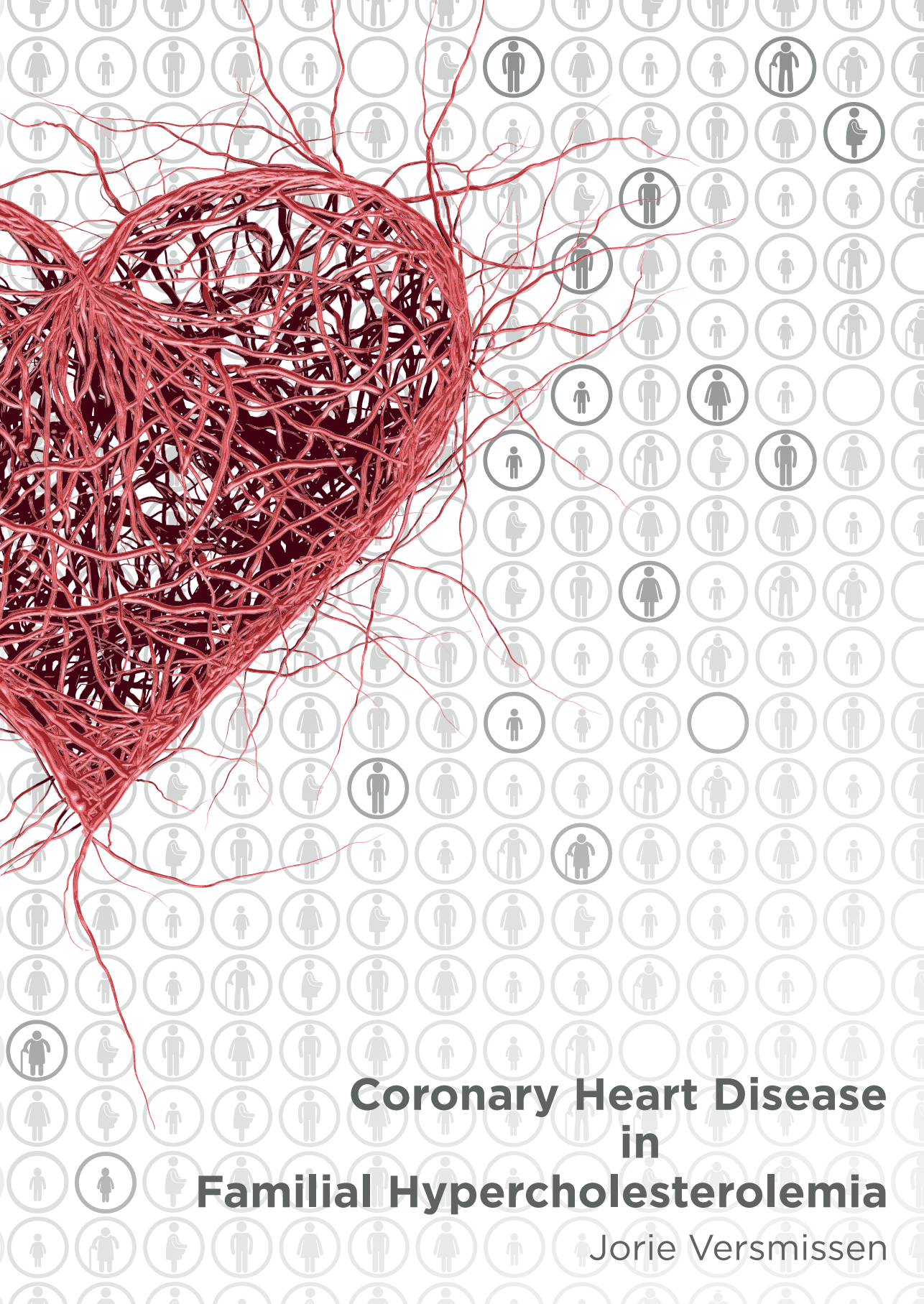

Coronary Heart Disease in Familial Hypercholesterolemia

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Overige leden:	Prof.dr. A.G. Uitterlinden
	Prof.dr.ir. C.M. van Duijn
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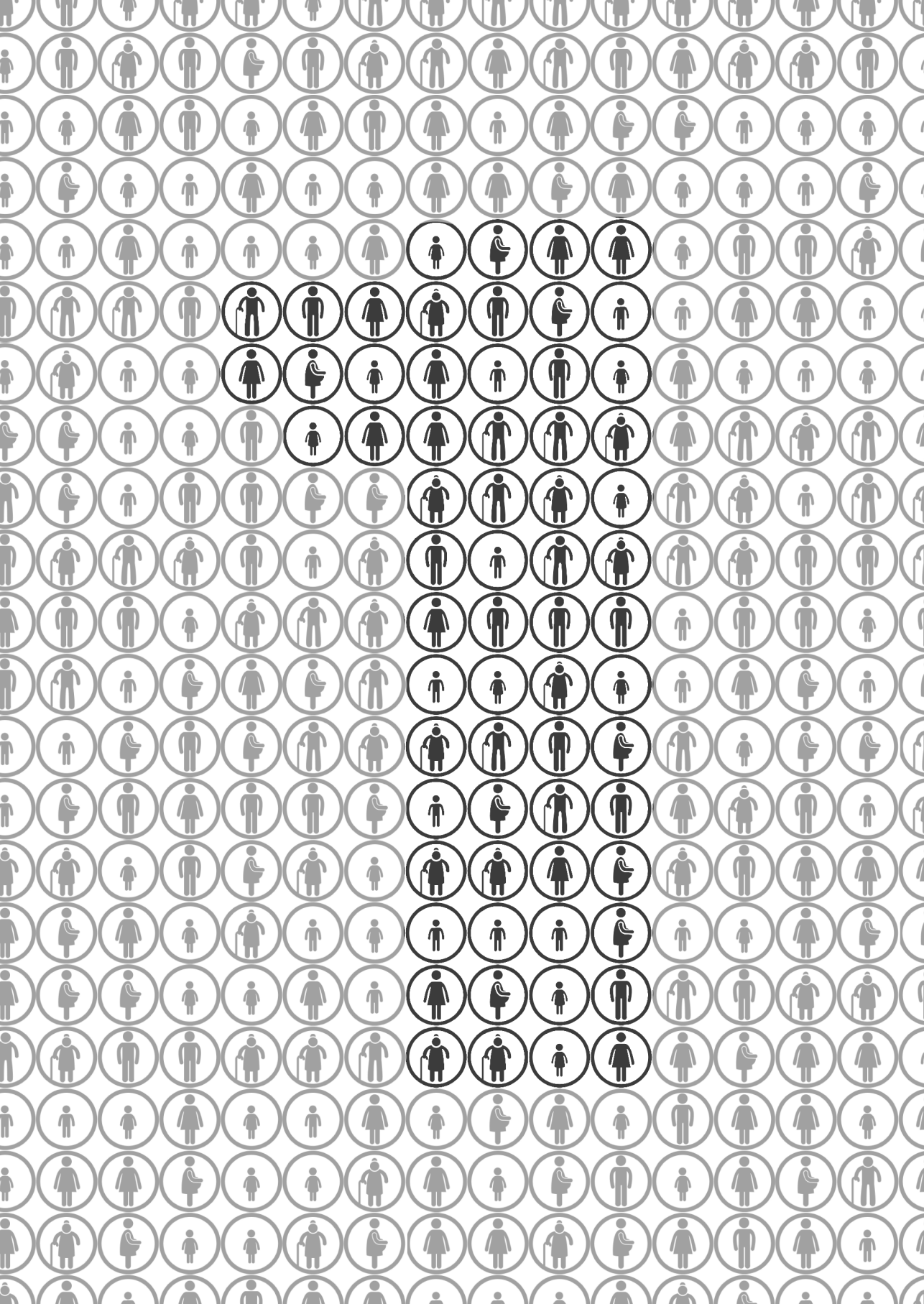

1 Introduction to the thesis

Based on

Daniëlla M Oosterveer†
Jorie Versmissen†
Arend FL Schinkel
Janneke G Langendonk
Monique Mulder
Eric JG Sijbrands

†These authors contributed equally to this work

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FAMILIAL HYPERCHOLESTEROLEMIA

Familial hypercholesterolemia (FH) (OMIM #143890) is an autosomal dominant disorder present in 1:500 Caucasians. FH is caused by defective low-density lipoprotein (LDL) receptors, leading to a diminished uptake of LDL cholesterol by the liver. As a result, FH patients have high LDL cholesterol levels and a high risk of contracting cardiovascular disease (CVD), mainly coronary heart disease (CHD).[1] FH can be diagnosed on the basis of clinical criteria (Table 1) or by detection of the causal mutation in the LDL-receptor gene.[2-4]

Despite the homogenous background of hypercholesterolemia, the onset and severity of CHD among FH patients varies considerably. This introduction provides a short update of the classic and genetic factors influencing CHD risk in heterozygous FH patients. Besides the importance of CHD risk prediction for FH patients, FH being the most common monogenetic disorder, FH can also be considered a high-risk model population in which CHD risk factors can more easily be detected. Risk factors identified in FH patients might consequently be translated to the general population.

