatment, while 82 participants (79.6%)of the pooled placebo group finished the blinded treatment period.

Table 1: Baseline characteristics

	Mipomersen 200 mg once weekly (n = 104)	**Mipomersen 70 mg thrice weekly** (n = 102)	**Pooled placebo** (n = 103)
Gender male, n (%)	46 (44.2)	48 (47.1)	47 (45.6)
Age (years), mean (SD)	56.4 (9.8)	53.1 (11.92)	54.9 (10.18)
Weight (kg), mean (SD)	78.43 (15.6)	79.88(16.73)	79.29 (16.28)
BMI (kg/m^2), mean (SD)	28.25 (4.65)	28.08 (4.6)	28.25 (4.32)
Race, n (%)			
- White	83 (79.8)	91 (89.2)	90 (87.4)
- Black	3 (2.9)	2 (2.0)	3 (2.9)
- Asian	13 (12.5)	7 (6.9)	7 (6.8)
- Other	4 (3.8)	2 (2.0)	3 (2.9)
Ethnicity, n (%)			
- Hispanic or Latino	4 (3.8)	4 (3.9)	10 (9.7)

Table 1: (Continued)

	Mipomersen 200 mg once weekly (n = 104)	**Mipomersen 70 mg thrice weekly** (n = 102)	**Pooled placebo** (n = 103)
- Not Hispanic or Latino	99 (95.2)	95 (93.1)	91 (88.3)
- Unknown	1 (0.9)	3 (2.9)	2 (1.9)
Tobacco users, n (%)			
- Current	16 (15.4)	23 (22.5)	22 (21.4)
- Former	30 (28.8)	29 (28.4)	27 (26.2)
- Never	57 (54.8)	50 (49.0)	64 (62.1)
Alcohol, n (%)			
- Current	40 (28.8)	45 (44.1)	37 (35.9)
- Former	6 (5.8)	14 (13.7)	14 (13.6)
- Never	58 (55.8)	43 (42.2)	52 (50.5)
HoFH, n (%)	1 (1.0)	2 (2.0)	4 (3.8)
CHD, n (%)	81 (77.9)	75 (73.5)	80 (77.7)
- MI n (%)	36 (34.6)	35 (34.3)	35 (34.0)
- CABG	23 (22.1)	22 (21.6)	23 (22.3)
- PCI or alternative revascularization	36 (34.6)	29 (28.4)	37 (35.9)
Other atherosclerotic disease (peripheral, carotid, abdominal aortic aneurysm), n (%)	32 (30.8)	36 (35.3)	43 (41.7)
Hypertension, n (%)	72 (69.2)	60 (58.8)	66 (64.1)
Diabetes, n (%)	23 (22.1)	11 (10.8)	15 (14.6)
Statin use, n (%)	91 (87.5)	89 (87.3)	85 (82.5)

Baseline characteristics of all participants. The pooled placebo group consists of patients receiving placebo once weekly and thrice weekly. BMI = body-mass index, HoFH = homozygous familial hypercholesterolemia, CHD = coronary heart disease, MI = myocardial infarction, CABG = coronary artery bypass grafting, PCI = percutaneous coronary intervention.

Efficacy

Mipomersen 200 mg once weekly and mipomersen 70 mg thrice weekly significantly lowered LDL-C by 21.0% and 18.8%, respectively, compared to placebo (p <0.001). The time course of changes in LDL-C levels during the 60-week blinded treatment period is shown in Figure 2 per treatment arm and per cohort. ApoB decreased by 22.1% with mipomersen 200 mg once weekly and 21.7% with mipomersen 70 mg thrice weekly compared to placebo (p <0.001). Lp(a) was significantly lowered after 60 weeks of treatment with mipomersen 70 mg thrice weekly but not with the once-weekly dosing regimen. The efficacy of mipomersen on LDL-C, apoB, and Lp(a) is summarized in Table 2 for both cohorts combined. The active treatment

regimens did not differ significantly from each other in their efficacy of lowering LDL-C and apoB.

In cohort 1 (severe HeFH) and cohort 2 (high-risk HeFH), mipomersen 200 mg once weekly significantly lowered LDL-C, apoB, TC, and VLDL-C compared with placebo after 60 weeks of treatment (Supplementary Table 3 and Supplementary Table 4). Lp(a) levels were not significantly changed by this mipomersen regimen compared with placebo in both cohorts. Mipomersen 70 mg thrice weekly did not significantly reduce LDL-C compared with placebo in severe HeFH, although the reduction in apoB was nominally significant (p=0.020). In contrast, in the high-risk HeFH cohort, this regimen was effective in lowering LDL-C, apoB, and TC. Mipomersen 70 mg lowered Lp(a) by 17.1% and 54.4% compared to placebo in the severe and high-risk HeFH cohorts, respectively.

Table 2: Change in key lipids from baseline to primary efficacy timepoint

Lipid	**Once weekly dosing**					
	Mipomersen (n = 104)		**Placebo** (n = 51)		**Difference mipomersen vs placebo**	
	Baseline	Mean % change	Baseline	Mean % change	Difference % change	*p*-value
LDL-C	232 (93.1)	-29.06 (4.358)	229 (72.3)	-8.10 (5.278)	-20.96 (5.117)	<0.001
ApoB	157 (47.0)	-27.34 (3.834)	158 (34.6)	-5.23 (4.700)	-22.11 (4.685)	<0.001
Lp(a)	32 (12, 64)	-22.50 (5.899)	30 (13, 70)	-17.83 (7.445)	-4.67 (7.988)	0.560
	Thrice weekly dosing					
	Mipomersen (n = 102)		**Placebo** (n = 52)		**Difference mipomersen vs placebo**	
LDL-C	240 (76.7)	-27.49 (4.263)	229 (81.4)	-8.69 (4.765)	-18.80 (5.104)	<0.001
ApoB	165 (38.7)	-25.78 (3.736)	157 (44.3)	-4.07 (4.283)	-21.71 (4.705)	<0.001
Lp(a)	29 (12, 66)	-27.79 (5.421)	25 (9, 73)	-0.14 (6.543)	-27.65 (7.586)	<0.001

Efficacy of mipomersen 200 mg once weekly subcutaneously (SQ) and mipomersen 70 mg thrice weekly SQ on LDL-C, apoB, and Lp(a) after a 60-week blinded treatment period for all subjects enrolled in this trial. Baseline values are in mg/dL and expressed as mean (SD), except for Lp(a) which is expressed as median (IQR). Mean % (SE) change between baseline and PET and Difference % change (SE) between mipomersen and placebo are calculated with a mixed model for repeated measurements (MMRM) with terms for baseline value, geopgraphic region, gender, statin use, treatment group, study visit, and study visit by treatment group interaction. Including patients who at least received one treatment dose but discontinued treatment early. LDL-C = low density lipoprotein cholesterol, apoB = apolipoprotein B, Lp(a) = lipoprotein (a), SD = standard deviation, IQR = interquartile range, SE = standard error.

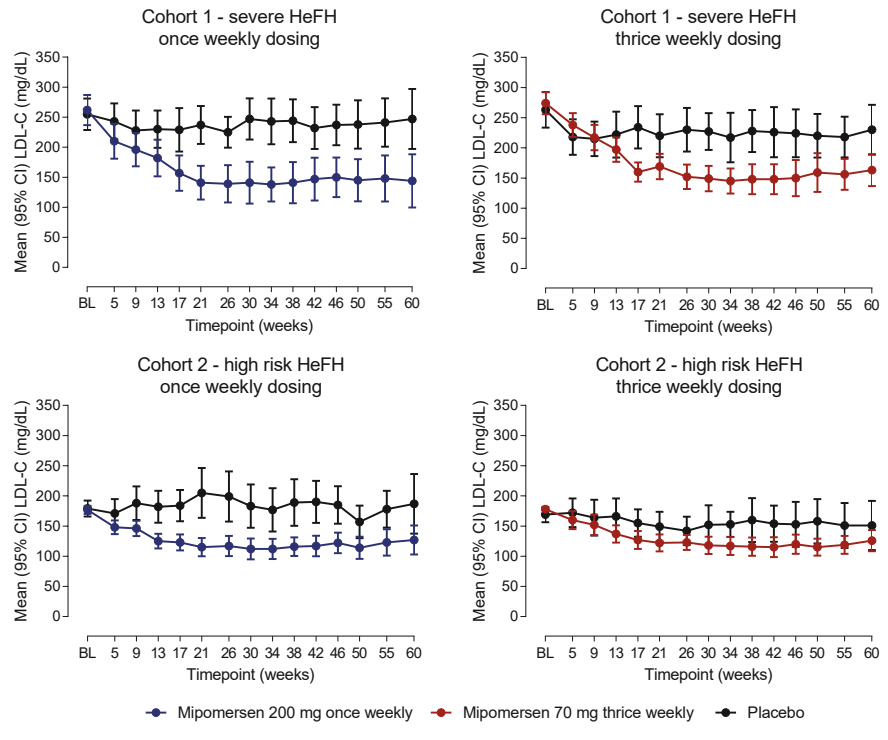

Figure 2: Mean LDL-C over time per cohort and mipomersen regimen
Mean LDL-C levels over time per cohort and per treatment regimen. Error bars represent 95% confidence interval.

Safety

A total of 632 AEs occurred in 91 (87.5%) patients receiving mipomersen 200 mg once weekly, 554 events in 87 (85.3%) patients receiving mipomersen 70 mg thrice weekly, and 459 events in 81 (78.6%) patients on placebo (Table 3). Flu-like symptoms were the most frequently reported AE, with an incidence of 42.3% in the mipomersen 200 mg group, 25.5% in the mipomersen 70 mg group, and 19.4% in the pooled placebo group. The most frequent injection-site reactions were erythema, pain, swelling, and pruritus and were more frequent in patients receiving active treatment than placebo (Table 3). The occurrence of flu-like symptoms or injection-site reactions led to discontinuation in 22 of 104 (21.2%) patients on mipomersen 200 mg once weekly, compared to 18 of 103 (17.6%) subjects in the 70 mg thrice-weekly group, and 7 of 102 (6.8%) subjects in the pooled placebo group.

A total of 41 SAEs occurred among all treatment arms. The number of MACE was low in the three treatment groups: 6 subjects out of 103 on placebo (5.8%), 8 subjects out of 102 (7.8%) on mipomersen 70 mg thrice weekly, and 0 out of 104 subjects on mipomersen 200 mg once weekly had one or multiple MACE (Table 3). Five subjects in the placebo group and one subject on mipomersen 200 mg developed neoplasms during the blinded treatment period; the latter subject reported a hemangioma. Two subjects died during the blinded treatment period; one of a lymphoma (receiving placebo once weekly) and one of an unknown cause (receiving mipomersen 200 mg once weekly).

Hepatobiliary disorders included cholelithiasis (1 placebo , 2 mipomersen 200 mg, 1 70 mg), chronic cholecystitis (1 placebo), gallbladder polyp (1 mipomersen 200 mg), hepatomegaly (1 mipomersen 200 mg, hyperbilirubinemia (1 placebo), and jaundice (1 placebo. Hepatic steatosis was diagnosed on serial magnetic resonance imaging in 10 (9.6%) patients receiving mipomersen 200 mg once weekly, in 9 (8.8%) patients receiving mipomersen 70 mg thrice weekly, and in 2 (1.9%) patients receiving placebo.

Laboratory assessments

Mean platelet levels remained stable during the study period in all patient groups. A platelet count of <100,000 occurred in 1 patient receiving mipomersen 200 mg and in 1 patient receiving mipomersen 70 mg. Another subject in the mipomersen 200 mg group had a platelet count of <50,000 during the study treatment period.

Table 3: Adverse events and discontinuation during blinded treatment period

	Mipomersen 200 mg once weekly (n = 104)	**Mipomersen 70 mg thrice weekly** (n = 102)	**Pooled Placebo** (n = 103)
Patients with AEs, n(%)	91 (87.5)	87 (85.3)	81 (78.6)
Serious AEs, n(%)	9 (8.7)	17 (16.7)	15 (14.6)
AEs leading to death, n(%)	1 (1.0)	0	1 (0.9)
Discontinuations due to AEs, n(%)	37 (35.6)	24 (23.5)	13 (12.6)
Most common AEs (>5%), n(%)	44 (42.3)	26 (25.5)	20 (19.4)
- Flu like symptoms	8 (7.7)	13 (12.7)	2 (1.9)
- Injection site erythema	13 (12.5)	7 (6.9)	1 (1.0)
- Injection site pain	4 (3.8)	8 (7.8)	2 (1.9)
- Injection site swelling	3 (2.9)	7 (6.9)	2 (1.9)
- Injection site pruritis	7 (6.7)	25 (8.1)	11 (10.7)
- Nasopharyngitis	5 (4.8)	9 (8.8)	8 (7.8)
- Influenza	2 (1.9)	7 (6.9)	6 (5.8)
- Upper respiratory tract infection	5 (4.8)	3 (2.9)	6 (5.8)
- Bronchitis	9 (8.7)	7 (6.9)	5 (4.9)
- Nausea	0	0	4 (3.9)
- Lower abdominal pain	8 (7.7)	5 (4.9)	3 (2.9)
- Myalgia	5 (4.8)	3 (2.9)	3 (2.9)
- Pain in extremity	2 (3.9)	9 (8.8)	12 (15.5)
- Headache	8 (7.7)	3 (2.9)	2 (1.9)
- Dizziness	5 (4.8)	5 (4.9)	3 (2.9)
- Cough	3 (2.9)	4 (3.9)	8 (7.8)
- Hypertension	10 (9.6)	9 (8.8)	2 (1.9)
- Hepatic steatosis			
Injection site reactions or flu like symptoms leading to discontinuation	22 (21.2)	18 (17.6)	6 (5.8)
MACE events, any n(%)			
- CV death	0	0	0
- (acute) myocardial infarction	0	3 (2.9)	2 (1.9)
- Ischaemic stroke	0	1 (1.0)	0
- Unstable angina	0	4 (3.9)	4 (3.9)

Overview of adverse events during the 60-week blinded treatment period for all participants. The pooled placebo group consists of patients receiving placebo once weekly and thrice weekly. AE = adverse event, MACE = major adverse cardiac event, CV death = cardiovascular death.

Hepatic transaminase elevations were more common in subjects receiving mipomersen 200 mg and mipomersen 70 mg than in those receiving placebo. Maximum ALT levels of ≥3 × ULN at least once were seen in 22 patients in both mipomersen groups (21.2% and 21.6%, respectively) compared to 1 (1.0%) in the placebo group. Maximum AST levels reached ≥3 × ULN at least once in 13

patients (12.5%) on the mipomersen 200 mg regimen and 9 patients (8.8%) on the mipomersen 70 mg regimen (Table 4) compared to 2 (1.9%) in the placebo group. Two consecutive ALT levels of ≥3 × ULN, at least 7 days apart, occurred in 21 (20.2%) patients on mipomersen 200 mg and 17 (16.7%) patients on mipomersen 70 mg compared to 0 patients in the placebo group. AST levels of ≥3 × ULN at two consecutive measurements was seen in 7 (6.7%), 3 (2.9%), and 0 patients in the respective groups. No cases of hepatic injury defined by Hy's Law were observed (elevated ALT levels [≥3 × ULN] in combination with elevated total bilirubin levels [defined as ≥1.5 × ULN]). Following the predetermined liver chemistry stopping rules, elevated liver enzymes led to adjusting of study medication doses in a total of 28 patients, 15 on mipomersen 200 mg and 13 on mipomersen 70 mg. Mipomersen 200 mg was temporarily stopped in 12 patients and permanently stopped in 4 patients because of liver rule events. Mipomersen 70 mg was temporarily stopped in 5 patients and permanently in 2 patients.

Table 4: Liver function test abnormalities

	Mipomersen 200 mg once weekly (n = 104)	**Mipomersen 70 mg thrice weekly** (n = 102)	**Pooled placebo** (n = 103)
ALT ≥3x ULN, n (%)	22 (21.2)	22 (21.6)	1 (1.0)
ALT ≥5x ULN, n (%)	6 (5.8)	3 (2.9)	0
ALT ≥8x ULN, n (%)	1 (1.0)	0	0
ALT ≥3 x ULN, two consecutive results (≥7 days apart), n (%)	21 (20.2)	17 (16.7)	0
AST ≥3x ULN, n (%)	13 (12.5)	9 (8.8)	2 (1.9)
AST ≥5X ULN, n (%)	2 (1.9)	1 (1.0)	1 (1.0)
AST ≥8x ULN, n (%)	1 (1.0)	1 (1.0)	0
AST ≥3 x ULN, two consecutive results (≥7 days apart), n (%)	7 (6.7)	3 (2.9)	0

Liver function tests during the blinded treatment period of all enrolled participants. The pooled placebo group consists of patients receiving placebo once weekly and thrice weekly. Numbers depict participants with at least one elevation of transaminases above a certain threshold (e.g., ≥3x ULN). ALT = alanine transaminase, AST = aspartate transaminase, ULN = upper limit of normal.

DISCUSSION

The FOCUS-FH study is the largest and longest randomized, placebo-controlled trial with mipomersen in FH patients to date. Two different regimens of mipomersen SQ (200 mg once weekly and 70 mg thrice weekly) and placebo were tested for 60 weeks in 309 patients with severe HeFH or with HeFH and a history of CVD. Mipomersen 200 mg once weekly and mipomersen 70 mg thrice weekly both produced significant LDL-C and apoB lowering of around 20% on top of maximally tolerated standard lipid-lowering regimens in both cohorts combined. Lp(a) was significantly lowered only with the thrice-weekly dosage for unclear reasons. The lipid-lowering effects of the two regimens per cohort were heterogeneous. The efficacy of mipomersen 200 mg once weekly in lowering multiple lipid parameters was consistent in severe and high-risk HeFH patients and ranged from 15% to 30% reductions. However, mipomersen 70 mg did not show significant LDL-C–lowering effects in severe HeFH, but did in high-risk HeFH for unclear reasons. In general, active treatment with mipomersen was less well tolerated than placebo. As previously described,[15] the most common AEs were injection-site reactions and flu like symptoms, of which the latter was less frequently present in the mipomersen 70 mg treatment arm. Elevated liver enzymes were more frequently observed in both active treatment arms compared to placebo.

Previous research has shown that every decrease of 1 mmol/L (39 mg/dL) in LDL-C levels results in a 19% reduction in coronary mortality as well as a 22% reduction in MACE.[17] This in combination with the fact that many FH patients do not reach their LDL-C guideline-recommended goals with standard lipid-lowering therapies clearly shows the need for additional or alternative LDL-C–lowering therapies for FH patients. Mipomersen may fulfil this role, as the 200 mg dose was an effective LDL-C–lowering agent in this study and others.[11,12,15] However, this might not be the case for mipomersen 70 mg thrice weekly because of its inconsistent effect on LDL-C lowering in the current study, although it is possible that noncompliance related to the higher frequency of injections may have interfered with LDL-lowering efficacy.

Since significant Lp(a) lowering during treatment with mipomersen 200 mg SQ weekly has been documented in previous clinical trials, it is surprising that Lp(a) was lowered significantly only in subjects treated with mipomersen 70 mg thrice weekly and not in those treated with mipomersen 200 mg once weekly. The discrepancy in efficacy between the two regimens might be explained by a lack of statistical power in the current study and a wide range in Lp(a) levels within the included subjects. In contrast, a pooled analysis of four randomized clinical trials has shown a 26.4% (95% confidence interval –42.8, –5.4) decrease in Lp(a) levels by mipomersen 200 mg once weekly vs. placebo.[20] Lp(a) has been shown to be an independent risk factor for the development of CVD[21], and until recently, few therapeutic options for lowering plasma levels of Lp(a) were available. The potential Lp(a)-lowering efficacy of mipomersen adds to effective treatment options for high Lp(a) levels and could theoretically contribute to lowering CVD risk in these vulnerable patients.

Despite the efficacy of mipomersen on atherogenic lipid levels, the benefits are tempered by lower tolerability compared with placebo. One concern is the increased occurrence of hepatic steatosis and elevated liver enzyme levels during mipomersen treatment, which are mechanism-based side effects. Elevated liver enzymes can be attenuated by reducing the mipomersen dose, reducing dosing frequency, or temporarily stopping mipomersen, as documented in this study and others.[12,15,19] However, 6 out of 205 participants stopped permanently because of increased liver enzymes in the current study in accordance with prespecified liver safety rules. While the frequency of ALT and AST elevation was not different between the two mipomersen dosing regimens, temporary and permanent discontinuations of treatment occurred less frequently in subjects treated with mipomersen 70 mg thrice weekly compared to 200 mg weekly. Whether increased ALT/AST levels and hepatic steatosis are a major problem for patients treated with mipomersen for longer periods remains to be elucidated, although data from previous studies suggested that the level of hepatic steatosis and occurrence of transaminase elevations stabilized after the first year during up to 4 years of follow-up.[15,23] Despite the relatively higher risk of steatosis in patients on mipomersen,[24] mipomersen has not been associated with fibrosis in the small number of subjects studied with liver biopsy.[14,15,25] Elevated AST and ALT levels have been shown to

stabilize over time and trend towards baseline in a long-term follow-up study after 2 years of treatment with mipomersen 200 mg once weekly.[15]

Approximately 42% of the mipomersen-treated participants, compared with 20% of the placebo group, discontinued treatment independently of dose frequency before the end of the blinded treatment period at 60 weeks. Most discontinuations were due to adverse events. Interim results of an open-label extension study have shown that 55% of patients treated with mipomersen discontinued treatment within the first 2 years.[15] Hence, one might conclude that the majority of discontinuations occur within the first year of treatment. Injection-site reactions and flu-like symptoms were the main reasons for patients to discontinue treatment. In the current study, approximately 50% of discontinuations of treatment were attributed to these AEs. The mipomersen70 mg thrice weekly regimen led to fewer discontinuations because of flu-like symptoms compared with the 200 mg once weekly regimen. In contrast, discontinuations due to injection-site reactions were higher in the mipomersen 70 mg thrice-weekly regimen compared with the 200 mg once-weekly regimen, possibly as a consequence of the 3-fold higher number of injections. The use of newer formulations of antisense oligonucleotides or short-interfering RNAs (siRNAs) specifically targeting the liver may attenuate injection-site reactions and flu-like symptoms and could thus improve tolerability. However, because of mipomersen's mode of action, targeting apoB production, elevated liver enzymes and hepatic fat accumulation would remain a concern. Mipomersen is currently not available for clinical use.

This study was sufficiently powered to draw conclusions about the efficacy and safety of mipomersen in HeFH patients in regards to the effects on lipids and lipoproteins. Its strengths are the relatively long blinded-treatment period of 60 weeks and the large number of FH subjects. However, some potential limitations of this study are present. First, the current results are mostly applicable to Caucasians, who comprised the majority of subjects in this study (80–90%, Table 1). Further research is needed to see if the effects of mipomersen in other ethnic groups with HeFH are the same. Second, it is unknown what proportion of subjects maintained blinded treatment during the study. Despite the double-blinded study design, a 1-year duration of blinded lipid profile testing is very long among subjects with very

high CHD risk. For this reason, it is possible that subjects may have inadvertently had surreptitious lipid profile testing performed through their primary care provider. In this situation, the mean on-treatment LDL-C concentration of roughly 200 mg/dL during the study could have contributed to premature study discontinuation from AEs. Third, since PCSK9 inhibitors are currently more frequently prescribed than mipomersen, it is uncertain what the effect of mipomersen will be when coadministered with PCSK9 inhibitors. One could speculate that there could still be a sufficient additional LDL-C–lowering effect of mipomersen, since it is believed to reduce production of apoB-containing lipoproteins, in contrast to drugs targeting increased clearance through up-regulation of LDL receptors (e.g., statins and PCSK9 inhibitors). Fourth, this study was not designed or powered to show benefit of treatment with mipomersen on cardiovascular outcomes. Therefore, longer and larger studies are needed to see if treatment with mipomersen reduces CVD risk and mortality, although the results of a recent prospective before-and-after analysis suggested that mipomersen may reduce the incidence of MACE during 2 years of treatment compared to 2 years prior to study entry.[26] Lastly, long-term follow-up studies are needed to evaluate mipomersen's long-term adverse effects, especially hepatic steatosis, and its long-term clinical course.

In conclusion, mipomersen 200 mg SQ once weekly and 70 mg SQ thrice weekly showed similar LDL-C–lowering effects; however, more consistent and significant efficacy was seen with the standard FDA-approved once-weekly dosage. Mipomersen is currently approved only for patients with HoFH; however, this and other studies demonstrate the LDL-C–lowering efficacy of mipomersen in patients with HeFH and persistent LDL-C elevation. The benefit of mipomersen is counterbalanced by limited tolerability in some subjects and the requirement for hepatic monitoring for transaminase elevations. In this light, a thrice-weekly regimen with mipomersen 70 mg might provide minor benefits, such as a lower prevalence of flu-like symptoms and slightly lower requirement for dosage adjustments due to elevated liver enzymes, although more injection-site reactions were seen with this regimen.

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CONFLICT OF INTEREST

Laurens F. Reeskamp has nothing to disclose. John J.P. Kastelein was a consultant to Ionis Pharmaceuticals during the FOCUS-FH study and his institution received the research grants for the clinical study. Patrick M. Moriarty reports: Regeneron: Speaker Fee—Consultant, Speaker; Sanofi: Speaker Fee—Speaker Bureau, Consultant; Amgen: Speaker Fee—Speaker, Consultant; Duke: Consultant Fee—Consultant; Esperion: Consultant Fee—Consultant, Executive Committee; Eliaz Therapeutics: Stock Options—Advisory Board; Kaneka: Consult Fee—Consultant; RegenXBio: Consulting—Consultant; Kastle Therapeutics: Consulting—Consultant; Amarin: Speaker Fee—Speaker; Gemphire Therapeutics: Consulting—Consultant; Aegerion: Advisory Board; Stage II Innovations: Consulting—Consultant; Ambry Genetics: Speaker Fee—Speaker; Novartis: Consulting—Consultant; Regeneron, Sanofi, Amgen, Ionis, Pfizer, Novartis, Kaneka, Stage 2 Innovations, University of Penn, Zydus Discovery, Gemphire, Kowa, Akcea, FH Foundation: Grant Research Support—Research P. Barton Duell reports consultant/institutional research grants from Esperion, Regeneron, Retrophin, RegenxBio; consultant: Akcea, Daiichi-Sankyo, Kastle. Alberico L. Catapano reports grants from Pfizer, Sanofi, Regeneron, Merck, Mediolanum, SigmaTau, Menarini, Kowa, Recordati, Eli Lilly, and personal fees from Astrazeneca, Genzyme, Bayer, SigmaTau, Menarini, Kowa, Eli Lilly, Recordati, Pfizer, Sanofi, Mediolanum, Pfizer, Merck, Sanofi, Aegerion, Amgen. Raul D Santos has received honoraria related to consulting, speaker activities, and research from: Amgen, Astra Zeneca, Biolab, Akcea, Esperion, Novo Nordisk, Merck, Sanofi/Regeneron. Christie M. Ballantyne has received grant/research support (all paid to institution, not individual) from Akcea, Amarin, Amgen, Esperion, Novartis, Regeneron, Sanofi-Synthelabo, NIH, AHA, ADA, and has been a consultant for Akcea, Amarin, Amgen, Astra Zeneca, Eli Lilly, Esperion, Matinas BioPharma Inc, Merck, Novartis, Regeneron, Sanofi-Synthelabo.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1: Summary of liver chemistry safety monitoring and stopping rules

Test and levels	Action	Monitoring
ALT and/or AST ≥3 x ULN but <5 x ULN		
First occurrence during study	Temporarily discontinue dosing	Repeat liver chemistry tests ASAP, max. 9 days
Single result, not first occurrence	Continue current dosing regimen	Repeat liver chemistry tests ASAP, max. 9 days
2 consecutive results or single result presumed confirmed	Conduct diagnostic evaluations after first occurrence is confirmed/presumed confirmed. Restart dosing at adjusted dose.	Repeat liver chemistry tests every 14 days, max. 16 days, until 2 consecutive results are <3 x ULN
Prolonged duration (e.g., >3 months)	Recommend referral to specialist/hepatologist for evaluation and consideration of liver biopsy (to assess for clinically significant liver disease)	Consult with Medical Monitor to consider reducing frequency of liver chemistry tests to scheduled study visits
ALT and/or AST ≥5 x ULN but <8 x ULN		
Single result	Temporarily discontinue dosing	Repeat liver chemistry tests ASAP, max. 9 days
2 consecutive results or single result presumed confirmed	Conduct diagnostic testing after result is confirmed/presumed confirmed. Continue temporary discontinuation of dosing if confirmed	Repeat liver chemistry tests within 14 days, max. 16 days
3 consecutive results ≥5 x ULN over ≥3 weeks	Permanently discontinue dosing in the absence of alternative cause	Patient enters post-treatment period
ALT and/or AST ≥8 x ULN		
Single result	Temporarily discontinue dosing	Repeat liver chemistry tests ASAP, max. 9 days. Conduct diagnostic testing after result is confirmed/presumed confirmed
2 consecutive results or single result presumed confirmed with alternative cause identified and treated	Continue temporary discontinuation of dosing until results approach normal, then consult Medical Monitor regarding dosing	Repeat liver chemistry tests every 14 days, max. 16 days
2 consecutive results or single result presumed confirmed with no alternative cause identified	Permanently discontinue dosing	Patient enters post-treatment period
ALT and/or AST ≥3 x ULN plus appearance/worsening of symptoms assessed by the investigator to be potentially related to hepatic inflammation		
Single occurrence with symptoms of hepatic inflammation	Temporarily discontinue dosing	Repeat liver chemistry tests ASAP, max. 9 days
2 consecutive results or single result presumed confirmed with symptoms of hepatic inflammation	Permanently discontinue dosing	Patient enters post-treatment period
ALT and/or AST ≥3 x ULN plus total bilirubin 1.5 x ULN		
Single occurrence	Temporarily discontinue dosing	Repeat liver chemistry tests ASAP, max. 9 days
2 consecutive results or single result presumed confirmed and no alternative cause identified	Permanently discontinue dosing	Patient enters post-treatment period
ALT and/or AST ≥1.5 x ULN or 1.5 x baseline value at last study visit, whichever is higher		
At last study visit	NA	Recommendation to continue monitoring until results return to Baseline values (per Investigator)
ALT and/or AST <3 x ULN after being ≥3 x ULN		
2 consecutive results	Resume normal dosing	Resume monitoring per scheduled study visits

Supplementary Table 2: Baseline characteristics per cohort and regimen

	Cohort 1 – severe HeFH				Cohort 2 – high-risk HeFH			
	Mipomersen 200 mg once weekly (n=67)	**Placebo once weekly** (n=34)	**Mipomersen 70 mg thrice weekly** (n=66)	**Placebo thrice weekly** (n=33)	**Mipomersen 200 mg once weekly** (n=37)	**Placebo once weekly** (n=17)	**Mipomersen 70 mg thrice weekly** (n=36)	**Placebo thrice weekly** (n=19)
Gender male, n (%)	25 (37.3)	13 (38.2)	27 (40.9)	14 (42.4)	21 (56.8)	9 (52.9)	21 (58.3)	11 (57.9)
Age (years), mean (SD)	55.19 (10.06)	56.21 (10.77)	51.70 (12.75)	56.06 (8.93)	58.46 (8.97)	54.06 (10.04)	55.81 (9.83)	51.47 (11.14)
Weight (kg), mean (SD)	76.48 (14.20)	76.43 (16.62)	78.62 (15.65)	79.82 (15.39)	81.96 (17.46)	84.23 (16.74)	82.19 (18.56)	79.05 (16.90)
BMI (kg/m^2), mean (SD)	27.92 (4.40)	27.16 (4.13)	27.88 (4.53)	28.62 (4.07)	28.82 (5.07)	30.18 (4.53)	28.45 (4.78)	27.84 (4.53)
Race, n (%)								
- White	47 (70.1)	31 (91.2)	59 (89.4)	28 (84.8)	36 (97.3)	15 (88.2)	32 (88.9)	16 (84.2)
- Black	3 (4.5)	2 (5.9)	2 (3.0)	1 (3.0)	0	0	0	0
- Asian	12 (17.9)	0	5 (7.6)	4 (12.1)	1 (2.7)	2 (11.8)	2 (5.6)	1 (5.3)
- Other or unknown	4 (6.0)	1 (2.9)	0	0	0	0	2 (5.6)	2 (10.6)
Ethnicity, n (%)								
- Hispanic or Latino	2 (3.0)	2 (5.9)	3 (4.5)	2 (6.1)	2 (5.4)	2 (11.8)	1 (2.8)	4 (21.1)
- Not Hispanic or Latino	64 (95.5)	31 (91.2)	60 (90.9)	31 (93.9)	35 (94.6)	14 (82.4)	35 (97.2)	15 (78.9)
- Unknown	1 (1.5)	1 (2.9)	3 (4.5)	0	0	1 (5.9)	0	0
Tobacco use, n (%)								
- Current	9 (13.4)	6 (17.6)	14 (21.2)	6 (18.2)	7 (18.9)	5 (29.4)	9 (25.0)	5 (26.3)
- Former	18 (26.9)	9 (26.5)	18 (27.3)	9 (27.3)	12 (32.4)	5 (29.4)	11 (30.6)	4 (21.1)
- Never	39 (58.2)	19 (55.9)	34 (51.5)	18 (54.5)	18 (48.6)	7 (41.2)	16 (44.4)	10 (52.6)
Alcohol, n (%)								
- Current	24 (35.8)	16 (47.1)	26 (39.4)	8 (24.2)	16 (43.2)	9 (52.9)	19 (52.8)	4 (21.1)
- Non-current	4 (6.0)	4 (11.8)	8 (12.1)	8 (24.2)	2 (5.4)	0	6 (16.7)	2 (10.5)
- Never	39 (58.2)	14 (41.2)	32 (48.5)	17 (51.5)	19 (51.4)	8 (47.1)	11 (30.6)	13 (68.4)