TABLE 1. Diagnostic criteria of FH [4]

Criteria	Points
Family history	
First-degree relative with known premature (men < 55 years; women < 60 years) coronary and vascular disease, or fist degree relative with known LDL cholesterol > 95th percentile	1
1st degree relative with tendon xanthomas and/or arcus cornealis, or children aged less than 18 years with LDL cholesterol > 95th percentile	2
Clinical history	
Patient with premature (men < 55 years; women < 60 years) CHD	2
Patient with premature (men < 55 years; women < 60 years) cerebral or peripheral vascular disease	1
Physical examination	
Tendon xanthomas	6
Arcus cornealis < 45 years of age	4
Cholesterol levels (mmol/l)	
LDL cholesterol ≥ 8.5	8
LDL cholesterol 6.5-8.4	5
LDL cholesterol 5.0-6.4	3
LDL cholesterol 4.0-4.9	1
DNA analysis	
Functional mutation in the LDLR gene	8

Diagnosis definite FH : > 8 points; probable FH : 6-8 points; possible FH 3-5 points

CLASSIC RISK FACTORS

Most classic CHD risk factors clearly contribute to CHD risk in FH patients: age, male gender, body mass index (BMI), hypertension, diabetes mellitus and high-density lipoprotein (HDL) cholesterol levels are all associated with CHD in FH.[5-7] Smoking is associated with a higher CHD risk up to nine years after quitting.[8]

'BIOLOGICAL' RISK FACTORS

In addition to these well-known classic risk factors, the influence of several biomarkers on cardiovascular risk was studied. Similar to the situation in the general population, hsCRP, a marker of inflammation, is in FH patients an independent predictor of higher intima media thickness (IMT) and coronary calcium score, both surrogate markers of generalized atherosclerosis.[9, 10] An atheroprotective biomarker in the general population is adiponectin, a hormone that is excreted by fat tissue.[11, 12] Low plasma levels of this hormone also increased the risk of CHD in a FH population.[13] Both biomarkers, however, need to be replicated in prospective analyses.

Small-isoform lipoprotein a (Lp(a)) levels are associated with CHD risk in the general population and in FH, although it is still unknown whether this is a risk factor or a risk marker.[6, 14-17] The size of specific lipoproteins seems to influence CHD risk in FH as well.[18, 19] Small, dense LDL is highly atherogenic and higher levels are associated with an increased CHD risk.[20] HDL also exists in different forms and densities.[21, 22] Higher levels of small, discoidal pre-beta HDL levels seem to be associated with CHD, although the composition of the whole HDL plasma pool seems to be most important with regard to the cardioprotective capacities of HDL.[22-25]

GENETIC RISK FACTORS

Candidate gene studies

Because not all of the variance in the phenotype and complications of FH can be explained by the risk factors mentioned above, numerous studies have focused on identifying genetic risk variants other than the LDL receptor mutation to explain the variance of CHD risk. The genetic variants that were most significantly associated with atherosclerosis within different studied pathways are shown in Table 2.

Lipid metabolism

Clearly, genes involved in lipid metabolism pathways have been studied extensively. Variants in genes interacting with the LDL receptor, such as apolipoprotein B (*APOB*) and apolipoprotein E (*APOE*), were not associated with CHD in FH.[26-28] Variants in the

TABLE 2. Most significant genetic CVD and CHD risk variants in FH patients

Gene	Genetic variant	Risk allele/ genotype	Study size	MAF	End-point	Effect	Ref
Lipid metabolism							
Lipoprotein lipase	Rs268 (A>G)	G allele	1,045	0.07	CVD	OR 3.88, *p* 0.006	[38]
Inflammation & LDL oxidation							
5-lipoxygenase activating protein (ALOX5AP)	haplotype rs17216473-rs10507391-rs9315050-rs17222842	A-A-A-G	1,817	0.07	CHD	HR 1.48, *p* 0.001	[47]
Blood pressure regulation							
Angiotensiongen (AGT)	haplotype rs5049 (G>A)-rs4762 (C>T)-rs699 (T>C)-rs7079 (C>A)	G-C-C-C	1,785	0.15	CHD	RR 1.45, *p* 0.006	[49]
Coagulation and hemostasis							
Prothrombin (F2)	Rs1799962 (G>A)	G allele	1,940	0.01	CVD	HR 2.44, *p* <0.001	[27]
Replication of GWA studies							
Near cyclin-dependent kinase 2A/B (CDKN2A/B)	Rs10757274 (A>G)	GG genotype	2,145	0.47	CHD	HR 1.39, *p*<0.001	[60]

Abbreviations: CHD coronary heart disease; CVD cardiovascular disease; MAF minor allele frequency; OR odds ratio; HR hazard ratio; ref reference

PCSK9 gene were associated with a more favorable lipid profile and lower CHD risk, and PCSK9 inhibitors are now tested in clinical trials.[29-31] Reverse cholesterol transport is the mechanism by which cholesterol that has accumulated in macrophages is returned to the liver.[25] This is facilitated by adenosine triphosphate binding cassettes A1 and G1 (ABCA1 and ABCG1). ABCA1 and ABCG1 enhance the cholesterol flux from macrophages to HDL particles. Then cholesteryl ester transfer protein (CETP) transfers this cholesterol from the HDL particles to LDL particles, which are cleared by LDL-receptors on the liver. Genetic variants in *ABCA1* and *CETP* influenced CHD risk but often an effect on HDL-C levels could not be established.[32-36]

The metabolism of chylomicrons and very-low-density lipoproteins is controlled by apolipoprotein AIV, apolipoprotein CIII and lipoprotein lipase (LPL). Genetic variants in these genes are associated with CVD in FH.[28, 37-39] Genetic variants in the adenosine triphosphate binding cassette G8 (ABCG8) transporter were examined because mutations in this gene cause siterosterolemia, resulting in impaired plant sterol metabolism and premature CVD. One of the two variants examined was associated with higher risk of CHD in FH patients.[40]

LDL oxidation and inflammation

LDL is more atherogenic when it is oxidized.[41] Paraoxonase 1 and 2 (PON1 and PON2) hydrolyze oxidized LDL, and may therefore protect against CVD. Variants in these genes are indeed associated with both IMT and CVD.[27, 42-44]

Inflammation is a major pathogenetic pathway in atherosclerosis in FH.[45, 46] A haplotype in one of the genes involved, encoding the 5-lipoxygenase-activating protein (ALOX5AP) important for the biosynthesis of the pro-inflammatory leukotrienes, increased the risk of CHD.[47] Complement factor H prevents uncontrolled complement activation and FH carriers of the CC genotype of the genetic variant rs1061170 are protected against CVD.[48]

Blood pressure regulation - Variation in genes known to be involved in blood pressure regulation have also been associated with CVD in FH, although an effect on blood pressure often could not be demonstrated. The main pathway studied is the renin-angiotensin-aldosterone system (RAAS). Several genetic risk factors were identified in the genes encoding angiotensin-converting enzyme (ACE), angiotensinogen (AGT) and angiotensin II receptor (AGTR1). [49-52]In addition to a moderately increased risk caused by one of these risk factors, FH patients carrying five or six genetic risk variants of the RAAS pathway had a 2.3 times higher risk of CHD than patients with no or only one risk variant.[53]

Coagulation and hemostasis

Coagulation and hemostasis are important pathogenetic pathways in atherosclerosis. One variant in the prothrombin gene is associated strongly with CHD in a large FH cohort (Table 2). [27] However, genetic variants in other genes involved in this pathway did not show a relationship with CHD.[27, 54]

Others

Risk variants were identified in a number of other genes. Examples of such genes are the estrogen receptor, the glucocorticoid receptor-α and others.[27, 55-58]

Genome-wide association (GWA) studies

In contrast to candidate gene studies, GWA studies test thousands of common genetic variants throughout the genome without a prior hypothesis. By studying patients with and without CHD, this method can lead to identification of unknown atherogenetic pathways. To limit false-positive results of GWA studies due to multiple-testing, it is important to replicate the results in independent populations.[59] In addition, genetic risk variants in the general population are not necessarily also associated with CVD risk in FH patients. Van der Net et al. tested ten genetic variants found previously in several GWA studies, in a large Dutch FH cohort, and could replicate only four of them (most significant one in Table 2).[60] However, the latest findings that were replicated in large consortia have not been tested in a large FH population so far.[61]

EPIGENETICS: EFFECTS OF HYPERCHOLESTEROLEMIA DURING PREGNANCY

Increasing evidence suggests that the risk of metabolic and cardiovascular disease later in life may be influenced by circumstances in utero, the so-called 'fetal origins of adulthood disease hypothesis'. [62, 63] In fact, elevated LDL-C in the mother related to fatty streak formation in the fetal aorta with rapid progression to atherosclerosis later in life.[64-66] Intrauterine conditions may lead to permanent non-genetic DNA changes, so-called epigenetic effects. These changes include chromatin modifications such as histone methylation, acetylation, and ubiquination that alter the regulation of genes that might influence the risk of CHD and mortality later in life.[67]

TREATMENT

Lipid-lowering treatment, more specifically statin treatment, has the greatest impact on CHD risk in FH patients. Before the widespread introduction of statins, treatment of FH patients mainly focused on reduction of cholesterol uptake in the intestine. These uptake-inhibitors, so-called bile-acid sequestrants, resulted in relatively moderate cholesterol lowering and CHD risk reduction.[68, 69] Due to gastrointestinal side effects compliance with this treatment was low, in contrast to the well-tolerated statins. Statins inhibit HMG-CoA reductase, the rate-limiting enzyme of cholesterol synthesis. This leads to up-regulation of LDL-receptors and therefore statins can be considered as causal treatment of FH.[70] Since it was considered to be unethical to withhold this therapy from FH patients, no hard endpoint clinical trials have been conducted and as a consequence it was for a long time unknown whether the risk in FH patients indeed had normalized.