Supplementary Table 2: (Continued)

	Cohort 1 – severe HeFH				Cohort 2 – high-risk HeFH			
	Mipomersen 200 mg once weekly (n=67)	**Placebo once weekly** (n=34)	**Mipomersen 70 mg thrice weekly** (n=66)	**Placebo thrice weekly** (n=33)	**Mipomersen 200 mg once weekly** (n=37)	**Placebo once weekly** (n=17)	**Mipomersen 70 mg thrice weekly** (n=36)	**Placebo thrice weekly** (n=19)
HoFH, n (%)	1 (2.9)	1 (2.9)	2 (6.1)	2 (6.1)	0	0	0	1 (5.3)
CHD, n (%)	50 (74.6)	24 (70.6)	47 (71.2)	25 (75.8)	31 (83.8)	15 (88.2)	28 (77.8)	16 (84.2)
- MI	21 (31.3)	9 (26.5)	22 (33.3)	9 (27.3)	15 (40.5)	7 (41.5)	13 (36.1)	10 (52.6)
- CABG	15 (22.4)	9 (26.5)	12 (18.2)	8 (24.2)	9 (24.3)	5 (29.4)	10 (27.8)	1 (5.3)
- PCI or alternative revascularization	22 (32.8)	8 (23.5)	19 (28.8)	11 (33.3)	14 (37.8)	9 (52.9)	10 (27.8)	9 (47.4)
Other atherosclerotic disease (peripheral, carotid, abdominal aortic aneurysm), n (%)	19 (28.4)	18 (52.9)	21 (31.8)	11 (33.3)	13 (35.1)	7 (41.2)	15 (41.7)	7 (36.8)
Hypertension, n (%)	46 (68.7)	21 (61.8)	36 (54.5)	21 (63.3)	25 (67.6)	14 (82.4)	24 (66.7)	10 (52.6)
Diabetes, n (%)	12 (17.9)	4 (11.8)	6 (9.1)	4 (12.1)	11 (29.7)	4 (23.5)	5 (13.9)	3 (15.8)
Statin use, n (%)	57 (85.1)	29 (85.3)	54 (81.8)	27 (81.8)	34 (91.9)	17 (100.0)	35 (97.2)	17 (89.5)

Baseline characteristics of all participants per cohort and per mipomersen regimen. BMI = body-mass index, HoFH = homozygous familial hypercholesterolemia, CHD = coronary heart disease, MI = myocardial infarction, CABG = coronary artery bypass grafting, PCI = percutaneous coronary intervention.

Supplementary Table 3: Efficacy endpoints cohort 1 – severe HeFH

Cohort 1 – Severe HeFH	Mipomersen 200mg once weekly (n = 67)			Placebo (n = 34)				
	Baseline	**PET**	**% Change**	**Baseline**	**PET**	**% Change**	**Mean difference in % change mipomersen vs placebo**	***p*-value**
LDL-C	262 (103.1)	156 (112.2)	-41.9 (20.20)	255 (75.0)	229 (82.0)	-13.1 (20.78)	-20.40 (6.70)	0.003
ApoB	172 (52.1)	108 (64.9)	-38.1 (22.57)	170 (35.0)	156 (41.0)	-8.2 (19.66)	-21.31 (6.31)	0.001
Total cholesterol	345 (100.9)	232 (110.9)	-33.1 (17.42)	338 (70.1)	312 (84.5)	-9.9 (17.50)	-15.95 (5.29)	0.004
non-HDL-C	293.33 (103.60)	179.31 (114.48)	-39.8 (20.31)	285.37 (74.53)	260.95 (79.30)	-10.8 (19.74)	-19.84 (6.52)	0.003
HDL-C	52 (18.1)	53 (17.5)	4.9 (24.47)	52 (17.6)	51 (20.3)	-2.2 (23.22)	8.84 (5.21)	0.094
Triglycerides	143 (101, 181)	107 (75, 159)	-19.5 (40.48)	144 (111, 193)	144 (104, 215)	13.2 (38.12)	-27.95 (7.59)	<0.001
VLDL-C	31 (14.9)	24 (11.1)	-19.6 (41.02)	31 (12.9)	32 (13.6)	8.2 (27.58)	-24.31 (7.14)	0.001
Lp(a)	30 (13, 69)	20 (11, 45)	-16.5 (65.12)	34 (20, 70)	25 (12, 50)	-6.8 (25.64)	-1.99 (11.07)	0.858
ApoA1	142 (30.9)	135 (28.0)	-2.2 (14.34)	144 (30.5)	140 (33.1)	-0.8 (17.28)	2.19 (3.19)	0.493
LDL/HDL Ratio	5.97 (4.74)	3.64 (4.96)	-43.5 (21.62)	5.69 (3.36)	5.14 (2.78)	-8.0 (26.87)	-26.77 (7.49)	<0.001
	Mipomersen 70mg thrice weekly (n = 66)			**Placebo** (n = 33)				
	Baseline	**PET**	**% Change**	**Baseline**	**PET**	**% Change**	**Mean difference in % change mipomersen vs placebo**	***p* -value**
LDL-C	274 (75.1)	184 (75.6)	-32.2 (22.83)	263 (82.9)	231 (95.4)	-7.4 (30.89)	-12.34 (6.41)	0.058
ApoB	182 (37.3)	124 (42.7)	-30.1 (21.17)	174 (45.2)	156 (49.3)	-6.0 (26.05)	-14.15 (5.97)	0.020
Total cholesterol	355 (77.5)	262 (78.6)	-25.2 (19.05)	345 (90.5)	306 (102.3)	-6.9 (24.53)	-9.24 (5.11)	0.074
non-HDL-C	307.05 (77.26)	210.05 (80.00)	-30.1 (22.41)	294.05 (88.45)	257.00 (98.88)	-7.1 (29.95)	-11.88 (6.24)	0.060
HDL-C	48 (10.8)	52 (13.0)	4.7 (18.01)	51 (17.1)	49 (18.1)	-2.4 (15.53)	5.90 (5.10)	0.250

Supplementary Table 3: (Continued)

Cohort 1 – Severe HeFH	Mipomersen 70mg once weekly (n = 66)			Placebo (n = 33)				
	Baseline	**PET**	**% Change**	**Baseline**	**PET**	**% Change**	**Mean difference in % change mipomersen vs placebo**	***p*-value**
Triglycerides	154 (100, 225)	104 (78, 177)	-7.9 (42.85)	151 (107, 189)	119 (93, 169)	-1.2 (37.94)	-7.45 (10.05)	0.461
VLDL-C	33 (15.1)	26 (15.4)	-9.0 (39.65)	31 (13.5)	27 (11.7)	-1.0 (38.05)	-10.42 (9.84)	0.292
Lp(a)	22 (11, 56)	17 (7, 37)	-23.8 (30.71)	29 (13, 79)	29 (8, 72)	-6.1 (24.85)	-17.10 (6.70)	0.013
ApoA1	136 (18.3)	136 (26.2)	-1.9 (14.07)	142 (25.2)	134 (26.9)	-2.9 (12.99)	3.36 (3.26)	0.307
LDL/HDL Ratio	6.09 (2.48)	3.81 (1.87)	-33.0 (27.32)	5.66 (2.69)	5.16 (2.77)	-0.8 (49.05)	-15.88 (8.60)	0.068

Efficacy of mipomersen 200 mg once weekly subcutaneously (SQ) and mipomersen 70 mg thrice weekly SQ compared to placebo during the 60 week blinded treatment period in cohort 1 (severe HeFH). All lipid parameters are measured in mg/dL, except for LDL/HDL ratio. Baseline and primary efficacy endpoint (PET) values are mean (SD), except for triglycerides and Lp(a) which are expressed as median (IQR). Lipid values at PET (week 60) and % change (SD) from baseline to PET are only displayed for patients who completed the full blinded treatment period. Mean difference in % change (SE) between active treatment and placebo group is based on a mixed model for repeated measurements with terms for baseline value, geographic region, gender, statin use, treatment group, study visit, and study visit by treatment group interaction, including patients who at least received one treatment dose but discontinued treatment early. LDL-C = low-density lipoprotein cholesterol, apoB = apolipoprotein B, = non-HDL-C = non–high-density lipoprotein cholesterol, HDL-C = high-density lipoprotein cholesterol, VLDL-C = very low density lipoprotein cholesterol, Lp(a) = lipoprotein (a), apoA1 = apolipoprotein A1, LDL/HDL ratio = low-density lipoprotein – high-density lipoprotein ratio, PET = primary efficacy endpoint (week 60), SD = standard deviation, IQR = interquartile range, SE = standard error.

Supplementary Table 4: Efficacy endpoints cohort 2 – high-risk HeFH

Cohort 2 – high risk HeFH	Mipomersen 200 mg once weekly (n = 37)			Placebo (n = 17)				
	Baseline	**PET**	**% Change**	**Baseline**	**PET**	**% Change**	**Mean difference in % change mipomersen vs placebo**	***p*-value**
LDL-C	177 (20.8)	112 (16.6)	-37.0 (12.85)	179 (25.6)	141 (34.2)	-20.2 (17.71)	-21.95 (8.93)	0.019
ApoB	131 (17.3)	87 (16.4)	-32.3 (14.60)	134 (17.0)	110 (23.1)	-18.0 (16.34)	-24.10 (7.36)	0.002
Total cholesterol	259 (26.6)	185 (20.5)	-27.3 (11.99)	264 (36.7)	218 (27.6)	-15.5 (10.56)	-19.25 (5.98)	0.003
non-HDL-C	207.70 (29.04)	130.38 (18.79)	-35.3 (13.33)	208.59 (26.47)	168.33 (35.82)	-19.3 (14.28)	-24.76 (7.53)	0.002
HDL-C	51 (11.1)	55 (10.4)	4.7 (15.52)	55 (18.3)	50 (16.6)	-2.1 (12.64)	1.87 (5.45)	0.734
Triglycerides	138 (96, 187)	84 (69, 100)	-20.9 (27.87)	148 (130, 185)	130 (108, 187)	-11.2 (21.18)	-27.30 (16.65)	0.109
VLDL-C	31 (16.1)	18 (7.9)	-21.5 (27.96)	30 (8.5)	28 (7.9)	-11.9 (21.07)	-24.73 (15.33)	0.115
Lp(a)	70 (9, 63)	46 (35, 51)	-15.1 (14.31)	23 (6, 45)	19 (10, 83)	2.2 (27.88)	-11.54 (9.74)	0.244
ApoA1	141 (19.7)	143 (14.7)	0.8 (12.07)	150 (28.2)	141 (36.4)	-4.5 (14.61)	-5.12 (4.76)	0.288
LDL/HDL Ratio	3.68 (1.10)	2.11 (0.51)	-39.2 (13.58)	3.51 (1.03)	3.33 (1.98)	-17.0 (23.01)	-26.04 (10.01)	0.013
	Mipomersen 70 mg thrice weekly (n = 36)			**Placebo** (n = 19)				
	Baseline	**PET**	**% Change**	**Baseline**	**PET**	**% Change**	**Mean difference in % change mipomersen vs placebo**	***p*-value**
LDL-C	178 (17.6)	134 (51.3)	-25.0 (27.71)	169 (26.6)	176 (73.2)	4.4 (36.52)	-30.03 (8.90)	0.002
ApoB	135 (16.3)	130 (30.5)	-22.3 (27.20)	128 (23.4)	104 (37.6)	5.9 (27.94)	-32.57 (8.06)	<0.001
Total cholesterol	260 (20.7)	212 (61.1)	-18.1 (22.28)	256 (33.0)	259 (76.1)	3.3 (28.95)	-22.66 (6.91)	0.002

Supplementary Table 4: (Continued)

Cohort 2 – high risk HeFH	Mipomersen 70 mg once weekly (n = 36)			Placebo (n = 19)				
	Baseline	**PET**	**% Change**	**Baseline**	**PET**	**% Change**	**Mean difference in % change mipomersen vs placebo**	***p*-value**
non-HDL-C	210.42 (22.31)	161.38 (57.57)	-23.2 (26.55)	198.71 (28.94)	204.00 (67.82)	5.2 (34.90)	-28.65 (8.66)	0.002
HDL-C	49 (16.0)	51 (18.8)	5.6 (23.59)	58 (23.5)	55 (18.9)	0.5 (18.96)	-0.80 (6.91)	0.909
Triglycerides	151 (101, 220)	109 (91, 189)	-6.0 (41.50)	144 (85, 204)	120 (85, 190)	11.1 (42.41)	-20.58 (11.48)	0.081
VLDL-C	33 (15.6)	27 (15.4)	-5.5 (41.43)	30 (15.8)	28 (12.8)	11.5 (42.03)	-21.50 (11.30)	0.064
Lp(a)	47 (20, 81)	28 (8, 54)	-28.6 (34.83)	14 (4, 43)	10 (3, 70)	10.0 (61.89)	-54.36 (17.24)	0.003
ApoA1	142 (28.8)	140 (39.4)	-1.2 (19.27)	149 (35.8)	144 (31.6)	1.3 (15.85)	-6.97 (5.33)	0.198
LDL/HDL Ratio	4.00 (1.306)	2.98 (1.59)	-27.1 (29.12)	3.41 (1.541	3.32 (1.38)	4.5 (36.53)	-29.98 (11.25)	0.011

Efficacy of mipomersen 200 mg once weekly subcutaneously (SQ) and mipomersen 70 mg thrice weekly SQ compared to placebo during the 60 week blinded treatment period in cohort 1 (severe HeFH). All lipid parameters are measured in mg/dL, except for LDL/HDL ratio. Baseline and primary efficacy endpoint (PET) values are mean (SD), except for triglycerides and Lp(a) which are expressed as median (IQR). Lipid values at PET (week 60) and % change (SD) from baseline to PET are only displayed for patients who completed the full blinded treatment period. Mean difference in % change (SE) between active treatment and placebo group is based on a mixed model for repeated measurements with terms for baseline value, geographic region, gender, statin use, treatment group, study visit, and study visit by treatment group interaction, including patients who at least received one treatment dose but discontinued treatment early. LDL-C = low-density lipoprotein cholesterol, apoB = apolipoprotein B, = non-HDL-C = non–high-density lipoprotein cholesterol, HDL-C = high-density lipoprotein cholesterol, VLDL-C = very low density lipoprotein cholesterol, Lp(a) = lipoprotein (a), apoA1 = apolipoprotein A1, LDL-HDL ratio = low-density lipoprotein – high-density lipoprotein ratio, PET = primary efficacy endpoint (week 60), SD = standard deviation, IQR = interquartile range, SE = standard error.

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Transintestinal cholesterol excretion in humans

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ABSTRACT

Purpose of review: To discuss recent insights into the measurement and cellular basis of transintestinal cholesterol excretion (TICE) in humans, and to explore TICE as a therapeutic target for increasing reverse cholesterol transport.

Recent findings: TICE is the net effect of cholesterol excretion by the enterocyte into the intestinal lumen and is the balance between input and output fluxes through the enterocytes. These fluxes are: cholesterol excretion into the intestinal lumen mainly via ABCG5/8, cholesterol absorption from the intestine by NPC1L1, the uptake of plasma lipoproteins by enterocytes at the basolateral membrane and the excretion of cholesterol in chylomicrons into the lymph. Multiple studies have shown that TICE contributes to fecal neutral sterol excretion in humans. TICE can be targeted with plant sterols, LXR agonists, bile acids, ezetimibe, and proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors.

Summary: TICE contributes significantly to fecal neutral sterol excretion in humans, independently of the biliary pathway. Knowledge about its underlying cellular mechanisms surges through *in vivo* and *in vitro* studies in mice and humans. TICE might be an interesting therapeutic target for increasing cholesterol disposal with the feces. Albeit multiple therapeutic options are available, studies showing clinical benefit are still needed.

INTRODUCTION

Cholesterol is essential for life in humans and most animals. It serves multiple functions in the human body since it is a crucial part of cell membranes and serves as a precursor for bile acids (BAs), steroid hormones, and vitamin D. Cholesterol metabolism is tightly regulated, with a major role for the liver and intestine. Oral intake serves as an essential source of cholesterol, however most cells are capable of cholesterol synthesis itself, explaining the modest effects of altering dietary cholesterol intake on plasma cholesterol levels [1]. Unfortunately, an excess of cholesterol (i.e. hypercholesterolemia) can accumulate in the arterial wall and lead to the development of atherosclerotic cardiovascular diseases (CVD), the cause of ~25% of deaths globally [2]. This process is predominantly caused by uptake of cholesterol rich low-density lipoproteins (LDL) particles by arterial wall macrophages and consequently the formation of foam cells, leading to atherosclerosis [3,4].