AIM AND OUTLINE

As discussed above, it is still not possible to predict which FH patient will prematurely develop CHD and which will not. As a consequence, all FH patients are treated similarly. Fortunately, statin treatment has shown to be an effective treatment, reducing CHD risk tremendously. It would be of great interest to predict who will develop CHD and who will not, and to know who will respond best to statin treatment. Both outcomes will result in personalized treatment and potentially reduce CHD risk more effectively.

The **general aim** of this thesis is to identify unknown CHD risk factors and underlying mechanisms. In addition, we show the efficacy of the most important CHD risk-reducing factor, statin treatment, and studied pharmacogenetics to identify genetic risk factors modifying CHD risk reduction by statins.

Part I

In part I, we describe both genetic and other biological CHD risk modifiers. In **chapter 2,** we show that *APOE4*, a well known CHD risk increasing haplotype in the general population, might be protective against CHD in the presence of an LDL receptor mutation, indicating a major role for the LDL-receptor-APOE interaction in the etiology of the increased CHD risk associated with ApoE4. In **chapter 3**, we present a GWA study in a unique case control design comparing the eldest FH patients without symptomatic CHD with the youngest FH patients with symptomatic CHD. We could replicate two polymorphisms in one other FH cohort and in three cohorts from the general population. One of these polymorphisms appeared to be associated with expression of a gene 400kb downstream of the identified polymorphisms. In **chapter 4** we show that variants identified in genetic association studies might indeed link to a gene hundreds of kilobases away from the polymorphism, so-called synthetic associations. By comparing an FH cohort with a cohort from the general population, we demonstrate that mutations in the LDL receptor gene can cause such associations.

In **chapter 5**, in a cholesterol efflux study using plasma of FH patients we show that HDL function rather than quantity seems to be most important in CHD risk prevention. The last biological risk factor studied in **chapter 6** was the effect of maternal hypercholesterolemia during pregnancy on the fetus. Only maternal inheritance of FH exposes a fetus to effects of parental hypercholesterolemia. Using an unique design by analyzing a large pedigree over seven generations, we were able to study the effects of maternal inheritance of FH on the offspring.

Part II

In part II, we discuss the efficacy of statin treatment and pharmacogenetics. In **chapter 7** we show that CHD risk indeed nearly has normalized in clinically identified FH patients. In **chapter 8** we present the effects of treatment on people with FH identified by genetic cascade screening. It would be of great interest to predict response to therapy according to genetic variation. However, not many convincing genetic risk modifiers of statin response have been identified so far. In a large cohort study described in **chapter 9,** we found that the C69T variant of the *ABCA1* gene associates with CHD independently of HDL levels, but in statin-treated patients no difference in CHD risk was seen, most likely due to the higher increase in HDL-C levels in statin-treated TT individuals.

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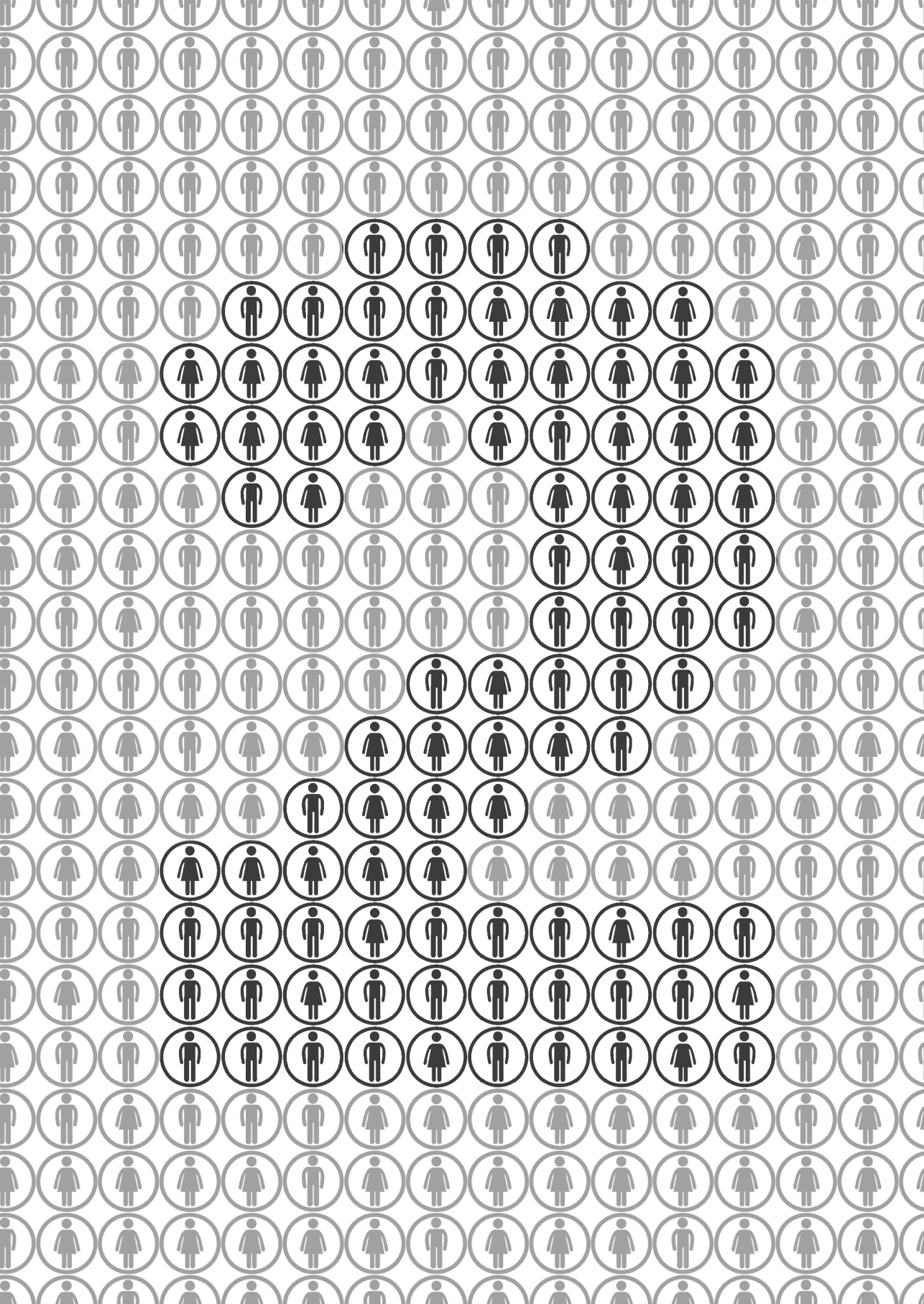
PART I

CHD RISK MODIFIERS IN FH



2

Apolipoprotein isoform E4 does not increase coronary heart disease risk in carriers of low-density lipoprotein receptor mutations

Jorie Versmissen
Daniëlla M Oosterveer
Menno Hoekstra
Ruud Out
Jimmy FP Berbée
Adriana C Blommesteijn-Touw
Leonie van Vark-van der Zee
Ranitha Vongpromek
Tim Vanmierlo
Joep C Defesche
Monique Mulder
John JP Kastelein
Eric JG Sijbrands

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ABSTRACT

Background

In humans, the *E4* allele of the apolipoprotein E gene (*APOE*) is associated with increased coronary heart disease (CHD) risk. Surprisingly, in rodents, ApoE4 only accelerates the atherosclerotic process when transgenic for the human low-density lipoprotein (LDL) receptor protein. We therefore investigated whether the *LDLR* locus interacted with *APOE* genotype on CHD risk in patients clinically diagnosed with familial hypercholesterolemia (FH) with and without *LDLR* mutation. We investigated whether the presence of an *LDLR* mutation diminishing LDL receptor function was protective in *E4/E4* carriers.

Methods& Results

In a cohort of 2,400 patients clinically diagnosed with FH, we found an *LDLR* gene mutation in 1,383 patients whereas in 1,013 patients such mutation was not present. In 92 patients homozygous for *APOE4*, the presence of an *LDLR* mutation conferred lower CHD risk (hazard ratio (HR) 0.16; 95% confidence interval (CI) 0.05-0.58; p=0.005). Mirroring these results, the *APOE4/E4* genotype was also associated with lower CHD risk in FH patients with an *LDLR* mutation (HR 0.26 HR 0.08-0.80; p=0.02).

Conclusions

LDL receptor function is key to the detrimental effects of ApoE4 in humans. Kinetic studies in humans are now required to study the consequences of our observation for prevention of both CHD and Alzheimer's disease.