Since cholesterol cannot be catabolized to a major extent within the human body except for conversion to bile acids (BAs), the reverse cholesterol transport (RCT) pathway is an essential anti-atherogenic tool by facilitating the removal of excess cholesterol from the body via fecal excretion [5]. RCT is generally defined as the efflux of cholesterol from peripheral tissues (e.g. atherosclerotic plaques), transportation to the liver by high-density lipoproteins (HDL), and subsequent hepatobiliary secretion into the feces. Nowadays, a second RCT pathway is known to contribute to fecal excretion of cholesterol: the direct transintestinal cholesterol excretion (TICE) of plasma-derived cholesterol by enterocytes into the lumen of the small intestine. There is strong evidence of the existence of TICE in mice and rats [6–9] and accumulating evidence of existence of TICE in humans [10–12]. The purpose of this review is to discuss the recent insights into the measurement and molecular mechanism of TICE in humans. Furthermore, TICE's potential as a therapeutic target for the removal of excess cholesterol from the human body will be discussed.

QUANTITATION OF TICE

Four cholesterol fluxes contribute to TICE: cholesterol excretion into the intestinal lumen, cholesterol absorption from the intestine into enterocytes, the uptake of plasma lipoproteins by enterocytes at the basolateral membrane and the excretion of cholesterol, together with triglycerides, packed in chylomicrons into the lymph. Under *in vivo* conditions TICE flux cannot be determined directly and has to be calculated from fecal neutral sterol (FNS) excretion minus the contribution of biliary and dietary input. Subsequently, both fluxes have to be corrected for intestinal absorption. Van der Velde et al. circumvented this procedure by quantifying TICE directly through performing perfusion experiments in selected parts of the small intestine in mice [6]. This procedure successfully assessed TICE activity and demonstrated the strict dependency of TICE on a luminal cholesterol acceptor. The advantage of this method is the possibility to study the mechanism of TICE in a direct way. The disadvantage is that normal intestinal physiology (motility) is disrupted because of the surgery and for this reason cannot be performed in humans. Calculation of TICE under *in vivo* conditions is relatively straightforward in mice. Dietary intake can be measured accurately and biliary cholesterol secretion can be estimated by collecting bile. Using a dual (stable) isotope method cholesterol absorption can be quantified making calculation of TICE possible. A disadvantage of this relatively simple approach is that it does not take into account the cholesterol synthesized in the enterocytes and cholesterol directly excreted or shedded from dead cells. Computational modelling of the isotope decay curves can address this problem and successfully quantify the flux from the blood into the intestinal lumen. Jakulj et al. adapted this approach for the use in humans [11]. The cellular mechanisms, proteins, and transporters driving the fluxes present in TICE, will be discussed below.

TICE AT THE CELLULAR LEVEL

Intestinal cholesterol excretion

The ATP-binding cassette (ABC) G5 and G8 (ABCG5/8) heterodimer acts as efflux transporter at the apical membrane of the enterocyte and hepatocyte where they promote secretion of cholesterol and plant sterols [13]. After synthesis in the endoplasmic reticulum, ABCG5 and ABCG8 dimerize, followed by transport to the apical plasma membrane [13–15]. Dimerization is essential because the individual proteins do not reach the apical membrane. The importance of ABCG5/8 in TICE surfaced in a murine study demonstrating that on the one hand Liver X Receptor (LXR) activation increases both TICE and ABCG5/8 expression, whereas TICE is decreased in mice lacking ABCG5 [16]. Intestinal ABCG5/8, in mice lacking hepatic ABCG5/8, is capable to excrete cholesterol into the intestinal lumen, implicating that intestinal ABCG5/8 contributes to non-biliary cholesterol excretion [17]. Surprisingly, intestinal perfusion studies failed to detect the stimulating activity of ABCG5/G8 seen in *in vivo* experiments. Apparently, a stimulating factor is missing in the perfusate which is present *in vivo* [6].

TICE cannot be entirely attributed to the ABCG5/8 heterodimer since a significant amount of TICE is still active in mice lacking ABCG5 or ABCG8, suggesting that other apical cholesterol transporters are involved in TICE as well [16,18,19]. Le May et al. suggested that the multidrug transporter ABCB1a/b could be involved [10]. This protein is expressed at the apical side of enterocyte and may act as a cholesterol transporter [20]. It was demonstrated in mice lacking ABCB1a/b that TICE was decreased by 26.5%, and resulted in decreased FNS excretion. Using a selective pharmacological ABCB1a/b inhibitor, TICE was significantly reduced in wild type mice and not in the knock-out mice, indicating the involvement of ABCB1a/b in TICE [10]. Thus, an interplay of the apical transporters ABCG5/8 and ABCB1a/b in the enterocyte may be responsible for TICE activity in mice. However, it would be interesting to investigate whether TICE is still active in mice lacking ABCG5/8 and ABCB1a/b to ascertain if these transporters are the only apical transports involved in intestinal cholesterol excretion in TICE or that other mechanisms are also involved and how these findings can be translated to humans.

Intestinal cholesterol absorption

The major pathway by which dietary and biliary cholesterol are taken up by enterocytes is through the Niemann-Pick C1 Like protein 1 (NPC1L1; see Figure 1). NPC1L1 knock-out mice showed a 64-69% reduction in cholesterol absorption from the intestinal lumen [21,22]. The same effect is accomplished by blocking NPC1L1 with ezetimibe, a drug now widely used for LDL cholesterol (LDL-C) lowering in hypercholesteremic patients. Rodents receiving ezetimibe showed a 92 to 96% reduction of cholesterol absorption [23]. The discrepancy in reduction of cholesterol absorption between NPC1L1 knock-out mice and inhibition with ezetimibe has still to be elucidated, but one may speculate that the lack of NPC1L1 transporters in knock-out mice is compensated through another transporter. Treatment of humans with ezetimibe monotherapy results in less intestinal cholesterol absorption and lowers LDL-C plasma levels by 15-20%, despite an 89% increase in endogenous cholesterol synthesis [24]. The cholesterol absorption inhibiting effects of ezetimibe are nowadays well established in mice and humans. Interestingly, studies in mice have shown that ezetimibe not only affects cholesterol absorption in the intestinal lumen, but also stimulates cholesterol excretion from enterocytes through the ABCG5/G8 heterodimer [25]. Nakano et al. investigated this process in more detail and proposed a model in which cholesterol content in the Brush Border Membrane (BBM) of the enterocytes is the driving force for NPC1L1 dependent cholesterol uptake [26]. An increased cholesterol efflux from the BBM to the intestinal lumen was seen when NPC1L1 was inhibited with ezetimibe in mice. Moreover, it was shown that this efflux was higher in wildtype mice treated with ezetimibe than in ABCG5/8 knockout mice, suggesting a promoting role for ABCG5/8 in ezetimibe induced cholesterol excretion [26]. Albeit, the exact mechanism by which NPC1L1 transports cholesterol to the intracellular space is yet not clear, NPC1L1-endocytosis mediated by Numb has been proposed as the underlying mechanism. The absence of Numb, a clathrin-adaptor protein, *in vitro* and in NPC1L1 knock-out mice impaired cholesterol absorption [27]. However, later reports challenged the concept that cholesterol absorption is achieved by endocytosis [28]. Future research should address those contradicting findings.

Basolateral cholesterol uptake by enterocytes

Cholesterol excreted via TICE can originate from different sources; a) intestinal uptake of dietary and biliary cholesterol b) de novo synthesis in enterocytes c) uptake from the blood. Cholesterol balance studies in humans and mice indicate that when TICE is stimulated by pharmacological means most of the cholesterol is derived from the blood [9,15]. The pathway via which cholesterol enters the enterocytes is a matter of controversy. Since TICE may play an important role in the RCT pathway, uptake from HDL seemed an attractive option. Vrins et al. investigated this mechanism by following the fate of radiolabeled cholesterol incorporated in HDL in *Abca1/Sr-b1* double knock-out mice. These mice lack endogenous HDL and cannot clear HDL via the liver. No transport of cholesterol into the intestinal lumen could be discerned in these experiments suggesting that HDL could not be the carrier for TICE cholesterol [29]. In contrast, Le May et al. carried out experiments with intestinal explants embedded in Ussing chambers and could demonstrate cholesterol transport across the intestinal tissue from HDL as well as LDL [10]. The reason for the discrepancy between the data of Vrins et al. and Le May et al. is not clear. Le May et al. reported conflicting data regarding the role of the LDL receptor in mediating TICE flux. They could not find decreased TICE in LDL receptor knock-out mice, a result confirmed by both Temel and Brown [30] and our group (unpublished observations). Despite this seemingly lack of evidence for a role of the LDL receptor mediated pathway, Le May et al. reported that absence of the LDL-receptor modulating protein PSCK9 increased TICE and conversely intravenous injection of this protein in mice inhibited TICE. Taken together it cannot be excluded that LDL receptor knock-out mice compensate for the lack of this receptor and upregulate another receptor, or proprotein convertase subtilisin/kexin type 9 (PCSK9) blocks an alternative route of cholesterol import. When indeed both LDL and HDL can be excluded as donors of TICE cholesterol, VLDL seems an attractive alternative candidate. Marshall et al. tested this hypothesis by treating mice with antisense oligonucleotides (ASO) targeting microsomal transfer protein (MTP). This treatment strongly inhibited VLDL secretion and FNS excretion, particularly in liver specific transgenic NPC1L1 overexpressing mice. Since these mice do not show biliary cholesterol secretion, the effect on the fecal neutral sterols must have been due to a decrease in TICE. The authors conclude that, at least, VLDL secretion is required to feed this pathway with cholesterol [31].

TICE IN HUMANS

In 1959, two studies observed that in patients with complete biliary obstruction, the intestinal mucosa still excreted 250 to 400 mg cholesterol per day indicating that an alternative route of cholesterol excretion exists in humans [32,33]. Under more physiological conditions Simmonds et al., using intestinal perfusion of the jejunum in humans, estimated that ~44% of FNS originated from non-biliary pathways [34]. In 2013, new evidence for activity of TICE in humans was provided by Le May et al. using isolated intestinal explants in Ussing chambers as described above [10]. The existence of TICE in humans was further established recently with the publication of Jakulj et al. [11]. Under basal conditions, TICE contributed for 35% of FNS excretion in fifteen men with mild hypercholesterolemia, when using stable isotope based technology as described in this review. Subsequently, ten subjects were treated with ezetimibe 10 mg/day, resulting in a significant fourfold increase in TICE (from 252 +/- 46 mg/day to 1024 +/- 114 mg/day). Those results underline the TICE mechanisms in mice to be present in humans as well. Dugardin et al. studied transcellular cholesterol transport in the human Caco-2/TC7 cell line to demonstrate that differentiated Caco-2T7 enterocyte cells take up cholesterol at the basolateral membrane and then excrete the cholesterol through the apical membrane [12]. In line with earlier findings of Van der Velde et al. [6], they found that phosphatidylcholine/taurocholate micelles modify the intracellular distribution of cholesterol and facilitate its transport from sub-basolateral to subapical areas of the enterocyte. Furthermore, it was demonstrated that less TICE occurred when the cellular microtubulae were disrupted with colchicine and nocodazole, suggesting that the microtubule cytoskeleton is important for intracellular cholesterol transport.

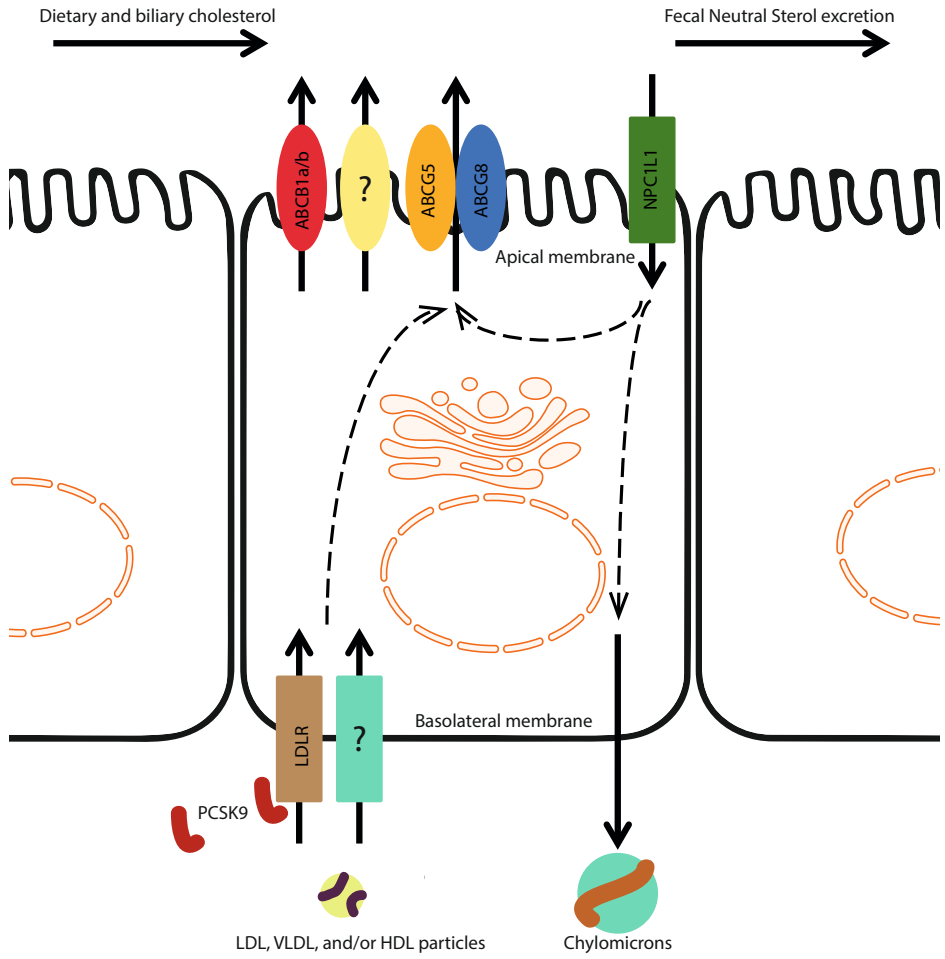

Figure 1: Cellular transporters and cholesterol fluxes involved in TICE
At the apical side of the enterocyte, dietary and biliary cholesterol are absorbed from the intestinal lumen by NPC1L1 whereas intracellular cholesterol is excreted by ABCG5/8, ABCB1a/b and possibly other unidentified mechanisms. Moreover, intracellular cholesterol is excreted in chylomicrons into the lymph at the basolateral side. Plasma cholesterol is derived from lipoprotein particles, possibly through endocytoses with the LDL receptor (LDLR) or other mechanisms. *Abbreviations*: ABCB1a/b, ATP-binding cassette B1 a and b; ABCG5/8, ATP-binding cassette G5 and G8; NPC1L1, Niemann-Pick C1 like protein 1; LDLR, low-density lipoprotein receptor; (V)LDL, (very) low-density lipoprotein; HDL, high-density lipoprotein; PCSK9, proprotein convertase subtilisin/kexin type 9.

TICE AS A THERAPEUTIC TARGET

As highlighted in this review, the TICE pathway is a major non-biliary contributor to FNS excretion and RCT in mice and humans. Compared to the stimulation of the hepatobiliary cholesterol excretion, which may promote gallstone formation [35], increasing intestinal cholesterol excretion by the TICE pathway is an attractive approach to remove the excess of cholesterol and prevent atherosclerosis. See Figure 2 for an overview of the therapeutic targets discussed below.

Recent research has shown that manipulation of the cycling of cholesterol between NPC1L1 and ABCG5/8 seems the most attractive option to stimulate TICE. Inhibition of NPC1L1 by ezetimibe is straightforward and stimulates TICE, whereas simultaneous activation of ABCG5/8 possible enhances TICE even more. LXR regulates the expression of hepatic and intestinal ABGG5/8 and could therefore be an interesting pharmacological target for TICE [16,36–38]. Studies have shown that activation of LXR, through cholesterol feeding, dietary plant sterols (PS), or agonist administration increases hepatic and intestinal ABCG5/8 expression [18,36,38] as well as biliary cholesterol concentrations in mice [36], whereas these effects were absent in mice lacking ABCG5/8 [37]. Moreover, murine studies demonstrated that TICE is directly stimulated with LXR agonists and that FNS excretion upon LXR activation is independent of hepatobiliary sterol secretion in mice [7,16]. To this end, administering a LXR agonist to increase intestinal ABCG5/8 expression in humans could be an interesting approach for targeting TICE in humans. However, an intestinal-specific agonist for LXR-induced TICE should be used to avoid hepatic side effects, such as increased hepatic lipogenesis, steatosis and hypertriglyceridemia [39,40]. Several first phase clinical trials with LXR agonists were performed with beneficial effects regarding RCT. However, one study had to be terminated early because of unexpected adverse neurological events [41], while other trials do not disclose the reasons for early termination and were not published [42].

Adding plant sterols (PS) to the diet has similar effects to LXR agonists. PS intake lowers total cholesterol and LDL-C in normolipidemic and hypercholesterolemic subjects without affecting HDL-cholesterol [43], via competition with cholesterol for incorporation in mixed micelles. This leads to a decreased fractional cholesterol

absorption and increased fecal cholesterol excretion [44]. PS may be able to activate LXR followed by the upregulation of ABCB1 and ABCG5 [45–47]. Brufau et al. investigated whether PS feeding stimulates FNS via TICE on a ABCG5/8 dependent manner. They found that PS feeding increased FNS in mice mainly by an increase in TICE and that ABCG5/8 plays a non-exclusive role in the preservation of TICE [18].

Recently, de Boer et al. reported that TICE is also regulated by intestinal FXR via induction of its target gene FGF15 in mice and FGF19 in rats and humans [19] . Bile acids are ligands for FXR and binding results in the release of FGF19 from the small intestine into the circulation, which contributes to regulation of bile acid synthesis and postprandial metabolism [48,49]. Stimulation of the FXR-FGF15 pathway with an FXR agonist and ezetimibe in mice led to an estimated excretion of 60% of their total body pool of cholesterol content each day [19]. Moreover, administration of a FXR agonist or FGF19 induced the muricholate:cholate ratio in bile, creating a more hydrophilic bile acid pool, resulting in secretion of cholesterol into the intestinal lumen via ABCG5/8. Notably, the increase in TICE induced by the FXR agonist was independent of changes in cholesterol absorption via NPC1L1. Thus, a therapy that combines the effect of FGF19 induction on ABCG5/8 by bile acids and simultaneous inhibition of intestinal cholesterol absorption by ezetimibe would supposedly lead to an increase in TICE. Wang et al. found that ursodiol increases hepatic ABCG5/8, biliary secretion and FNS elimination and acts additively to ezetimibe to increase FNS excretion in mice [17]. Remarkably, the combination of ursodiol and ezetimibe also increased FNS excretion in ABCG5/8-deficient mice, leading to the conclusion that this combination increases ABCG5/8 expression and FNS excretion, but the latter effect was ABCG5/8 independent.

Another therapeutic modulator of TICE might be PCSK9, an interesting target in the light that PCSK9 inhibition is more and more prescribed as a LDL-C lowering agent for the prevention of CVD. The PCSK9 protein binds the LDL receptor and enhances its intracellular lysosomal degradation, resulting in an impaired LDL-C uptake in the liver. Although the role of the LDL receptor in TICE is still unclear, PCSK9 has been shown to modulate TICE [10,12]. When recombinant PCSK9 is added to Caco-2/TC7 cells at the basolateral compartment, transcellular cholesterol transport is decreased by 28% [12]. PCSK9 knock-out mice showed increased TICE, which could

be blocked by an injection of recombinant PCSK9 [10]. Data in humans is still lacking, but it can be hypothesized that PCSK9 inhibition with the now available antibodies might increase FNS in addition to its plasma LDL-C lowering effect via the liver.

CONCLUSION

TICE is the net effect of cholesterol excretion by the intestine exclusively, and serves as a second, non-biliary, RCT pathway in humans. The rate of TICE depends on the balance between four fluxes; intestinal cholesterol excretion through ABCG5/8 and ABCB1a/b, cholesterol absorption through NPC1L1, cholesterol uptake and cholesterol excretion at the basolateral membrane of enterocytes. Targeting TICE to enhance RCT, might be an attractive target to attenuate CVD risk and might be accomplished with plant sterols, LXR agonists, bile acids, ezetimibe, and PCSK9 inhibitors. Exciting times are ahead of us since our knowledge of TICE and its therapeutic role in CVD keeps expanding.

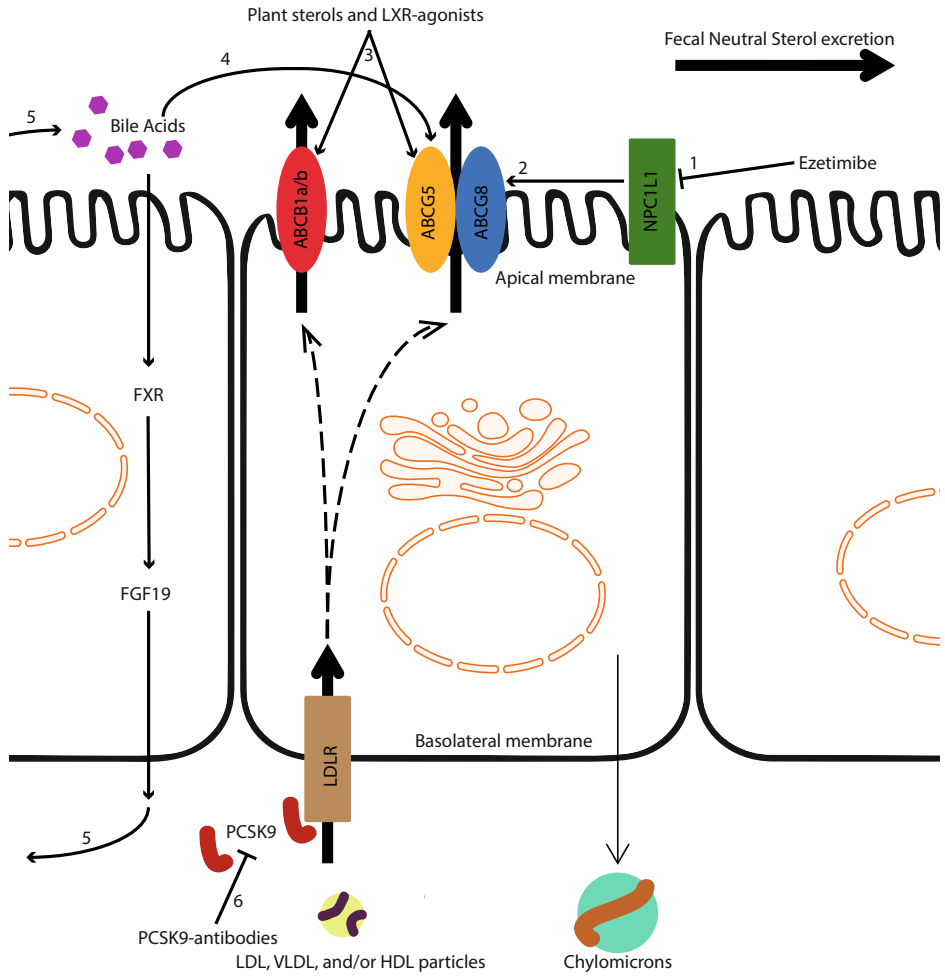

Figure 2: Therapeutic options for targeting TICE
Ezetimibe inhibits cholesterol absorption by blockage of NPC1L1 (arrow 1), subsequently leading to an increased cholesterol excretion through ABCG5/8 via the brush border membrane(arrow 2). Plant sterols and LXR-agonists stimulate cholesterol excretion by ABCG5/8 and ABCB1a/b (arrow 3). Furthermore, bile acids have a direct stimulating effect on ABCG5/8 (arrow 4), function as cholesterol acceptors, and activate FXR in the enterocyte, leading to the transcription of FGF19 (arrow 5). FGF19 downregulates hepatic bile acid synthesis and creates a more hydrophilic bile acid pool, which promotes ABCG5/8 cholesterol excretion. PCSK9 antibodies inhibit PCSK9-mediated degradation of the LDL receptor at the basolateral membrane, resulting in increased cholesterol uptake from the plasma (arrow 6). *Abbreviations:* see legend of Figure 1; FXR, farnesoid X receptor; FGF19, fibroblasts growth factor 19; LXR, liver X receptor.

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Summary, discussion, and future perspectives

SUMMARY AND DISCUSSION

The studies described in this thesis are all focused on the molecular analysis of patients with clinical characteristics of Familial Hypercholesterolemia (FH) and novel therapeutic targets to lower LDL-C levels in these patients. In **chapter 1**, a general introduction about FH and these new lipid lowering therapies is provided. Moreover, the importance of early detection and early treatment of FH to prevent accelerated atherosclerotic plaque development in these patients is underlined.

Part I A: Diagnosing familial hypercholesterolemia

In **part I A** I describe the current clinical practice for molecularly diagnosing FH by focusing on the three 'classical' FH genes: *LDLR*, *APOB*, *PCSK9*. In **chapter 2** an overview of the current diagnostic yield of molecular analysis in The Netherlands is provided. We show that despite extensive next-generation sequencing of clinical FH patients, only 14.9% of patients is found to carry an FH causing variant in one of the three 'classical' FH genes. Unsurprisingly, the yield of sequencing increases with increasing severity of the FH phenotype to over 50% in patients with LDL-C levels above 8 mmol/L. However, this is still markedly lower compared to the 92% of patients with LDL-C >8 mmol/L that was found to carry a variant in these genes as described by Wang and colleagues.[1] We speculate that this low yield is possibly due to patient selection applied by referring physicians. Interestingly, the yield increases markedly when the validated clinical FH criteria (i.e. Dutch Lipid Clinics Network [DLCN] criteria) are applied. For example, patients with a probable or definite FH score according to the DLCN criteria have >50% chance of carrying an FH variant. This suggests that implementation of stricter clinical criteria when considering sequencing may be warranted in order to decrease the number of sequenced individuals per diagnosed FH patient. However, the low diagnostic yield may also be caused by other FH mimicking or novel genetic causes of FH. For example, high levels of lipoprotein (a) (Lp(a)) have been shown to interfere with laboratory calculated LDL-C levels, are genetically determined, and thus give rise to a suspicion of FH[2], but are not routinely measured in Dutch clinical practice.

In the next two chapters, **chapter 3 and 4**, new pathogenic variants causing FH in largely neglected regions, the intronic regions of *LDLR,* are investigated. Our

journey into the world of intronic variants started with the in-depth sequencing of members of a family with clinical FH following an autosomal dominant pattern of inheritance (**chapter 3**). Although we applied whole genome sequencing in this family to look for new genes involved in FH, we were surprised to find a deep intronic variant causative of FH in *LDLR*. In **chapter 3**, we therefore, conclude that for future sequencing strategies coverage of intronic regions of this gene should be considered. This latter concept was put into practice in the study described in **chapter 4**, where we utilize routinely obtained, but discarded sequences of *LDLR* introns, in order to discover other FH causing intronic variants among 909 subjects with clinical FH in whom no exonic variant was identified. The application of multiple filtering steps, which included the selection of intronic variants in these clinical FH patients but not present in molecularly proven FH patients, as well as *in silico* assessment, greatly reduces the number of variants of which the effect have to be assessed in subsequent cDNA studies. In the end, one novel intronic variant is identified, which is the deepest intronic FH causing variant currently known and causes the first ever reported pseudo-exon inclusion in *LDLR*. However, this study also shows that intronic variants in *LDLR* causing FH are rare and/or that newer and broader sequencing methods are needed to discover more pathogenic variants in this gene. In our study, only 36% of the intronic regions of *LDLR* is on average covered. To overcome this, whole *LDLR* gene sequencing in combination with whole gene mRNA analysis could be utilized to simultaneously identify variants in currently uncovered *LDLR* regions and assess their effect on *LDLR* mRNA splicing.

Part I B: Novel diagnostic targets

In **part I B** we focus on the identification of novel genetic causes of familial hypercholesterolemia. In **chapter 5**, the genes *ABCG5* and *ABCG8* (ATP-binding cassettes G5 and G8) are assessed for rare variants in patients with (clinical) FH. The proteins encoded by these genes form a heterodimer and are involved in transmembrane cholesterol transport into bile. Common and rare variants in these genes are associated with LDL-C levels and cardiovascular disease (CVD).[3,4] In our cohort, consisting of 3031 clinical FH patients, 1.48% and 0.96% are found to be carrier of loss-of-function variants in *ABCG5* and *ABCG8*, respectively. Patients carrying such genetic variants have significant lower LDL-C levels compared to "classical" FH patients with a *LDLR* pathogenic variant (6.2±1.7 vs 7.2±1.7 mmol/L).

Next, a segregation analysis in three families of one *ABCG5* and two *ABCG8* variants does not show co-segregation of the variant with the FH phenotype. It is, therefore, concluded that *ABCG5* and *ABCG8* variants might play a role in the hypercholesterolemic phenotype in FH patients, especially in those with moderately elevated LDL-C, but are unlikely to cause autosomal dominant FH. We speculate that these variants predispose to hypercholesterolemia under certain conditions, for example in the presence of dietary disturbances. Further studies are needed to unravel the relationship between FH, *ABCG5*, *ABCG8,* and lifestyle factors.

In **chapter 6**, the role of *STAP1* (signal transducing adaptor family member 1), another gene earlier described to cause FH, is investigated. Although, the STAP1 protein has no apparent role in cholesterol homeostasis and is mainly expressed in immune cells, it was associated with FH in two families from the Netherlands.[5] To further understand this association, whole-body *Stap1* knockout mice were generated, which did not show any changes in lipid levels compared with control mice. Additional immunological analyses were performed, including a bone marrow transplantation of *Stap1*$^{-/-}$ to *Ldlr*$^{-/-}$ mice and co-culturing of peripheral blood mononuclear cells of human *STAP1* variant carriers with HepG2 liver cells. None of these studies reveal an effect of the immune system on cholesterol homeostasis caused by STAP1 depletion. Lastly, *STAP1* variant carriers in four additional hypercholesterolemic families do not have higher LDL-C levels compared to their family controls. It is therefore concluded that *STAP1* is very likely not an FH associated gene. This conclusion is also supported by other recent studies confirming the lack of cosegregation of *STAP1* variants with the FH phenotype in hypercholesterolemic families.[6,7]

The previous chapters focused on monogenic causes of FH. However, a much less researched field is epigenetic regulation of genes in hypercholesterolemic patients. In **chapter 7**, it is hypothesized that differentially methylated DNA might be associated with FH and is investigated in clinical FH patients. To control for the effects of hypercholesterolemia on DNA methylation itself, DNA methylation of FH mutation-negative patients is compared to FH patients with a deleterious defect in *LDLR*. This study shows that there is no apparent difference in DNA methylation in FH genes and other selected genes related to lipid metabolism between the

two groups. However, in the same study, a machine learning approach reveals a difference on a genome-wide scale, with important model features for *PRDM16* and *GSTT1*. Both genes were previously associated with LDL-C levels.[8,9] Although differential methylation of specific genes is likely not a cause of FH per se, we speculate that cumulative small-effect DNA methylation may cause elevated LDL-C in clinical FH patients. This phenomenon might resemble the polygenic causes of hypercholesterolemia in which elevated LDL-C levels are not explained by individual genetic variants, but rather by the sum of effects of common genetic variants.[10] Obviously, further research into DNA methylation is needed to further elucidate its relationship to hypercholesterolemia and lipid metabolism in general.

Part II: The role of ANGPTL3 in dyslipidemia

In **part II** and **part III** the studies in which we nvestigated novel therapeutic targets for lowering LDL-C are described. **Part II** solely focusses on ANGPTL3, a natural inhibitor of lipoprotein lipase (LPL) and endothelial lipase (EL) which both accelerate lipoprotein lipolysis and thus accelerate clearance of lipoproteins from the circulation.[11] A pilot study with evinacumab, a monoclonal antibody against ANGPTL3, in nine homozygous FH (hoFH) patients showed a LDL-C reducing efficacy of 50% after a single infusion.[12] In **chapter 8**, we review the data to support the notion that targeting ANGPTL3 not only reduces LDL-C levels but also has the promise to reduce CVD risk. Given the effect of ANGPTL3 inhibition on apolipoprotein B levels, the main structural protein of lipoproteins, and the overwhelming evidence of a beneficial effect of low levels of ANGPTL3 both in mice models and genetic studies we anticipate the ANGPTL3 lowering agents to result in CVD risk reduction. Next, **chapter 9** describes a randomized placebo-controlled trial in which evinacumab is tested in 65 hoFH patients and is found to effectively reduce LDL-C by 49% in these hard to treat patient population. HoFH patients usually require multiple LDL-C lowering therapies, with limited efficacy especially in patients with no residual LDLR activity left, to reach guideline recommended targets.[13] Since evinacumab lowers LDL-C to a similar extent in patients with and without residual LDLR activity left, it is a promising drug for this hard to treat patient population. Based on these data the food and drug administration (FDA) has recently approved the use of evinacumab in hoFH patients in the USA.[14]

In a subsequent case-series, described in **chapter 10**, we show that aggressive treatment with evinacumab results in nearly complete atherosclerotic plaque regression in two adolescent hoFH patients. Although, this finding itself is extremely relevant for abolishing CVD risk in these two patients, it is also valuable evidence for the notion that young atherosclerotic plaques possess the ability to regress in contrast to plaques in older patient.[15] This is of particular interest since these hoFH patients had a cumulative LDL-C exposure equal to an average 40 year old male[16] and only a 1% plaque regression is observed after intensive lipid lowering in older non-FH patients with advanced atherosclerosis.[17] This finding further substantiates that early treatment of hypercholesterolemia might be the most effective prevention strategy for atherosclerosis and could be guided by (frequent) plaque imaging.