BACKGROUND

Complications of atherosclerotic vascular disease are the most common causes of death and morbidity in the Western world, with coronary heart disease (CHD) as it's most prominent manifestation.[1] Hereditary predisposition plays an important role in the pathobiology of atherosclerosis. One of the genes best known for it's association with CHD risk is the one coding for the low-density lipoprotein receptor (LDLR) protein. Mutations in this gene cause an autosomal dominant disorder called familial hypercholesterolemia (FH), which is characterized by severe hypercholesterolemia and premature CHD[2] Another important gene known to influence CHD risk is Apolipoprotein E (*APOE)*.[3] Mice completely lacking the ApoE protein are severely hypercholesterolemic and develop extensive atherosclerotic lesions, a process which is accelerated when they are fed a high-fat diet.[4, 5] Homozygous deficiency of the *APOE* gene in humans is extremely rare and is also characterized by atherogenic lipid abnormalities and premature CHD.[6, 7]

In humans, three main haplotypes of the *APOE* gene have been identified: APO*E2*, APO*E3* and APO*E4*. The encoded ApoE2, ApoE3 and ApoE4 proteins differ in their amino acid sequences at positions 112 and 158. Although these differences are not located in the LDL receptor binding domain, affinity to the LDL receptor differs between genotypes: ApoE2 has the lowest affinity for the LDL receptor while ApoE4 has the highest.[8] This leads to lower LDL-cholesterol levels in *APOE2* carriers due to up-regulation of LDL receptors and higher LDL-cholesterol levels in *APOE4* carriers due to down-regulation of LDL receptors.[9, 10] While the *APOE4* allele is best known for its strong association with Alzheimer`s disease, it is also consistently associated with a 26-42% higher CHD risk.[11, 12] This increased CHD risk in *APOE4* carriers cannot merely be explained by small differences in LDL-cholesterol levels. The LDL receptor-APOE interaction is, however, central to the increased CHD risk associated with ApoE4: atherosclerosis resulting from specific *APOE* genotypes can be replicated in rodents, but only when human LDL receptors are also abundant.[13, 14] Notably, mice expressing human ApoE4 develop no substantial atherosclerosis, but they display fulminant disease when the human LDL receptor is also introduced.[14, 15] To date, the interaction between ApoE and the LDL receptor has not directly been examined in humans. A suitable group of patients for investigating this interaction would consist of patients who have a genetic defect in the *LDLR* gene that results in lower residual function of the LDL receptor protein. In fact, in two earlier studies of modest study size in FH patients, the *APOE4* allele was not associated with CHD risk. We therefore hypothesized that carriers of the *E4/E4* genotype might benefit from a reduction in functional LDL receptors.[16, 17] In a large population of persons clinically diagnosed with FH, we investigated whether the presence of an *LDLR* mutation that reduces LDL receptor function was protective in *E4/E4* carriers.

MATERIALS AND METHODS

FH Cohort

During 1989-2002, we recruited a cohort of 2,400 patients with severe hypercholesterolemia from 27 lipid clinics as described in detail previously.[18] We selected 2,400 unrelated subjects who fulfilled the internationally established FH diagnostic criteria. A well-trained team of 13 data collectors reviewed medical records to establish extensive phenotypic data including CHD events.[19] Total cholesterol, high-density lipoprotein cholesterol (HDL) and triglyceride levels were measured by standard methods in fasting patients who had been withdrawn from lipid-lowering medication at least 6 weeks before blood collection. LDL-cholesterol concentration was calculated with the Friedewald formula.[20] The promoter region and all exons (including exon-intron boundaries) of the *LDLR* gene were sequenced in all patients and multiplex ligation-dependent probe amplification (MLPA) technique was used to identify large re-arrangements. Exon 26 and 29 of the APOB gene, encoding the major LDL receptor binding sites, were also sequenced. All known mutations were tested in duplo and sequencing and MLPA was performed twice when no previously known mutation was identified. Therefore, false negative results for *LDLR* mutation assessment were unlikely (around 0.06%). Genetic variants without clear effect on LDL receptor function were filtered using pedigree data from the Dutch screening program as published earlier: if a potential mutation did not segregate with hypercholesterolemia, pathogenicity of this variant is questionable. In the current study, such variants were not considered as LDLR mutation. Carriers of such mutations were considered to have no *LDLR* mutation.[21] In a later stage, *PCSK9* was sequenced in samples in which no mutation was identified but this analysis did reveal only a limited number of mutations in patients with an FH phenotype in general and none in this cohort.[22] The DNA of 2145 unrelated patients was available for *APOE* genotyping, performed in a multilocus genotyping assay.[23] All patients gave informed consent, and the ethics institutional review board of each hospital approved the protocol.

CHD outcome measures

CHD was defined as the presence of at least one of the following: *(i)* myocardial infarction, proved by at least two of the following: *(a)* classical symptoms (>15 minutes), *(b)* specific ECG abnormalities, and *(c)* elevated cardiac enzymes (>2x upper limit of normal); *(ii)* percutaneous coronary intervention or other invasive procedures; *(iii)* coronary artery bypass grafting; *(iv)* angina pectoris, diagnosed as classical symptoms in combination with at least one unequivocal result of one of the following: *(a)* exercise test, *(b)* nuclear scintigram, *(c)* dobutamine stress ultrasound, or *(d)* more than 70% stenosis on a coronary angiogram.

TABLE 1. General characteristics per genotype with and without LDLR mutation

	No *LDLR* mutation				*LDLR* mutation present			
	APOE3/E3	***APOE3/E4***	***APOE4/E4***	***p***	***APOE3/E3***	***APOE3/E4***	***APOE4/E4***	***p***
Number	**502**	**289**	**44**		**676**	**291**	**49**	
Male (%)	258 (51.4)	157 (54.3)	18 (40.9)	*0.24*	319 (47.2)	126 (43.3)	16 (32.7)	*0.10*
Age first visit lipid clinic (years)	48.9±11.8	48.2±11.7	47.6±14.0	*0.64*	41.8±12.5	41.4±12.1	42.1±12.8	*0.89*
Follow-up time (years)	50.0±12.0	49.7±11.7	49.1±13.5	*0.89*	45.4±11.8	45.1±12.2	46.3±12.9	*0.82*
Smoking ever (%)	363 (80.7)	212 (80.6)	32 (84.2)	*0.86*	425 (69.9)	178 (68.7)	29 (67.4)	*0.90*
Hypertension (%)	51 (10.2)	40 (14.2)	4 (9.3)	*NS*	58 (8.7)	24 (8.3)	3 (6.1)	*NS*
Diabetes (%)	35 (7.0)	16 (5.5)	6 (13.6)	*NS*	33 (4.9)	14 (4.8)	1 (2.0)	*NS*
Coronary heart disease (%)	186 (37.1)	97 (33.6)	16 (36.4)	*NS*	170 (25.1)	75 (25.8)	3 (6.1)	*0.001**
Statin at baseline (%)	118(23.5)	75 (26)	13(29.5)	*0.55*	257 (38)	116 (39.9)	19 (38.8)	*0.86*
Age statin started (years)	46.0±11.7	46.2±11.0	46.9±12.8	*0.33*	38.8±11.3	39.7±11.2	41.4±11.9	*0.59*
BMI (kg/m2)	25.3±3.4	25.5±3.3	25.6±4.0	*0.78*	24.4±3.3	24.5±3.6	24.4±3.2	*0.79*
Total cholesterol (mmol/l)	8.48±1.28	8.56±1.36	8.45±1.02	*0.70*	10.06±2.07	9.95±1.98	9.97±1.95	*0.78*
LDL-cholesterol (mmol/l)	6.26±1.16	6.31±1.21	6.26±1.01	*0.88*	7.92±1.97	7.67±1.93	7.67±1.75	*0.35*
HDL-cholesterol (mmol/l)	1.21±0.32	1.18±0.32	1.27±0.52	*0.24*	1.12±0.33	1.15±0.32	1.15±0.27	*0.49*
Triglycerides (mmol/l)	1.72±0.79	1.75±0.87	1.74±0.73	*0.86*	1.42±0.71	1.41±0.72	1.45±0.85	*0.94*
Lp(a) (mg/l)	193±257	166±215	149±224	*0.27*	183±227	138±173	124±147	*0.02*
Lp(a)>3mg/dl	166(43.6)	85 (37)	15 (41.7)	*0.27*	163 (35.7)	57(28.8)	5 (17.9)	*0.05*

BMI: Body mass index; CHD: Coronary heart disease; NS: non-significant; *: APOE4/E4 vs APOE3/E3; Values are mean±standard deviation; Follow-up time from birth to event or censoring

Statistical analysis

General characteristics were compared using analysis of variance (ANOVA) for continuous variables (Statistical analyses of substanially skewed data were performed after logarithmic tranformation) and X^2 test for categorical variables. Variables with low frequencies (hypertension, diabetes, CHD) were analyzed using the Fisher's exact test comparing each genotype separately with *APOE3/E3*. In Table 1, p-values are given when comparing *E3/E3*, *E3/E4* and *E4/E4*, while in the supplemental Table 2 overall p-values comparing all genotypes are given. Firstly, we investigated the effect of having an *LDLR* mutation on CHD risk in this FH study population using Cox proportional hazards models adjusted for year of birth, sex and smoking as the Cox proportional hazards modelling seems most powerful in genetic studies.[24] Follow-up started at birth and ended at the first occurrence of CHD. Patients without CHD were censored at the date of the last lipid clinic visit or at the date of death attributable to causes other than CHD. The proportional hazards assumption was tested by drawing log minus log plots of the survival function and was met for all Cox proportional hazard models used. Next, we stratified by *APOE* genotype. We also studied the effect of *APOE* genotypes on CHD risk in the whole study population as well as stratified according to presence of an *LDLR* mutation. Interaction was tested by introducing an interaction term in Cox regression analyses. A p-value less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS 15.0.

TABLE 2. Protective effect of an *LDLR* mutation on CHD risk in *APOE4/E4* FH patients

	N	*Model 1*			*Model II*		
		HR	**95% CI**	***p-value***	**HR**	**95% CI**	***p-value***
Overall	2,400	0.88	0.76-1.02	*0.10*	0.85	0.71-1.02	*0.07*
APOE3/E3	1,178	0.84	0.68-1.04	*0.11*	0.84	0.67-1.05	*0.12*
APOE3/E4	580	1.01	0.74-1.37	*0.96*	0.96	0.70-1.33	*0.81*
APOE4/E4	93	0.16	0.05-0.58	*0.005*	0.13	0.03-0.48	*0.002*

Model I Adjusted for gender, year of birth; Model II additionally adjusted for smoking

RESULTS

In 1,383 patients an *LDLR* mutation was identified while in 1,017 patients no pathogenic mutation in the *LDLR* or *APOB* gene was found by sequencing the complete *LDLR* gene or by using the multiplex ligation-dependent probe amplification technique. Comparison of individuals with and without *LDLR* mutation in this cohort showed significant differences as published earlier by Van Aalst-Cohen et al.: FH patients without LDLR mutation had higher triglyceride levels (1.71 vs 1.39 mmol/l), body mass index (25.6 vs 24.7 kg/m^2) and systolic blood pressure (137 vs 133 mm Hg) and were more likely to have ever smoked (79.5 vs 68.7%), while FH patients with *LDLR* mutation had higher LDL-C levels (8.18 vs 6.61 mmol/l).[25] Successful genotyping for *APOE* was assessed in 2,061 patients of whom

1,150 had an *LDLR* mutation. The polymorphisms at positions 112 and 158 were in Hardy Weinberg equilibrium both in the whole population as in the group with an *LDLR* mutation. Genotype frequencies are shown in supplemental Table 1. General characteristics are shown in Table 1. For the sake of clarity, we only list *APOE3/E3*, *E3/E4* and *E4/E4* and p-values given are for this comparison; patients carrying genotypes consisting of one or two *APOE2* alleles were not significantly different except that triglyceride levels appeared to be increased by the APOE2 allele in the group without *LDLR* mutation (supplemental Table 2; p-values comparing all genotypes). HDL-C levels also appeared significantly different in the group without *LDLR* mutation but this association disappeared after adjustment for triglyceride levels.

In the total cohort successfully genotyped for *APOE*, 603 CHD events occurred of which 547 were in the *E3/E3, E3/E4* and *E4/E4* genotyped individuals (Table 1) Classical risk factors and lipid levels were not significantly different between subjects carrying the different *APOE* genotypes except Lp(a) levels in the analysis restricted to FH patients with an *LDLR* mutation: *E4/E4* genotyped FH patients with an *LDLR* mutation displayed the lowest Lp(a) levels while *E3/E4* genotyped FH patients with a mutation were intermediate between *E3/E3* and *E4/E4*. The most striking difference was the prevalence of coronary heart disease: this was lowest in the *APOE4/E4* genotyped patients, but only in the group with an *LDLR* mutation (*E4/E4* 6.1% vs 25.1% in *E3/E3*; p=0.009; group without *LDLR* mutation 36.4% vs 37.1%; p=0.61).

LDL receptor mutation protective in patients expressing ApoE4

The effect of the presence of an *LDLR* mutation on CHD risk in these FH patients is shown in Table 2. Within the entire cohort, the presence of an *LDLR* mutation was not significantly associated with CHD (HR 0.88; 95% CI 0.76-1.02; p=0.10). The borderline significant protective effect of having an LDLR mutation appeared to be due to the larger burden of classical risk factors such as smoking and hypertension in FH patients without LDLR mutation; as published earlier, when adjusting for these additional risk factors the apparent difference in CHD risk difference did no longer exist.[25] However, in *E4/E4* genotyped FH patients a strong protective effect of an *LDLR* mutation was observed (HR 0.16; 95% CI 0.05-058; p=0.005). Since the groups with and without *LDLR* mutation differed with regard to classical risk factors such as smoking and hypertension, we added different covariates to the analyses. None did influence the protective effect of the *LDLR* mutation in *APOE4/E4* genotyped individuals in this cohort of individuals with the clinical diagnosis of FH.

APOE4/ E4 protective in the presence of an LDLR mutation?

Since it appeared that the lowest number of events occurred in *E4/E4* genotyped patients with an *LDLR* mutation (Table 1), we tested whether the *APOE4* allele was associated with a lower CHD risk using a Cox proportional hazards model (Table 3). In the complete study population, the *APOE4/E4* genotype was not significantly related to CHD (*E4/E4* vs *E3/E3* HR 0.66; 95% CI 0.42-1.05; p=0.94). However, separate analyses of patients with and without *LDLR* mutation showed that a CHD protective effect was restricted to *APOE4/E4* homozygotes who had an *LDLR* mutation (no *LDLR* mutation: HR 1.16; 95% CI 0.69-1.95; p=0.57; with *LDLR* mutation: HR 0.26 HR 0.08-0.80; p=0.02). We confirmed the interaction by analyzing *LDLR* mutation status, *APOE* genotype and interaction terms for *LDLR* mutation with *APOE* genotype. Indeed, the interaction term *APOE4/E4*LDLR* mutation was highly significant (HR 0.22; 95% CI 0.06-0.76; p=0.016). CHD risk in carriers of *APOE2* containing genotypes was not significantly different from CHD risk in *APOE3/E3* genotyped FH patients (data not shown).

TABLE 3. Protective effect *APOE4/E4* genotype in FH patients with an *LDLR* mutation

	Without ***LDLR*** mutation				With ***LDLR*** mutation			
	N	**HR**	**95% CI**	***p***	**N**	**HR**	**95% CI**	***p***
APOE3/E3	447	(ref)			603	(ref)		
APOE3/E4	260	0.83	0.64-1.07	*0.15*	258	0.97	0.72-1.30	*0.83*
APOE4/E4	38	1.16	0.69-1.95	*0.57*	43	0.26	0.08-0.80	*0.02*

Adjusted gender, birth year, smoking

DISCUSSION

We show for the first time in humans that the LDL receptor plays a key role in the detrimental consequences of Apolipoprotein E4 carriership that is known to lead to the development of CHD. Our data reveal a protective role for *LDLR* mutations in FH subjects carrying the APO*E4/E4* genotype; in fact, this genotype seems to even reduce CHD risk in FH patients, in contrast to the increased risk that ApoE4 confers in the general population.[11] It should be stressed that having an *LDLR* mutation is detrimental for CHD risk in the first place, and since our study is restricted to analyses within a severely hypercholesterolemic population we cannot say that CHD risk in FH patients with an LDLR mutation is normalized by the APOE4*/E4* genotype; most likely CHD risk is still increased but to a lesser extent in comparison with other *APOE* genotypes. This CHD risk reduction might partly be explained by lower Lp(a) levels, but in all likelihood this cannot be directly deduced from our study.

ApoE3 and ApoE4 differ by only one amino acid at position 112, but the effects on the risk of CHD and Alzheimer's disease show that the consequences of such a change can be immense. In humans, the interaction between ApoE and the LDL receptor has been

studied only indirectly. Three small studies have previously shown that *APOE4/E4* is not a genetic risk factor for CHD in FH patients despite the observed increased risk in *APOE4/E4* carriers in the general population.[16, 17, 26] Similarly, two polymorphisms in the *LDLR* gene were linked to Alzheimer's disease, but only in patients carrying at least one *APOE4* allele.[27] To the best of our knowledge, a role of the ApoE-LDL receptor interaction in the etiology of atherosclerosis in human subjects is a novel concept.

Most studies investigating the interaction between the LDL receptor and ApoE have been performed in murine models in which the *ldlr* gene was either knocked out or over-expressed by replacing the mouse *ldlr* gene by the human *LDLR* gene. *Ldlr* knockout mice homozygous for the human *APOE4* haplotype (*Ldlr*$^{-/-}$ *Apoe*$^{4/4}$) display less atherosclerosis in response to a Western-type diet than both *Apoe*$^{4/4}$ mice over-expressing the human LDL receptor and *Apoe*$^{4/4}$ mice with physiological murine ldl receptor levels.[14, 15] Mice homozygous for the *APOE3* isoform did not show different responses to variations in LDL receptor expression.

We recognize a number of weaknesses of our study. Firstly, all patients in our cohort had hypercholesterolemia, which means that patients in this cohort without an *LDLR* mutation cannot be considered as healthy controls; in fact, they are individuals having another primary lipid disorder, most likely familial combined hyperlipidemia (FCH).[25] On the other hand, this fact implies that hypercholesterolemia per se cannot be responsible for the interaction. The fact that in our study the *APOE4/E4* genotype was not associated with CHD in patients without an *LDLR* mutation, in contrast to a strong and consistent association in earlier studies and meta-analyses, suggests that the contribution of the *APOE4/E4* genotype in patients with severe dyslipidemia is small.[11, 12] If the control group would have consisted of persons without hypercholesterolemia, the *APOE4/E4* genotyped individuals would most likely have been identified as having an increased CHD risk. The distributions of the different *LDLR* mutations were similar among *APOE* genotypes. However, we did not measure residual LDL receptor activity in all individuals.