Interestingly, the exact mechanism by which ANGPTL3 inhibition leads to lower LDL-C plasma levels remains partly unclear. Since evinacumab is an ANGPTL3 inhibitor, triglyceride hydrolysis in the circulation by LPL increases. This is also seen in homozygous FH patients treated with evinacumab in **chapter 11**. In this small study, four patients underwent an apoB kinetic study before and after evinacumab infusion. After a single infusion of evinacumab a dramatic increase in fractional catabolic rates of apoB in IDL and LDL subfractions was observed compared to baseline, indicating that these were cleared faster from the circulation. This is possibly due to faster particle uptake by the liver and this process is believed to be LDLR independent since hoFH with null/null variants experience the same magnitude of LDL-C lowering effect (**chapter 9**). A recent study showed that evinacumab is only effective for this purpose in the presence of EL in mice, suggesting a role for EL in hepatic uptake of remnant and low-density lipoproteins.[18] More research is needed to investigate EL's role in humans.

In addition to the incomplete understanding of the LDL-C lowering effects of ANGPTL3 inhibition, the regulation of ANGPTL3 production and secretion is poorly understood. To this end we conducted a study described in **chapter 12** where we explored the effect of statins in hepatocellular cell lines on ANGPTL3 production, as well as the association between statin use and plasma ANGPTL3 levels in hypercholesterolemic patients. Through a series of experiments we show that

plasma ANGPTL3 levels are 15% lower in FH patients on statin therapy compared to FH patients who are not using a statin. This effect is likely due to reduced ANGPTL3 production due to decreased oxysterol liver receptor X (LXR) activation. The relation between statin use and ANGPTL3 is of particular interest, since patients that are likely to be treated with ANGPTL3 inhibition (e.g. patients with FH) presumably also use statins. Of note is that we do not observe a difference in efficacy of evinacumab between hoFH patients who are on- and off-statin therapy in **chapter 9**, although the numbers of patients are small (61 vs 4 patients on and off statins, respectively).

Part III: Novel therapeutic options for hypercholesterolemia

In **part III** we zoom in on other potential therapeutic targets for FH patients; apoB production and transintestinal cholesterol excretion (TICE). The former has been considered a target for LDL-C lowering given its role as the main structural protein in atherogenic lipoproteins, including LDL. As described in **chapter 13**, mipomersen, an antisense oligonucleotide against *APOB* mRNA, lowers plasma levels of LDL-C with ±20%, depending on the exact dosing regimen (200mg once weekly vs 70mg thrice weekly) compared to placebo in heterozygous FH patients. Although effective, inherent to intrahepatic apoB production inhibition, is the accumulation of triglycerides in the liver. It was previously observed that mipomersen administration does result in increased liver fat content in 25% of treated FH patients[19]. In our study its use is associated with increased ALT levels at least once of ≥3 times the upper limit of normal in >20% of included patients. Unsurprisingly, the food and drug administration in the USA approved mipomersen only for homozygous FH patients. Mipomersen was recently removed from the market by its producer.

TICE, on the other hand, remains a puzzling phenomenon. The underlying mechanisms of TICE and its potential as a pharmacological target is extensively reviewed in **chapter 13**. It was recently shown that TICE is active in humans and accounts for 35% of cholesterol excretion in the feces.[20] Moreover, it was shown that TICE can be increased by the use of ezetimibe[20] and even further accelerated when a hydrophilic bile acid pool is present in the intestine of mice.[21] Whether these observations in mice are also true in humans, and whether TICE is an attractive target for LDL-C lowering needs to be confirmed with robust clinical trials in hypercholesterolemic individuals.

FUTURE PERSPECTIVES

The research projects described in this thesis only entails a small part of currently ongoing research into FH and improvements in treatment of dyslipidemias. It is therefore worthwhile to look forward and place the small steps made with the studies described here in a bigger context: the future of the fight against cardiovascular disease burden.

Improving FH diagnostics

As described in **chapter 2**, the current yield of genetic testing for FH in The Netherlands is around 15%, a number that is considered to be low compared to other countries.[1] This finding likely reflects the easy access of patients and physicians to genetic testing by not requiring extensive phenotyping or cumbersome referrals to specialized lipid clinics. Although testing accessibility is a great good and no other country has proportionally as many diagnosed FH patients as The Netherlands[22], some improvements can be made to, for instance, lower the costs associated with extensive genetic testing. For example, obtaining Lp(a) levels and correcting LDL-C for its cholesterol content in Lp(a), would lower an patient's LDL-C value that is used in clinical FH criteria such as the DLCN criteria.[2] Moreover, obtaining multiple LDL-C measurements might exclude patients with incidental or secondary hypercholesterolemia. In one study, 70% of patients did not fulfill the MedPed criteria for FH anymore after a second blood draw, albeit that some of the subjects might have started a lipid lowering treatment in the meantime.[23]

Next, the molecular analyses methods should be improved in order to diagnose patients with FH. Current strategies focus on sequencing the coding regions of known FH genes with great precision (i.e., sequencing depth of >600x). As shown in this thesis, it might be feasible to also assess variants in intronic regions of *LDLR* and if we take our findings one step further likely also other FH genes. Although we were only able to assess variants that were accidently captured with the used exome centered next-generation sequencing platform, it would be relatively easy to expand the coverage to include intronic regions. Intronic variants of interest should than be assessed for their pathogenicity by obtaining mRNA from patients carrying such an intronic variant in order to study their effects on *LDLR* splicing.

An algorithm to identify variants of interest (for example with *in silico* analyses as described in **chapter 4**) is needed to prevent unnecessary analyses of variants that are unlikely to cause FH and could be relatively easy implemented.

Expanding genetic analyses for precision medicine

As described above, the current sequencing strategies focus on identifying single rare variants with a large effect in patients with a severe phenotype (i.e., FH). However, genetic testing could be expanded to also include a number of small effect variants (single nucleotide polymorphisms [SNPs]) to obtain data on cumulative genetic exposure originating from these variants. These so-called genetic risk scores could explain the occurrence of less severe and less clear phenotypes of hypercholesterolemia, as well as the complex genetic architecture of coronary heart disease in general. Moreover genetic risk scores can also be used for risk stratification in these patients.[10] Measuring SNPs is relatively cheap (approximately 30 Euro) compared to diagnostic next-generation sequencing (approximately 2000 Euro) and many studies have investigated the additional value of genetic risk scores for hypercholesterolemia and risk for ASCVD. For example, it has been shown in one landmark study that individuals of the general population, who have a genetic risk score in the top 8% for coronary heart disease consisting of >6.6 million SNPs had a >3 fold increased risk for coronary heart disease (CHD)[24], which is comparable to the increased risk observed in FH in some studies. However, the prevalence of FH is only 0.3% in the general population, and as such polygenic risk might have a larger impact on total cardiovascular disease burden[25] Moreover, not every FH patient will suffer from coronary heart disease and it appears that CHD polygenic risk scores have indeed additional risk stratification value for FH patients: FH patients with a low or high polygenic risk score for CHD (<20th and >80th percentile, respectively) had odds ratios of 1.31 (95%-CI 0.39-4.20) and 12.61 (95%-CI: 2.96-53.62), respectively for developing CHD compared to non-FH patients with an intermediated polygenic risk score.[26] This means that even patients with a profound genetic alteration (i.e., an FH causing variant) are subject to the effect of additional genetic risk factors for ASCVD. While this information is currently not used in clinics for risk stratification within FH and non-FH patients, it is relatively easy to include polygenic risk scores (consisting of a limited number of SNPs) in

FH sequencing arrays or to incorporate existing SNP arrays in clinical practice to assess this new ASCVD risk factor.

The advantage of genetic analyses over other "-omics" modalities, such as proteomics or imaging modalities, is that it reflects a risk factor that is present from birth and can thus be used for very early identification of high-risk patients, before they even develop an measurable risk factor for ASCVD. However, further clinical validation of its added value is needed before mass genetic testing can be implemented in clinical practice.

Towards personalized medicine in hypercholesterolemia

Currently, every FH patient is treated to obtain a guideline recommended LDL-C level (<1.4 mmol/L)[27] and in many patients this goal will not be achieved, given the severity of the disease combined with the overall effect of the limited therapeutic agents available (i.e. statins, ezetimibe, monoclonal antibodies directed against PCSK9). Further characterization of FH patients by means of the aforementioned genetic approaches, but also by, for example, imaging could lead to the identification of FH patients that are at a very high risk of developing CHD and patients that have a moderate or low risk. Reflecting these risk categories, different LDL-C goals can be set and thus, often expensive, novel therapies (e.g., ANGPTL3 inhibition; estimated yearly costs 450.000 US dollars in homozygous FH patients[14]) can be allocated to those at highest need for aggressive LDL-C lowering. The treatment of FH patients can be further personalized by evaluating the effect of treatment on actual atherosclerotic plaque progression and regression. As we showed in two hoFH patients (**chapter 10**), early aggressive treatment in young patients can lead to plaque reversal and it would be interesting to investigate if further LDL-C lowering treatments can be adjusted to mitigate the formation of plaque burden during the following years (image-tailored treatment algorithms).

Utilizing the DNA as a therapeutic target

We are currently at the start of an era where DNA is not only the source of the health problems for FH patients, but might also harbor its cure. For example, inclisiran, a silencing RNA therapeutic against PCSK9, was recently approved by the FDA and European Medicines Agency (EMA) for clinical use. This drug showed a

48% LDL-C lowering efficacy in HeFH patients, only has to be administered twice a year, and blocks the translation of the PCSK9 protein from mRNA in the liver.[28] But not only in the world of CVD disease are we capable of utilizing the building blocks of life (i.e. DNA and RNA). In the currently ongoing COVID-19 pandemic, millions of people are being vaccinated with the first in its class RNA vaccines.[29,30]

An interesting next step, would be to correct FH at the DNA level and effectively abolish the need for any LDL-C lowering therapy. Although this is in theory possible, for example by CRISPR-cas9 correction of the FH causing mutation or the introduction of correct *LDLR* RNA into the liver cell for translation, this approach requires much more research and ethical discussion before it is put into practice. It might not be feasible due to the amount of different therapies that need to be developed to target all >1600 known FH causing mutations. In contrast to FH causing variants, there are genetic variants known that effectively lower LDL-C. For example, LOF mutations in *PCSK9* and *ANGTPL3* are associated with lower LDL-C levels and a lower risk for CVD.[31,32] This opens the door to a, maybe counter-intuitive, approach for CVD protection in FH patients: currently it is being investigated whether artificially introducing a LDL-C lowering mutation on top of the known LDL-C increasing variant is safe and effective.[33]

CONCLUSIONS

In conclusion, DNA does not only harbor the culprit of severe diseases such as FH, but may also provide inroads to its solution, which, in the end, may result in the prevention of ASCVD. Especially, if the current strict distinction between the search for rare LOF variants and common variants fades away, the DNA's full potential can be used for refined risk prediction (e.g., genetic risk scores for ASCVD[24]) and novel drug target discovery as was shown for PCSK9 and ANGPTL3 inhibition.[31,34] Moreover, it is likely that we will enter an era in which treatments blocking the RNA or targeting the DNA will become part of our armamentarium in the battle against CVD.

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APPENDICES

NEDERLANDSE SAMENVATTING

De studies beschreven in dit proefschrift zijn allemaal gericht op de moleculaire analyse van patiënten met klinische kenmerken van Familiaire Hypercholesterolemie (FH) en nieuwe therapeutische doelen om het LDL-C bij deze patiënten te verlagen. In **hoofdstuk 1** wordt een algemene inleiding gegeven over FH en deze nieuwe lipidenverlagende therapieën. Bovendien wordt het belang onderstreept van vroege detectie en vroege behandeling van FH om het versneld ontstaan van atherosclerotische plaques bij deze patiënten te voorkomen.

Deel I A: Diagnose van familiaire hypercholesterolemie

In **deel I A** beschrijf ik de huidige klinische praktijk voor moleculaire diagnose van FH door te focussen op de drie 'klassieke' FH genen: *LDLR*, *APOB*, *PCSK9*. In **hoofdstuk 2** wordt een overzicht gegeven van de huidige diagnostische opbrengst van moleculaire analyse in Nederland. We laten zien dat ondanks uitgebreide next-generation sequencing van klinische FH patiënten, slechts 14.9% van de patiënten drager blijkt te zijn van een FH veroorzakende variant in één van de drie 'klassieke' FH genen. Het zal geen verbazing wekken dat het rendement van sequencing toeneemt met toenemende ernst van het FH-fenotype tot meer dan 50% bij patiënten met LDL-C waarden boven 8 mmol/L. Dit is echter nog steeds duidelijk lager vergeleken met de 92% van de patiënten met LDL-C >8 mmol/L bij wie een variant in deze genen werd gevonden, zoals beschreven door Wang en collega's.[1] Wij speculeren dat deze lage opbrengst mogelijk te wijten is aan patiënten selectie door verwijzende artsen. Interessant is dat de opbrengst duidelijk toeneemt wanneer gevalideerde klinische FH criteria (in dit geval de Dutch Lipid Clinics Network [DLCN] criteria) worden toegepast. Patiënten met een waarschijnlijke of zekere FH score volgens de DLCN criteria hebben bijvoorbeeld >50% kans om een FH variant te dragen. Dit suggereert dat de toepassing van strengere klinische criteria bij het overwegen van sequencing gerechtvaardigd kan zijn om het aantal individuen dat geanalyseerd wordt per gediagnosticeerde FH-patiënt, te verminderen. De lage diagnostische opbrengst kan echter ook worden veroorzaakt door andere FH-mutaties of nieuwe genetische oorzaken van FH. Zo is bijvoorbeeld aangetoond dat hoge niveaus van lipoproteïne (a) (Lp(a)) interfereren met in het laboratorium berekende LDL-C waarden, genetisch bepaald zijn, en dus aanleiding geven tot een

verdenking van FH[2], maar in de Nederlandse klinische praktijk niet routinematig worden gemeten.

In de volgende twee hoofdstukken, **hoofdstuk 3 en 4**, worden nieuwe pathogene varianten die FH veroorzaken in grotendeels genegeerde regio's, de intronische regio's van *LDLR*, onderzocht. Onze reis in de wereld van intronische varianten begon met de diepgaande sequencing van leden van een familie met een autosomaal dominant overervingspatroon van klinisch FH (**hoofdstuk 3**). Hoewel we in deze familie whole genome sequencing toepasten om te zoeken naar nieuwe genen die betrokken zijn bij FH, waren we verrast een diepe intronische variant te vinden die FH veroorzaakt in: *LDLR*. In **hoofdstuk 3** concludeerden we daarom dat voor toekomstige sequencing strategieën dekking van intronische regio's van dit gen moet worden overwogen. Dit laatste concept is in praktijk gebracht in de studie beschreven in **hoofdstuk 4**, waar we routinematig verkregen, maar genegeerde sequenties van *LDLR* introns gebruikten om andere FH veroorzakende intronische varianten te ontdekken onder 909 personen met klinische FH bij wie geen exonische variant was geïdentificeerd. De toepassing van meerdere filterstappen, waaronder de selectie van intronische varianten in deze klinische FH patiënten die niet aanwezig waren in moleculair bewezen FH patiënten, alsmede in silico beoordeling, heeft het aantal varianten waarvan het effect in latere cDNA studies moest worden beoordeeld sterk verminderd. Uiteindelijk werd één nieuwe intronische variant geïdentificeerd, die momenteel de diepst bekende FH veroorzakende intronische variant is en die de allereerste gerapporteerde pseudo-exon insluiting in *LDLR* veroorzaakt. Deze studie toont echter ook aan dat intronische varianten in *LDLR* die FH veroorzaken zeldzaam zijn en/of dat nieuwere en bredere sequencing methoden nodig zijn om meer pathogene varianten in dit gen te ontdekken. In onze studie werd gemiddeld slechts 36% van de intronische regio's van *LDLR* bestreken. Om dit te verhelpen kan sequencing van het gehele *LDLR*-gen in combinatie met mRNA-analyse van het gehele gen worden gebruikt om tegelijkertijd varianten te identificeren in momenteel nog niet ontdekte *LDLR*-regio's en hun effect op *LDLR* mRNA-splicing te beoordelen.