A number of artefacts could underlie our remarkable findings. Survival bias was not likely in view of the Hardy Weinberg equilibrium and similar ages of the different genotype groups. We confirmed the *APOE* genotype distribution in an additional cohort of FH patients but the number was too small to consider this as a true replication (n=464; data not shown). Classical CHD risk factors, most importantly smoking and untreated total and LDL-cholesterol levels, did not differ between *APOE* genotypes with or without *LDLR* mutation. Earlier studies suggested an interaction between *APOE4* and smoking.[28, 29] Adjustment for smoking did not change our results. Differences in risk profiles, especially untreated lipid levels, might have led to earlier statin treatment and consequent risk reduction. However, as expected from the lack of differences in terms of classical risk factors, age of starting statins was similar between different *APOE* genotypes with or without *LDLR* mutation.

The substitution at position 112 of a cystein in ApoE3 by an arginin in ApoE4 causes structural variations. Firstly, the interaction between the N-terminal and C-terminal domain leads to a more compact structure with lower thermal and chemical stability

making ApoE4 more prone to aggregation and the formation of molten globules and subsequently to increased degradation in the unfolded protein pathway.[30-32] Secondly, ApoE4 displays altered preference for lipoproteins and increased affinity to the LDL receptor.[8, 9, 33-36] There is substantial evidence that the higher binding affinity of ApoE4 to the LDL receptor leads to unfavourable 'trapping' of ApoE, enhancing sequestration of VLDL at the hepatocyte surface, which consequently delays internalization leading to an increased conversion at the hepatocyte surface of VLDL and intestine-derived chylomicrons into atherogenic remnants.[13, 14, 37]

Our findings might therefore be explained by the interaction between ApoE4 and less functional LDL receptors. Alternatively, increased binding to other receptors, such as related LDLR family members or proteoglycans, could also explain why the presence of an LDLR mutation might be beneficial for *APOE4/E4* genotyped persons.[38-42] Many of these receptors display favourable effects on atherosclerosis, which might be more pronounced if more ApoE is able to bind to these receptors.[38-43]

In conclusion, we show that an *LDLR* mutation is protective in FH patients with the *APOE4/E4* genotype, and that this genotype even seems to reduce CHD risk in FH patients with an *LDLR* mutation instead of increasing it as observed in the general population. This risk reduction might involve reduced Lp(a) levels. Further studies are needed to unravel the biological basis of our finding and to find therapeutic approaches using this interaction in the prevention of CHD. These results might be extrapolated to an important role of this interaction in Alzheimer disease as well

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SUPPLEMENTAL TABLE 1. Genotype frequencies

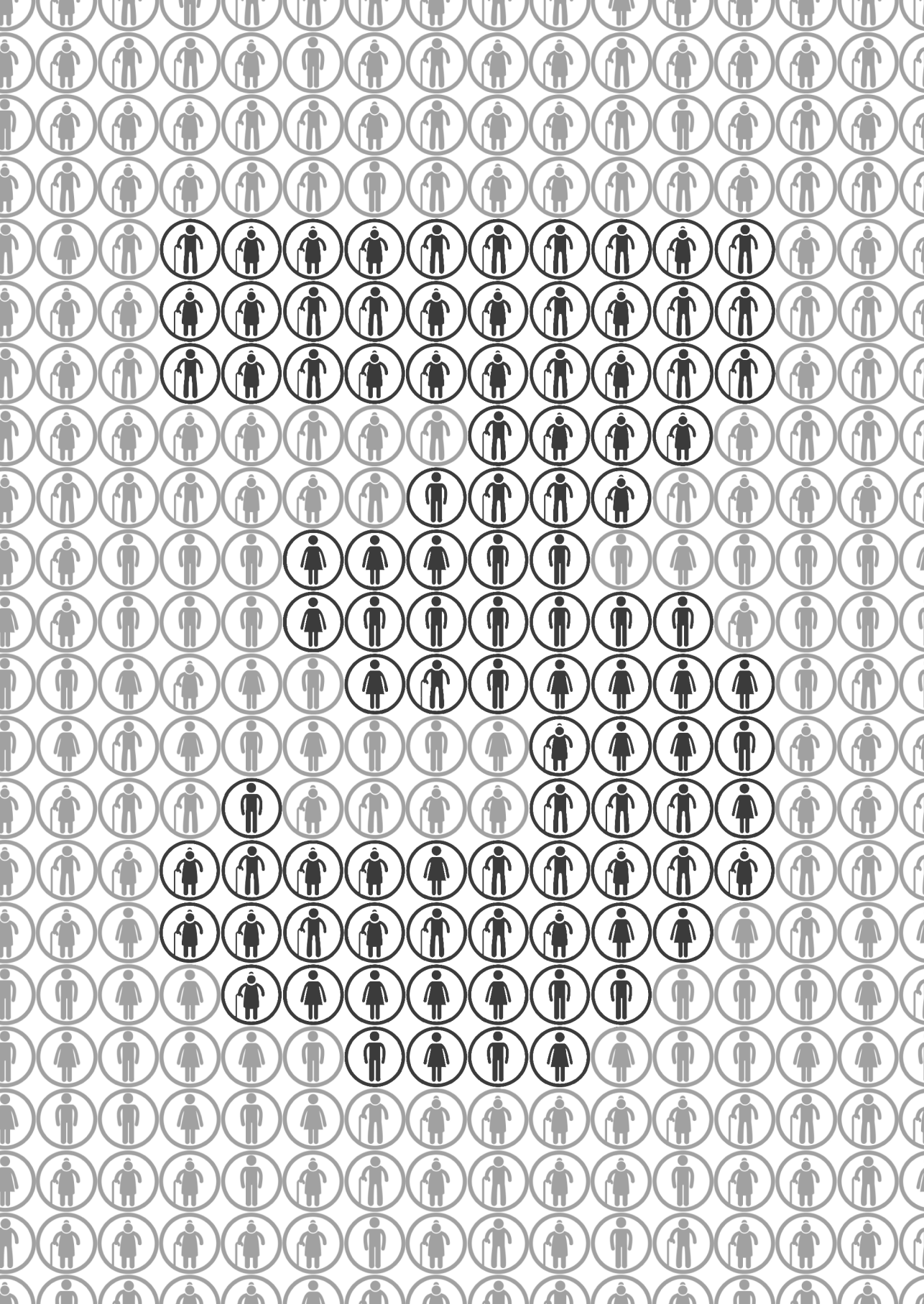
	Total	No *LDLR* mutation	*LDLR* mutation present
APOE3/E3	1178 (57.2)	502 (55.1)	676 (58.8)
APOE2/E3	140 (6.8)	48 (5.3)	92 (8)
APOE3/E4	580 (28.1)	289 (31.7)	291 (25.3)
APOE2/E4	61 (3.0)	21 (2.3)	40 (3.5)
APOE2/E2	9 (0.4)	7 (0.8)	2 (0.2)
APOE4/E4	93 (4.5)	44 (4.8)	49 (4.3)

Values are count (percentage)

SUPPLEMENTAL TABLE 2. General characteristics per *APOE2* containing genotypes with and without *LDLR* mutation

	No *LDLR* mutation				*LDLR* mutation present			
	APOE2/E2	***APOE2/E3***	***APOE2/E4***	***p-value***	***APOE2/E2***	***APOE2/E3***	***APOE2/E4***	***p-value***
Male (%)	3 (42.9)	29 (60.4)	13 (61.9)	0.39	1 (50)	41 (44.6)	19 (47.5)	0.45
Age first visit lipid clinic	57.0±12.8	49.0±13.3	50.5±13.3	0.42	51.5±1.3	43.3±14.3	43.1±12.2	0.66
Follow-up time	56.9±13.7	51.0±13.9	50.1±14.4	0.71	54.9±2.9	46.3±14.1	47.1±12.6	0.73
Smoking ever (%)	3 (50)	33 (76.7)	17 (89.5)	0.38	1 (100)	57 (67.9)	26 (70.3)	0.98
Hypertension (%)	2 (28.6)	7 (15.2)	3 (14.3)	0.35	0	7 (7.6)	3 (8.1)	0.98
Diabetes (%)	1 (14.3)	2 (4.2)	1 (4.8)	0.39	0	5 (5.4)	4 (10)	0.67
Coronary heart disease (%)	4 (57.1)	15 (31.3)	8 (38.1)	0.72	0	20(21.7)	9 (22.5)	0.06
Statin at baseline	2 (28.6)	11 (22.9)	5 (23.8)	0.96	1 (50)	31 (33.7)	16 (40)	0.90
Age statin started	56.9±11.4	46.9±12.9	44.7±13.8	0.24	46.5±6.1	40.5±13.8	39.9±12.6	0.72
BMI (kg/m2)	25.3±3.8	25.0±3.0	24.3±3.3	0.69	24.3±2.7	24.9±3.7	24.5±2.9	0.81
Total cholesterol (mmol/l)	9.08±2.34	8.21±1.26	8.8±1.06	0.39	16.10	9.60±2.35	10.34±2.17	0.08
LDL-cholesterol (mmol/l)	5.94±1.30	6.08±1.10	6.28±0.88	0.90	13.42	7.22±2.49	7.95±1.82	0.04
HDL-cholesterol (mmol/l)	1.34±0.22	1.11±0.35	1.03±0.23	0.02	1.47	1.20±0.34	1.09±0.29	0.39
Triglycerides (mmol/l)	2.88±1.33	1.99±0.91	2.25±1.26	0.01	2.89	1.52±0.82	1.76±0.98	0.14
Lp(a) (mg/l)	77±89	107±151	147±239	0.05	122±72	122±152	134±144	0.02
Lp(a)>3mg/dl	1 (14.3)	12 (30)	5 (33.3)	0.22	0	20 (27)	6 (23.1)	0.11

BMI: Body mass index; CHD: Coronary heart disease; Values are mean±standard deviation; Follow-up time from birth to event or censoring



3 Identifying genetic risk variants for coronary heart disease in familial hypercholesterolemia: an extreme genetics approach

Jorie Versmissen†
Daniëlla M Oosterveer†
Mojgan Yazdanpanah†
Abbas Dehghan
Hilma Hólm
Jeanette Erdman
Yurii S Aulchenko
Gudmar Thorleifsson
Heribert Schunker
Ranitha Vongpromek
André G Uitterlinden
Joep C Defesche
Cornelia M van Duyn
Monique Mulder
Tony Dadd
Hróbjartur D Karlsson
Jose Ordovas
Iris Kindt
Amelia Jarman
Albert Hofman
Leonie van Vark-van der Zee
Adriana C Blommestijn-Touw
Jaap Kwekkeboom
Anho H Liem
Frans J van der Ouderaa
Sebastiano Calandra
Stefano Bertolini
Maurizio Averna
Leiv Ose
Emilio Ros
Fatimá Almagro
Peter W de Leeuw
Fernando Civiera
Jorge Ordoñez
Luis Masana
Xavier Pintó
Maarten L Simoons
Arend FL Schinkel
Martin R Green
Aeilko H Zwinderman
Keith J Johnson
Arne Schaefer
Andrew Neil
Steve E Humphries
Jacqueline CM Witteman
John JP Kastelein
Eric JG Sijbrands

† *These authors contributed equally to this work*

MANUSCRIPT IN PREPARATION

ABSTRACT

Background

Mutations in the low-density lipoprotein (LDL) receptor gene cause familial hypercholesterolemia (FH), characterized by coronary heart disease (CHD) at young age. We aimed to apply an extreme sampling method to enhance the statistical power to identify novel genetic risk variants for CHD in FH.

Methods

We selected cases and controls with an extreme contrast in CHD risk out of 17000 FH patients, whose functional LDL receptor mutation had been identified. A genome-wide association (GWA) study (ARGOS) consisted of 249 very young FH cases with CHD and 217 old FH controls without CHD (above 65 years for males and 70 years of age for females). The GWA study was performed using the Illumina HumanHap 550K chip. In the next stage, two independent samples of FH patients were used as replication samples. The first one, MARCH, included 190 FH cases and 226 FH controls based on the same sampling method (although less extreme) from Norway, Spain and the United Kingdom. The second consisted of 2022 FH patients without selection on outcome or age from a large FH Follow-up cohort (FHFU).

Results

In the initial GWA analysis we identified 29 independent single nucleotide polymorphisms (SNPs) with suggestive association with premature CHD ($p<1x10^{-4}$). We examined the association of these SNPs with risk of CHD in the replication samples. After Bonferroni correction, none of the SNPs either replicated or reached genome-wide significance after combining the discovery and replication samples.

Conclusions

Applying an 'extreme genetics' approach did not help to identify previously unknown genetic risk variants influencing CHD risk.

INTRODUCTION

Coronary heart disease (CHD) is one of the leading causes of death.[1] Multiple genetic risk variants with small to moderate effects on the susceptibility to CHD have so far been identified in genome-wide association (GWA) studies, however, these variants explain only a small fraction of the heritable component of the risk of CHD.[2, 3] Therefore, many genetic variants remain to be discovered. Amongst the discovered genes, many are related to lipid metabolism.[4, 5] Populations who already have high lipid levels are suitable to search for genes that increase the risk of CHD beyond dyslipidemia.[6] Therefore, we performed a GWA study in a selected sample of familial hypercholesterolemia (FH) patients having severe hypercholesterolemia caused by mutations in the low-density lipoprotein (LDL) receptor gene.[7]

Traditional GWA studies are performed on extremely large sample sizes including tens of thousands of individuals.[8] This approach was not achievable for our study given the fact that the number of FH patients is limited. Therefore, we decided to use an 'extreme approach to enhance the statistical power. In this method only the individuals with an extreme of the phenotype are genotyped. In our study, we genotyped FH patients who had CHD at very young age as cases and elderly patients who, despite their high LDL cholesterol level, had experienced no CHD as controls. We hypothesized that using this design our statistical power would enhance to identify genetic risk variants that affect the risk of CHD in FH. The results of such study in FH could potentially be translated to the general population.

METHODS

The schematic study design is shown in Figure 1. We performed the study in two stages. Stage I included a GWA study in the Dutch 'Association of CHD Risk in a Genome-wide

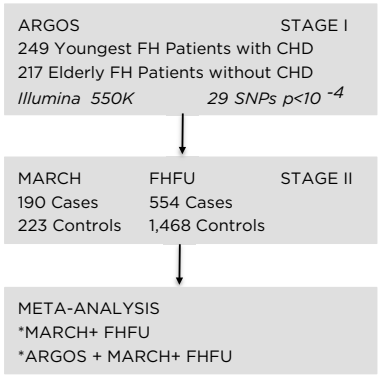


FIGURE 1. Study design.
Abbreviations: CHD coronary heart disease; FH familial hypercholesterolemia; GWA genome-wide association; SNP single nucleotide polymorphism.

Old-versus-young Setting' (ARGOS) sample. Stage II consisted of genotyping of a second case-control sample we refer to as MARCH and a large FH cohort we refer to as FHFU. All patients were of Caucasian descent. In ARGOS this was confirmed by multi-dimensional scaling of identical-by-state pairwise distances. All patients gave informed consent and the local ethics committees approved the protocol.

Description of the populations

Gene-finding stage: ARGOS

The ARGOS sample consisted of 500 patients, who were selected from 13,000 Dutch FH patients with a mutation in the LDL receptor gene. They were recruited from 38 lipid and cardiology clinics in the Netherlands in the nationwide molecular screening program of the 'Stichting Opsporing Erfelijke Familiare Hypercholesterolemie (StOEH)'.[9] Phenotypic data (including CHD events) were acquired from general practitioners and by reviewing medical records at the lipid and cardiologic clinics. We selected the 264 youngest patients with premature CHD and the 236 oldest patients without any CHD, stratified for sex. The maximum age of the female cases was 60 and that of the male cases 45 years. The minimum age of the controls was 65 for males and 70 for females. First and second degree family members were excluded. CHD was defined as the presence of at least one of the following: *(i)* myocardial infarction (MI), proved by at least two of the following: *(a)* classical symptoms (>15 minutes), *(b)* specific abnormalities on electrocardiography (ECG), and *(c)* elevated cardiac enzymes (>2x upper limit of normal); *(ii)* percutaneous coronary intervention or other invasive procedures; *(iii)* coronary artery bypass grafting (CABG). Patients with angina pectoris were excluded because in the majority of cases, this diagnosis could not be assured by objective data.

MARCH

The MARCH study group consisted of 413 FH patients (190 cases, 223 controls), from Italy, Norway, Spain, and the United Kingdom. A few patients were born in another country but all were Caucasian. All patients had clinically proven FH and a mutation in the *LDLR* or *APOB* gene. The maximum age of the female cases was 59 and that of the male cases 45 years. The minimum age of the controls was 50 for males and 60 for females. For the cases in this cohort, the same CHD definition was applied as described above for the ARGOS sample with the addition of *(iv)* angina pectoris (AP), since this phenotype was accurately addressed in this cohort. AP was diagnosed as classical symptoms in combination with at least one unequivocal positive result of one of the following: *(a)* exercise test, *(b)* nuclear scintigram, *(c)* dobutamine stress ultrasound, or *(d)* more than 70% stenosis on a coronary angiogram. The controls had no manifest CHD.

FHFU Study

The second replication cohort consisted of Dutch clinically proven heterozygous FH patients who were recruited from 27 lipid clinics in the Netherlands between 1989 and 2002.[10, 11] For the cases in this cohort, the same CHD definition was applied as described above for the ARGOS sample. The controls had no manifest CHD. The DNA of 2,073 unrelated patients was available for the present analysis. A total 51 FH patients had already been inlcluded in the ARGOS sample and they were removed, leaving 2,022 for analyses.

Additional cohorts

Besides these three FH cohorts, selected SNPs were also genotyped in population-based cohorts described in detail previously: The Rotterdam Study, deCODE and GerMIFSII.[12-14]

Genotyping in ARGOS

The samples of participants of the ARGOS study were assayed with Illumina Infinium HumanHap550K Chips (Illumina, San Diego, USA) at Erasmus University Medical Center in Rotterdam, the Netherlands. Samples were processed according to the Illumina Infinium II manual. In brief, each sample was whole-genome amplified, fragmented, precipitated and resuspended in the appropriate hybridization buffer. After hybridization, these denatured samples were processed for the single-base extension reaction and were stained and imaged on an Illumina Bead Array Reader. Normalized bead-intensity data obtained for each sample were loaded into the Illumina Beadstudio software where the fluorescent intensities were converted into SNP genotypes.