Deel I B: Nieuwe diagnostische doelwitten

In **deel I B** richten we ons op de identificatie van nieuwe genetische oorzaken van familiaire hypercholesterolemie. In **hoofdstuk 5** worden de genen *ABCG5* en *ABCG8* (ATP-binding cassettes G5 en G8) onderzocht op zeldzame varianten in patiënten met (klinische) FH. De eiwitten die gecodeerd worden door deze genen vormen een heterodimer en zijn betrokken bij het transmembraan cholesterol transport naar gal toe. Veel voorkomende en zeldzame varianten in deze genen zijn geassocieerd met LDL-C niveaus en cardiovasculaire aandoeningen (CVD).[3,4] In ons cohort, bestaande uit 3031 klinische FH patiënten, bleken respectievelijk 1,48% en 0,96% drager te zijn van loss-of-function varianten in *ABCG5* en *ABCG8*. Patiënten die drager waren van dergelijke genetische varianten bleken significant lagere LDL-C niveaus te hebben in vergelijking met "klassieke" FH patiënten met een *LDLR* pathogene variant (6,2±1,7 vs 7,2±1,7 mmol/L). Vervolgens toonde een cosegregatie analyse in drie families van één *ABCG5* en twee *ABCG8* varianten geen cosegregatie aan van de variant met het FH fenotype. Daarom wordt geconcludeerd dat *ABCG5* en *ABCG8* varianten mogelijk een rol spelen in het hypercholesterolemisch fenotype bij FH patiënten, vooral bij die met matig verhoogde LDL-C, maar dat het onwaarschijnlijk is dat ze autosomaal dominante FH veroorzaken. Wij speculeren dat deze varianten onder bepaalde omstandigheden predisponeren voor hypercholesterolemie, bijvoorbeeld in de aanwezigheid van dieetstoornissen. Verdere studies zijn nodig om de relatie tussen FH, *ABCG5*, *ABCG8*, en levensstijl factoren te ontrafelen.

In **hoofdstuk 6** wordt de rol van *STAP1* (signal transducing adaptor family member 1), een ander gen waarvan eerder is beschreven dat het FH veroorzaakt, onderzocht. Hoewel het STAP1 eiwit geen duidelijke rol heeft in de cholesterol homeostase en voornamelijk tot expressie komt in immuuncellen, werd het geassocieerd met FH in twee families uit Nederland.[5] Om deze associatie verder te begrijpen, werd een knock-out muismodel gegenereerd, dat geen veranderingen in lipidengehalten liet zien vergeleken met controlemuizen. Aanvullende immunologische analyses werden uitgevoerd, waaronder een beenmergtransplantatie van *Stap1*$^{-/-}$ naar *Ldlr*$^{-/-}$ muizen en een co-cultuur van perifere bloed mononucleaire cellen van menselijke *STAP1* variant dragers met HepG2 levercellen. Geen van deze studies toont een effect aan van het immuunsysteem op de cholesterolhomeostase veroorzaakt door STAP1 depletie. Tenslotte bleken *STAP1* variant dragers in vier hypercholesterolemische

families geen hogere LDL-C niveaus te hebben vergeleken met hun familiecontroles. Daarom werd geconcludeerd dat *STAP1* zeer waarschijnlijk geen FH-geassocieerd gen is. Deze conclusie wordt ook ondersteund door andere recente studies die het ontbreken van cosegregatie van *STAP1* varianten met het FH-fenotype in hypercholesterolemische families bevestigen.[6,7]

De vorige hoofdstukken concentreerden zich op monogenetische oorzaken van FH. Echter, een veel minder onderzocht gebied is de epigenetische regulatie van genen in hypercholesterolemische patiënten. In **hoofdstuk 7** wordt verondersteld dat gedifferentieerd gemethyleerd DNA geassocieerd zou kunnen zijn met FH en dit wordt onderzocht bij klinische FH patiënten. Om te controleren voor de effecten van hypercholesterolemie op de DNA methylering zelf, wordt de DNA methylering van FH mutatie-negatieve patiënten vergeleken met FH patiënten met een pathogeen defect in de *LDLR*. Uit deze studie blijkt dat er tussen de twee groepen geen duidelijk verschil bestaat in DNA methylering in FH-genen en andere geselecteerde genen die verband houden met het lipidenmetabolisme. In dezelfde studie onthult een machine learning benadering echter een verschil op genoombrede schaal, met belangrijke modelkenmerken voor *PRDM16* en *GSTT1*. Beide genen werden eerder geassocieerd met LDL-C niveaus.[8,9] Hoewel differentiële methylering van specifieke genen waarschijnlijk niet per se een oorzaak is van FH, speculeren wij dat cumulatieve klein effect DNA methylering een verhoogde LDL-C kan veroorzaken bij klinische FH patiënten. Dit fenomeen zou kunnen lijken op de polygenetische oorzaken van hypercholesterolemie, waarbij verhoogde LDL-C niveaus niet worden verklaard door individuele genetische varianten, maar eerder door de som van effecten van veel voorkomende genetische varianten.[10] Het is duidelijk dat verder onderzoek naar DNA methylering nodig is om de relatie met hypercholesterolemie en het lipidenmetabolisme in het algemeen verder op te helderen.

Deel II: De rol van ANGPTL3 in dyslipidemie

In **deel II** en **deel III** worden de studies beschreven waarin wij nieuwe therapeutische doelwitten voor de verlaging van LDL-C hebben onderzocht. **Deel II** concentreert zich uitsluitend op ANGPTL3, een natuurlijke remmer van lipoproteïnelipase (LPL) en endotheellipase (EL), die beide de lipoproteïnelipolyse versnellen en zo de klaring van lipoproteïnen uit de circulatie versnellen.[11] Een proef

studie met evinacumab, een monoklonaal antilichaam tegen ANGPTL3, in negen homozygote FH (hoFH) patiënten toonde een LDL-C verlagende werkzaamheid van 50% na een eenmalige infusie.[12] **In hoofdstuk 8** bespreken we de gegevens die de hypothese ondersteunen dat het remmen van ANGPTL3 niet alleen LDL-C niveaus verlaagt, maar ook de belofte in zich draagt om het CVD risico te verminderen. Gezien het effect van ANGPTL3 remming op apolipoproteïne B niveaus, het belangrijkste structurele eiwit van lipoproteïnen, en het overweldigende bewijs van een gunstig effect van lage niveaus van ANGPTL3 zowel in muizenmodellen als in genetische studies, verwachten we dat de ANGPTL3 verlagende middelen zullen resulteren in een vermindering van het CVD risico. Vervolgens wordt in **hoofdstuk 9** een gerandomiseerde placebo-gecontroleerde studie beschreven waarin evinacumab wordt getest bij 65 hoFH patiënten en blijkt dat het LDL-C effectief met 49% vermindert bij deze moeilijk te behandelen patiëntenpopulatie. HoFH-patiënten hebben gewoonlijk meerdere LDL-C-verlagende therapieën nodig, met beperkte werkzaamheid, vooral bij patiënten die geen residuele LDLR-activiteit meer hebben, om de door de richtlijn aanbevolen doelen te bereiken.[13] Aangezien evinacumab de LDL-C in vergelijkbare mate verlaagt bij patiënten met en zonder residuele LDLR-activiteit, is het een veelbelovend geneesmiddel voor deze moeilijk te behandelen patiëntenpopulatie. Op basis van deze gegevens heeft de Food and Drug Administration (FDA) onlangs het gebruik van evinacumab bij hoFH-patiënten in de VS goedgekeurd.[14]

In een volgende case-series, beschreven in **hoofdstuk 10**, laten we zien dat agressieve behandeling met evinacumab resulteert in bijna volledige regressie van atherosclerotische plaques bij twee adolescente hoFH patiënten. Hoewel deze bevinding op zich zelf uiterst relevant is voor het wegnemen van het CVD risico in deze twee patiënten, is het ook waardevol bewijs voor de notie dat jonge atherosclerotische plaques het vermogen bezitten om te verdwijnen in tegenstelling tot plaques in oudere patiënten.[15] Dit is van bijzonder belang omdat deze hoFH-patiënten een cumulatieve LDL-C-blootstelling hadden die gelijk was aan die van een gemiddelde 40-jarige man[16] en slechts 1% plaque-regressie wordt waargenomen na intensieve lipidenverlaging bij oudere niet-FH-patiënten met gevorderde atherosclerose.[17] Deze bevinding bevestigt verder dat een vroege behandeling van hypercholesterolemie de meest effectieve preventiestrategie voor

atherosclerose zou kunnen zijn en zou kunnen worden geleid door (frequente) plaque-beeldvorming.

Interessant is dat het exacte mechanisme waardoor remming van ANGPTL3 leidt tot lagere LDL-C plasmaspiegels deels onduidelijk blijft. Aangezien evinacumab een ANGPTL3-remmer is, neemt de triglyceridehydrolyse in de circulatie door LPL toe. Dit wordt ook gezien bij homozygote FH patiënten die behandeld worden met evinacumab in **hoofdstuk 11**. In deze kleine studie ondergingen vier patiënten een apoB kinetisch onderzoek voor en na evinacumab infusie. Na een eenmalige infusie van evinacumab werd een dramatische toename van de fractionele katabole snelheid van apoB in intermediate-density lipoproteins en LDL subfracties waargenomen in vergelijking met de onbehandelde snelheden, wat erop wijst dat deze sneller uit de circulatie werden verwijderd. Dit is mogelijk te wijten aan een snellere opname van deeltjes door de lever en aangenomen wordt dat dit proces LDLR onafhankelijk is aangezien hoFH met null/null varianten dezelfde omvang van het LDL-C verlagend effect ondervinden (**hoofdstuk 9**). Een recente studie toonde aan dat evinacumab alleen effectief is in aanwezigheid van EL bij muizen, hetgeen een rol suggereert voor EL bij de hepatische opname van remnant- en low-density lipoproteïnen.[18] Meer onderzoek is nodig om de rol van EL bij de mens te onderzoeken.

Naast het onvolledige begrip van de LDL-C verlagende effecten van ANGPTL3 remming, is de regulatie van ANGPTL3 productie en secretie slecht begrepen. Daarom hebben wij een studie uitgevoerd, beschreven in **hoofdstuk 12**, waarin wij het effect van statines in hepatocellulaire cellijnen op de productie van ANGPTL3 hebben onderzocht, evenals de associatie tussen statinegebruik en plasma ANGPTL3 niveaus in hypercholesterolemie patiënten. Door middel van een serie experimenten toonden wij aan dat plasma ANGPTL3 niveaus 15% lager zijn bij FH patiënten die statine gebruiken in vergelijking met FH patiënten die geen statine gebruiken. Dit effect is waarschijnlijk te wijten aan verminderde ANGPTL3 productie als gevolg van verminderde oxysterol leverreceptor X (LXR) activering. De relatie tussen statinegebruik en ANGPTL3 is van bijzonder belang, omdat patiënten die waarschijnlijk behandeld zullen worden met ANGPTL3 remming (b.v. patiënten met FH) waarschijnlijk ook statines gebruiken. Opmerkelijk is dat we in **hoofdstuk**

9 geen verschil zien in de werkzaamheid van evinacumab tussen hoFH patiënten die wel en geen statine therapie hebben, hoewel het aantal patiënten klein is (respectievelijk 61 vs 4 patiënten in de wel en geen statine groep).

Deel III: Nieuwe therapeutische opties voor hypercholesterolemie

In deel III zoomen wij in op andere potentiële therapeutische doelwitten voor FH-patiënten; apoB-productie en transintestinale cholesteroluitscheiding (TICE). ApoB-productie wordt beschouwd als een doelwit voor LDL-C-verlaging, gezien de rol van apoB als het belangrijkste structurele eiwit in atherogene lipoproteïnen, waaronder LDL. Zoals beschreven in **hoofdstuk 13**, verlaagt mipomersen, een antisense oligonucleotide tegen *APOB* mRNA, de plasmawaarden van LDL-C met ±20% bij heterozygote FH patiënten, afhankelijk van het exacte doseringsschema (200mg eenmaal per week vs 70mg driemaal per week) vergeleken met placebo. Hoewel effectief, is inherent aan de intrahepatische remming van de apoB-productie de ophoping van triglyceriden in de lever. Eerder werd vastgesteld dat toediening van mipomersen bij 25% van de behandelde FH-patiënten leidt tot een verhoogd vetgehalte in de lever.[19] In onze studie is het gebruik ervan geassocieerd met verhoogde ALAT-spiegels ten minste een keer ≥3x de bovengrens van normaal in >20% van de geïncludeerde patiënten. Het is geen verrassing dat de Food and Drug Administration in de VS mipomersen alleen heeft goedgekeurd voor homozygote FH-patiënten. Mipomersen werd onlangs door de producent ervan van de markt gehaald.

TICE, daarentegen, blijft een raadselachtig fenomeen. De onderliggende mechanismen van TICE en zijn potentieel als farmacologisch doelwit worden uitvoerig besproken in **hoofdstuk 13**. Recent is aangetoond dat TICE actief is bij de mens en 35% van de cholesteroluitscheiding in de feces voor zijn rekening neemt.[20] Bovendien is aangetoond dat TICE kan worden verhoogd door het gebruik van ezetimibe[20] en zelfs verder versneld wanneer een hydrofiele galzuurpool aanwezig is in de darm van muizen.[21] Of deze waarnemingen bij muizen ook opgaan voor de mens, en of TICE een aantrekkelijk doelwit is voor LDL-C verlaging moet worden bevestigd met robuuste klinische trials bij individuen met hypercholesterolemie.

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LIST OF PUBLICATIONS (NOT IN THIS THESIS)

Collard D, Nurmohamed NS, Kaiser Y, **Reeskamp LF**, Dormans T, Moeniralam H, Simsek S, Douma R, Eerens A, Reidinga AC, Elbers PWG, Beudel M, Vogt L, Stroes ESG, van den Born BH. Cardiovascular risk factors and COVID-19 outcomes in hospitalised patients: a prospective cohort study. BMJ Open. 2021 Feb 22;11(2):e045482.

Ibrahim S, **Reeskamp LF**, Stroes ESG, Watts GF. Advances, gaps and opportunities in the detection of familial hypercholesterolemia: overview of current and future screening and detection methods. Curr Opin Lipidol. 2020 Dec;31(6):347-355.

Larsen LE, Smith MA, Abbey D, Korn A, **Reeskamp LF**, Hand NJ, Holleboom AG. Hepatocyte-like cells derived from induced pluripotent stem cells: A versatile tool to understand lipid disorders. Atherosclerosis. 2020 Jun;303:8-14.

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Segboer C, Gevorgyan A, Avdeeva K, Chusakul S, Kanjanaumporn J, Aeumjaturapat S, **Reeskamp LF**, Snidvongs K, Fokkens W. Intranasal corticosteroids for non-allergic rhinitis. Cochrane Database Syst Rev. 2019 Nov 2;2019(11):CD010592.

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Gazzola K, **Reeskamp L**, van den Born BJ. Ethnicity, lipids and cardiovascular disease. Curr Opin Lipidol. 2017 Jun;28(3):225-230.

PORTFOLIO

Name PhD student: L.F. Reeskamp
PhD period: Nov 2016 – July 2020
Name PhD supervisor: G.K. Hovingh & E.S.G. Stroes

1. PhD training	Year	Workload (Hours/ ECTS)
General courses		
- eBrok	2017	28/1.0
- Practical Biostastistics	2017	40/1.4
- Research Data Management	2017	25/0.9
Specific courses		
- Genetic Epidemiology	2017	30/1.1
- Crash Course	2017	10/0.4
- Systems medicine	2017	40/1.4
- DNA Technology	2018	60/2.1
- Advanced Topics in Biostatistics	2019	60/2.1
Seminars, workshops and master classes		
- Masterclass Prof. Dr. Ira J. Goldberg	2018	2/0.1
Presentations		
- "A deep intronic variant in *LDLR* causing familial hypercholesterolemia: time to widen the scope?" Oral presentation - EAS 2018	2018	12/0.5
- "FH patients with unexplained low LDL-C: a role for Angptl3?" Oral presentation – ACS PhD retreat 2019	2019	12/0.5
- "FH patients with unexplained low LDL-C: a role for Angptl3?" Science at a glance presentation – EAS 2019	2019	12/0.5
- "ABCG5 and ABCG8 variants in Familial Hypercholesterolemia" Science at a glance presentation – EAS 2019	2019	12/0.5
- "Next-generation sequencing to confirm clinical FH in The Netherlands" Oral presentation Young Investigator Award session – ESC 2019	2019	24/1.0
- "Cholesterolverlaging door ANGPTL3 inhibitie" Oral presentation Young Lipid Talents – Nationale lipidendag	2020	5/0.2
(Inter)national conferences		
- Rembrandt symposium	2016	8/0.3
- The 85th European Atherosclerosis Society Congress	2017	32/1.1
- Transcard meeting	2017	10/0.4
- The 86th European Atherosclerosis Society Congress	2018	32/1.1
- ACS symposium 2018	2018	8/0.3
- Rembrandt symposium	2018	8/0.3
- ACS PhD retreat 2019	2019	28/1.0
- EAS satellite symposium: the imminent danger of Lp(a)	2019	8/0.3
- The 87th European Atherosclerosis Society Congress	2019	32/1.1
- ACS symposium 2019	2019	8/0.3
- European Society of Cardiology Congress	2019	32/1.1
- Nationale Lipiden Dag (NLA/DAS)	2020	8/0.3

Other		
- Advances in Lipid Management and Cardiovascular Risk Reduction: New Horizons for 2018 – Studiereis Harvard Medical School Boston	2017	16/0.6
- EAS Advanced Course on Epidemiology and Genetics of Atherosclerosis and Causality of Risk Factors – Copenhagen, Denmark	2018	18/0.7

2. Teaching	Year	Workload (Hours/ ECTS)
Lecturing		
- Nascholing FH-consulenten Landelijk Expertisecentrum Erfelijkheidsonderzoek Familaire hart- en vaatziekten (LEEFH)	2019	5/0.2
- Nascholing PACE Cardiovascular risk masterclass Madrid (Lipids & Genetics and Lipids & Novel therapies)	2019	40/1.6
- LEEFH educational video on diagnosing FH	2020	5/0.2
Supervising		
- Amber Korn: *"Induced pluripotent stem cells in lipid research"* Literature review master biomedische wetenschappen	2017	20/0.8
- Lotte Zandbergen: *"The role of ANGPTL3 in patients with familial hypercholesterolemia"* Bachelorthesis gezondheid en leven	2018	40/1.6
- Rogier Rutte: *"Epigenetic regulation and dyslipidemia"* Bachelorthesis geneeskunde	2018	20/0.8
- Douwe de Wijer: *"Lipid-lowering effects of RNA-based interventions: a scope on emerging therapies"* Bachelorthesis geneeskunde	2018	20/0.8
- Manon Balvers: *"Extensive bioinformatics analyses in genetically undiagnosed Familial Hypercholesterolemia patients to identify new pathogenic variants that affect splicing of the Low Density Lipoprotein Receptor transcript"*	2018-2019	40/1.6

3. Parameters of Esteem	
Grants	
- C.J. Vaillantfonds – FH sequencing HELIUS cohort	2019
- Stichting Atheros – Travelgrant Boston	2020
Awards and Prizes	
- Young Investigator Award – The 87th European Atherosclerosis Society Congress	2019
- Young Investigator Award - European Society of Cardiology Congress	2020
- Young Lipid Talent – Nationale Lipidendag	2020

A

DANKWOORD

Bladerend door een net ontvangen proefschrift gaat men in de regel als eerst op zoek naar het dankwoord. Kijken of je genoemd wordt. Logisch, zonder de hulp van velen (en dus van jou) was dit proefschrift er niet geweest. Geen enkel hoofdstuk in dit proefschrift is een soloprestatie, alles deed ik samen met anderen. In het bijzonder wil ik daarom de volgende personen bedanken.

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Beste Kees, ons avontuur begon toen ik een e-mail naar Max stuurde om 'wat onderzoek' te doen en ik prompt voor een verkapt sollicitatiegesprek werd uitgenodigd. Vanaf dat moment was het alsof ik op een hogesnelheidstrein sprong zonder ooit nog uit te willen stappen. De eerste paar jaar hadden we wekelijks intensief contact en konden we tal van studies opzetten. Dank voor de inspiratie die je verschafte, de directe en laagdrempelige begeleiding en het vele vertrouwen dat je had in mijn kunnen. Ik denk dat menig promovendus jaloers zou moeten zijn op de menselijke maat die jij in de begeleiding stopt. Het was inspirerend om je professor te zien worden, maar ook hoe je je hart achternaging en je uit de beklemmende wereld van een academisch centrumpje in Holland wist te onttrekken. Hopelijk kunnen we onze samenwerking de komende jaren blijven voortzetten.

Beste Erik, alhoewel je in het begin al mijn promotor was op papier, heb ik met name in het tweede deel van mijn PhD ontzettend veel aan je begeleiding gehad. Als Kees mij op een hogesnelheidstrein heeft gezet, dan ben jij de hyperloop die voorbijschiet. Wat een energie, scherpte en originaliteit weet je aan de dag te brengen. Ik denk dat er geen hoogleraar te vinden is die zo in de bres springt voor zijn promovendi. DANK!

Beste Aldo, ondanks dat je iets later naar het AMC bent gekomen, ben je van onschatbare waarde gebleken. Was je er maar geweest toen ik een jaar eerder nog hopeloos fibroblasten in het lab stond te kweken. Jij maakte het translationele karakter van dit proefschrift mogelijk en was daarnaast een uiterst fijne begeleider. Ik heb genoten van onze ANGPTL3 hypotheses. We zijn er nog niet helemaal, but the story continues!

Dear members of my defense committee, prof. dr. Kastelein, prof. dr. Mannens, prof. dr. Hennekam, prof. dr. ir. Kuivenhoven, prof. dr. ir. Kersten and prof. dr. Cariou, thank you very much for reading and assessing my thesis, as well as your willingness to serve as opponent during my defense.

Beste John, mijn eerste artikel als eerste auteur schreef ik onder jouw supervisie. Ook daarna heb ik de eer gehad om jouw handschrift te mogen ontcijferen als de correcties van artikelen mijn kant opkwamen. Nog steeds werkt er een leger promovendi door aan de onderzoekslijnen die jij ooit hebt opgezet en mag ik me gelukkig prijzen hier deel van uitgemaakt te hebben.

Beste Kuif, onze samenwerking begon binnen TRANSCARD, en leverde uiteindelijk de data op die nodig was voor de oprichting van www.lipidtools.nl. Dankjewel voor de fijne Groningse-Amsterdamse samenwerking.

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Beste co-auteurs, te veel om allemaal bij naam te noemen, dank voor alle inzichten, bijsturingen en snelle revisies van artikelen. Elke versie staat keurig gearchiveerd, het zijn er enkele honderden; dankjulliewel!

Dear international collaborators, thank you for all the calls, e-mails, and corrections. Some chapters in this thesis are solely there because of your contributions. Marina, you are a true inspiration when it comes to (homozygous) FH knowledge as well

as punctuality in publishing. Dear Amit, unfortunately I was unable to come to Boston, however I am sure that a new opportunity to visit Boston will occur in the near future. Andrea, I really enjoyed our collaboration in the six months that you were in Amsterdam, thank you!

Paranimfen, Wouter en Nick, jullie nemen een speciale rol in binnen mijn promotie en mijn leven. Wouter, wat begon als een onwennige kennismaking in een lokaaltje in het AMC leidde tot samen roeien, zeven jaar samenwonen, vele kapotte squashrackets, een schijnbaar eeuwig geduld voor mijn flauwe grappen en heul veul liters bier. Onze vriendschap is een never ending story. Weet dat Renske een mooie naam is! Nick, jij kwam als student de vasculaire wereld binnen, toen ik met het laatste jaar van mijn onderzoek bezig was. Het heeft inmiddels geleid tot vier (bijna) artikelen (waaronder voor mij de eerste als laatste auteur), een bedrijf met twee websites en een potentiële app, jouw vriendin en bovenal een mooie vriendschap. Nu nog even deze proefschriften bij de postkamer ophalen.

Experimentele vasculaire geneeskunde, G1, wat een prachtige groep mensen zijn jullie. Alinda, onder jouw kundige begeleiding zette ik de eerste stapjes in het lab. Ondanks het lot van onze projecten, wil ik je bedanken voor die mooie momenten. Het spijt mij dat ik niet beter kan presteren tijdens het bowlingtoernooi. Jorge, als er iets is dat je mij geleerd hebt, is het nanodroppen. Jij was degene die de eerste FH4 doorbraak forceerde en een nobelprijs voor intronomics binnen hengelde. Het was en is een feest om met je samen te werken! Bert, je bent een eeuwige bron van wetenschappelijke inspiratie, dank voor de samenwerking; TICE bestaat, ondanks de eerste resultaten van de EXCRETE. Geesje, dank voor je eeuwige aanwezigheid in het lab, je was onmisbaar en wordt, nu je met pensioen bent, nog steeds gemist. Xiang, it was an honor to serve as your paranymph! Manon, als student was je van onschatbare waarde en dat heeft geresulteerd in een mooie publicatie. Terecht dat je bij de vasculaire kon blijven. Dear Evgeni and Joao, thanks for our smooth collaboration on epigenetics!
Aan alle andere collega's van G1: nu F4 niet meer bestaat is G1 de leukste plek van het AMC. Geweldig om met jullie al die te gekke borrels, feesten, skireizen en congressen meegemaakt te hebben en dank voor alle mooie momenten in het lab zelf.

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Beste medewerkers van het trialbureau, studiearts zijn, wordt door sommigen gezien als corvee. Geheel onterecht, ik heb genoten van alle patiënten die we daar samen gezien hebben. Hans, jajajajaja, ik kon soms echt niet sneller prikken. Linda, tanner-expert, jouw zorg voor de FH-kinderen is ongeëvenaard. Daniela, zonder jou zou het trialbureau half zo efficiënt zijn, heel veel succes met je eigen PhD. En Tanja, ontzettend bedankt voor je ondersteuning gedurende mijn PhD, maar vooral ook nu, tijdens de laatste loodjes.

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Rowan, Megan en Michal, dank voor de prachtige 8 maanden wetenschappelijke stage die ik op de experimentele chirurgie bij jullie mocht doen. Zonder die leerzame tijd was mijn interesse in wetenschap nooit zo ver aangewakkerd dat het heeft geleid tot het moment waarop ik een dankwoord voor mijn eigen proefschrift zit te schrijven.

F4, oud-F4, en wannabe F4. F4 was en wordt ooit weer alles. De hele vasculaire kon alleen maar bestaan door de geweldige sfeer, kruisbestuiving en het harde werk dat we daar hebben verzet. Wat een prachtig zooitje ongeregeld waren we. Lotte en Renate, de absolute crême de la crême. Jullie hielden letterlijk de hele vasculaire (lab, CTU, F4 en staf) jarenlang bij elkaar. Ik heb genoten van alle koffiemomentjes en ik weet zeker dat we in de toekomst, als internisten, nog van heel veel koffie

kunnen gaan genieten en vlogs kunnen maken. Rutger, cardioleur in opleiding en specialist in vrouwen, heerlijk om bij jou op de kamer te mogen beginnen aan mijn promotie. Denk nog vaak met een glimlach terug aan dat eerste jaar. Guido, jouw toewijding aan het koffiezetapparaat (en schaken) is ongeëvenaard. Je was een fantastische kamergenoot. En dan de opperbever, Merel; er zijn maar weinig mensen met zoveel zelfspot en humor als jij. Ik werd dan wel "de rensdesk" door jou genoemd, maar andersom geldt dit ook. Zonder de introductie tot de vasculaire wereld die jij voor mij verzorgde, was dit hele proefschrift er niet geweest. Beste Kang, soms schrik ik nog wakker en ren ik schreeuwend door mijn kamer als ik jouw head-first botsing met een glazen wc-deur in een droom herbeleef. Yannick, Chaniqua, als het promoveren niet meer gaat, kan je misschien nog iets met fietsen gaan doen. Dank voor de gezelligheid en het invriezen van feces. Didier, wandelend statistiekhandboek, het is absoluut niet verrassend dat jij drie proefschriften tegelijk schrijft. Er gaat niks boven een biertje drinken terwijl we op vrijdagmiddag discussiëren hoe de lijnen van een linear mixed model met het juiste kleurtje en dikte in ggplot tevoorschijn kunnen komen.
Frits, het stelt mij teleur dat jij met je kennis en kunde van planten niet in staat was permanent waterkers te laten groeien uit je toetsenbord. Het is je vergeven. Laten we onze mooie gesprekken voorzetten in de toekomst. Floris, waar is mijn scheerapparaat gebleven? Het is een rare gewaarwording om tegelijk boos te zijn en helemaal kapot te gaan van het lachen. We drinken er vast binnenkort weer een biertje op, op de dijk!
Jeffrey en Jan, het was lachen, gieren, brullen op F4/G1, borrels, congressen en wintersport. Love your work, maar wees een beetje zuinig op je telefoon, Jef!
Emma, omdat dat je niet echt F4 was, maar het wel zou kunnen zijn, bedank ik je hier. Onze carrières lopen opvallend synchroon en iedere keer dat ze elkaar kruizen is het feest.
Mannelijke helden van de Kees-groep: Tycho, inmiddels FH4 goeroe, ik heb diep respect voor je kennis, scherpe blik en doorzettingsvermogen op moeilijke momenten. Het gaat zich uitbetalen! Arjen, ondanks dat je drie jaar later nog steeds zes manuscripten voor de NEJM hebt liggen, is de reis die je door de genetische epidemiologie hebt gemaakt ongekend. Je moest het jezelf allemaal eigen maken en dat is gelukt. Het gaat zich nu uitbetalen. Snel weer met Tycho whisky drinken.

Shirin, we gaan van jouw proefschrift een gouden boekwerk maken. We hadden geen betere start kunnen maken dan we hebben gedaan op wetenschappelijk en persoonlijk vlak. Je weet het, mijn naam begint niet voor niets met een R.

Alle overige oud-collega's die ik hier niet bij naam noem. Ik ben jullie niet vergeten: dank, dank, dank. Ik drink graag binnenkort weer een biertje met jullie.

Beste collega's uit het OLVG, we hebben zo veel borrels in te halen na de lockdown. De gezelligheid tijdens het werken in het OLVG doet vermoeden dat die een gekkenhuis worden.

Lieve vrienden van buiten de "geneeskunde", ondanks dat de afgelopen jaren vol in het teken stonden van het voltooien van dit boekwerk, was dat nooit gelukt zonder de afleiding die jullie van tijd tot tijd verschaften. Luuk, Christiaan, Casper, Wouter, Matthijs, Olivier, Djao, overige leden van Ad Fundum en de Ambtenaren, Roderik en Ferdinand, Bestuur '12, jullie houden mij op het rechte pad.
Knotsgekke familie, Reeskampjes en Knopsjes, we gaan een hoop inhalen na de lockdown!

Lieve Rob, Erline, Ruben, Rosa en Ralf, "schoonfamilie", inmiddels ken ik jullie bijna acht jaar. Er zijn maar weinig momenten zo rustgevend als bij jullie langskomen in Amsterdam, Bussum of Frankrijk. Ik geniet van ieder moment met jullie.

Lieve pa en ma, deze nerd was er nooit geweest zonder de juiste genenmix, opvoeding en liefde die jullie verschafte. Aan ieder van jullie draag ik 50% van dit proefschrift op. Maud en Emma jullie zijn awesome. Diezelfde genenmix, opvoeding en liefde hebben geleid tot drie totaal verschillende siblings die alles kunnen bereiken als we er maar in blijven geloven. Jullie boffen met Robbin en Sam.

En tot slot, lieve Tamara, jij bent de bijzonderste en belangrijkste van allemaal. Zonder jou was deze hele reis het überhaupt niet waard geweest. Ik kijk ontzettend uit naar de nieuwe avonturen die we in de toekomst gaan beleven.

CURRICULUM VITAE

Laurens Frans Reeskamp werd geboren op 21 maart 1991 te Blaricum. Hij groeide op in Naarden bij zijn ouders Sicco en Wilma, samen met zijn zusjes Maud en Emma. Hij behaalde in 2009 zijn gymnasiumdiploma met de profielen natuur en gezondheid en natuur en techniek aan het Sint Vituscollege in Bussum. Hierna startte hij met de studie geneeskunde aan de Universiteit van Amsterdam en ging op kamers in de hoofdstad. Gelijktijdig werd hij lid van de Algemene Amsterdamse studentenroeivereniging Skøll waar hij als gebouwcommissaris in het bestuur plaatsnam tijdens het collegejaar 2011-2012.

Zijn interesse in wetenschappelijk onderzoek werd gewekt tijdens het schrijven van zijn bachelorthesis bij de afdeling keel-, neus-, en oorheelkunde en werd verder versterkt tijdens zijn wetenschappelijke stage bij de afdeling experimentele chirurgie van het Academisch Medisch Centrum. Direct na het afronden van zijn semi-artsstage interne geneeskunde in het voormalige MC Slotervaart en het afleggen van zijn artseneed in 2016 ging Rens aan de slag als promovendus in de onderzoeksgroepen van prof. dr. Hovingh en prof. dr. Stroes, waarvan dit proefschrift het resultaat is.

Momenteel is Rens werkzaam als arts niet in opleiding tot specialist (ANIOS) bij de afdeling interne geneeskunde van het OLVG locatie oost. Hij ambieert het om internist te worden en dit te combineren met onderzoek naar de erfelijkheid van hart- en vaatziekten en lipidenmetabolisme.

Rens leerde bij Skøll zijn vriendin Tamara kennen. Met haar woont hij samen in Amsterdam. Naast squash, padel, lezen en ongevraagd advies geven, is Rens altijd te enthousiasmeren voor het drinken van een sociaal biertje met collega's, vrienden en familie.