Quality control filtering

After genome-wide genotyping, a call-rate threshold above 98% was used for inclusion of the samples. SNPs were excluded if they had *(i)* successful genotyping in less than 90% of the cases and controls, *(ii)* a minor allele frequency less than 1% in the population, or *(iii)* showed deviation from Hardy-Weinberg equilibrium (p-value < 0.0001).

Genotyping in MARCH and FHFU

In MARCH and FHFU, the genotypes of selected SNPs were determined using fluorescence-based TaqMan allelic discrimination assays and analyzed on an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). Reaction components and amplification parameters were based on the manufacturer's instructions using an annealing temperature of 60°C. Results were scored blinded to CHD status. SNPs were excluded following the same quality criteria as in the gene-finding stage.

Statistical analysis

For each SNP which passed the quality control in the ARGOS sample, the association with risk of CHD was examined in an additive genetic model using a logistic regression model adjusted for sex. Given the fact that age was an inclusion criterion to generate the contrast between cases and controls of the ARGOS study we did not further adjust for age.

We selected all SNPs that were associated with CHD with a p-value < 1.00×10^{-4} in ARGOS to analyze in the second stage. In the second stage the association with risk of CHD was examined using logistic regression in MARCH and the FHFU. In MARCH, we adjusted for sex only, since age was a selection criterion similar to ARGOS. In the FHFU cohort, we adjusted for age, sex and statin use since statin use was expected to be a confounder and, in contrast to ARGOS and MARCH, well-documented in the FHFU cohort. Using Bonferroni correction, significance threshold was 0.0017 (0.05/29). We performed a z-based meta-analysis to combine the results of MARCH and FHFU in the second stage. Furthermore, we combined the results of all three studies using z-based meta-analysis.

We used Plink version 1.03 to run GWA study in ARGOS, SPSS version 15 to run logistic regression models in MARCH, and FHFU and finally 'meta' and 'rmeta' packages running under R to perform the meta-analysis.[15-17]

Replication of well-known SNPs associated with CHD

To test the effect of earlier defined genetic risk variants on CHD in ARGOS, we analyzed the SNPs described in a large-scale meta-analysis in the CARDIoGRAM consortium(22,233 cases and 64,762 controls). In CARDIoGRAM, 9 out of 12 previously reported CHD loci were confirmed with a p-value of < 5.0×10^{-8} and 13 new ones identified (Supplemental Table 3). [5] Whenever the SNP was not available on the IlluminaHap550, we used a proxy as identified by SNAP.[18]

RESULTS

Characteristics of the ARGOS cohort

From over 17,000 Dutch FH patients with a known LDL receptor mutation, we selected the 264 youngest FH patients with CHD and the 236 oldest FH patients without any CHD. The mean±SD (range) age was 41.7±8.3 (23 to 59 years) in cases and 75.7±5.9 (65 to 92 years) in controls. A total of 249 cases and 217 controls were successfully genotyped. There were no significant differences in age, smoking and cholesterol levels between the patients who were and those who were not successfully genotyped (data not shown). General characteristics of the genotyped patients are shown in Table 1 and the age distribution in Supplemental Figure 1.

TABLE 1. Characteristics of ARGOS and the replication samples

		Males	Age	Smoking	Diabetes Mellitus	Hypertension	Total Cholesterol
Sample	**N**	(%)	(years)	(%)	(%)	(%)	(mmol/l)
Stage I: Genome-wide analysis							
ARGOS							
CHD cases	249	55.0	41.7 ± 8.4*	74.2*	2.4*	19.7*	11.3 ± 2.6*
Controls	217	47.9	75.6 ± 5.9*	51.2*	9.2*	30.0*	10.6 ± 2.8*
Stage II: Replication							
FHFU							
CHD cases	554	65.5*	48.9 ± 10.6*	83.0*	4.8*	16.7*	9.5 ± 2.1
Controls	1,468	43.5*	46.6 ± 12.7*	70.3*	1.7*	6.0*	9.4 ± 1.9
MARCH							
CHD cases	190	63.2	39.4 ± 7.4	49.5	10.0	36.3	11.0 ± 2.2*
Controls	223	60.1	63.6 ± 9.2	47.1	11.2	34.5	10.5 ± 1.7*

Continuous variables are given as mean ± standard deviation. Abbreviations: CHD coronary heart disease; FH familial hypercholesterolemia; N number of participants. * p < 0.05 for the difference between CHD cases and controls within the cohort.

Eighty-one cases (32.5%) had an LDL receptor negative mutation, i.e. a mutation leading to complete loss of function of the LDL receptor, while only 42 controls (19.4%) had a receptor negative mutation p=0.004. This was mainly due to an overrepresentation of the 1359-1 mutation, which was present in 46 cases and only in 21 controls. The other mutations were equally distributed (Supplemental Table 1). On average, the controls were 34 years older than the cases (p-value <0.001). Consequently, hypertension and diabetes mellitus were more often present in the controls than in the cases. More cases than controls were smokers (Table 1).

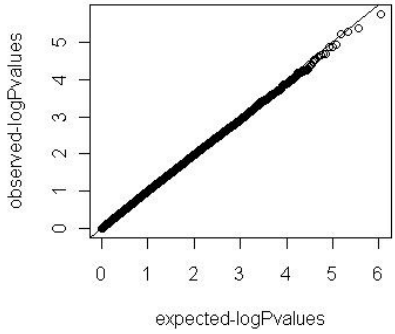


FIGURE 2. QQ Plot GWA in ARGOS.

Stage I (GWA analysis)

After quality-control filtering, we included 535,179 SNPs in the GWA analysis. A quantile-quantile plot of the observed against expected p-value distribution is shown in Figure 2. The genomic inflation factor (λgc) was 1.01 in the total sample. The Supplemental Figure 2 illustrates the primary findings from the GWA analysis in the ARGOS population and presents p-values for each of the interrogated SNPs across the chromosomes. A total of 40 SNPs clustered around 21 loci on all chromosomes except 3, 6, 12, 15, 16 and 18-21 exceeded a p-value threshold of $1x10^{-4}$ (Table 2). Of these, 11 were in complete linkage disequilibrium (LD) with a leading SNP in the same locus. Twelve out of 40 SNPs were located in a cluster on chromosome 11p15; they were located in four different LD blocks (Supplemental Figure 3) and could be tagged by six SNPs. Including these six, we took 29 SNPs to stage II.

Stage II

We successfully genotyped 28 SNPs in MARCH and all 29 SNPs in FHFU (Supplemental Table 2). None of the SNPs exceeded the Bonferroni corrected threshold of 0.017 either in MARCH or FHFU (Table 3). The smallest p-value was found for rs176388 (OR 0.33, p=0.042) in MARCH. Although the direction of the effect was the same in FHFU, the association was not significant (OR 0.80, p=0.36). We performed a meta-analysis to combine the results of the analysis in MARCH and FHFU. None of the SNPs reached the Bonferroni significant threshold after combining the results of MARCH and FHFU.

Finally, we combined the results of ARGOS, MARCH, and FHFU, however, none of the top SNPs identified in ARGOS reached the genome-wide significance level. The smallest combined p-value was 4×10^{-4} for of rs176388. Genotyping additional cohorts from the general population did not show any genome-wide significance.

Replication of well-known SNPs associated with CHD in the general population

To examine if the genetic risk variants identified in GWA studies in the general population also showed an effect in ARGOS and to test whether effect sizes were larger in our 'extreme genetics' population, we examined the association of 25 previously reported genetic risk factors (Supplemental Table 3). None of the studied SNPs were significantly associated with CHD in ARGOS. Lowest p-values were obtained for rs4977574 at 9p21 (p=0.05) and for the SNP in *PCSK9* that did not show genomic signficance in CARDIoGRAM (CARDIoGRAM OR 1.08, $p=9.1x10^{-8}$; ARGOS OR 1.52, p=0.01).

TABLE 2. SNPs influencing coronary heart disease risk in ARGOS (with p < 1.0x10-4)

SNP	Chr	Position	Allele	SNP type	Gene	Frequency Cases	Frequency Controls	OR	95% CI	p-value
rs12087224	1p22	83,902,972	A	intergenic	TTLL7	0.15	0.06	2.54	1.59-4.04	8.86x10-5
rs7581691	2p24	13,953,005	A	-	-	0.16	0.07	2.62	1.67-4.11	2.93x10-5
rs1406333	2q32	184,155,187	G	intergenic	NUP35	0.33	0.47	0.59	0.45-0.77	8.23x10-5
rs2412397	4q12	53,804,380	G	intronic	SCFD2	0.31	0.44	0.57	0.43-0.76	9.98x10-5
rs778940	4q13	62,926,680	G	intergenic	LPHN3	0.16	0.07	2.44	1.57-3.77	6.74x10-5
rs6835823	4q32	155,191,807	C	intergenic	DCHS2	0.48	0.37	1.77	1.36-2.30	2.11x10-5
rs4696207	4q32	155,488,838	A	intronic	DCHS2	0.50	0.36	1.80	1.36-2.38	3.36x10-5
rs7691894	4q32	167,900,239	G	intronic	SPOCK3	0.39	0.23	2.06	1.53-2.77	1.74x10-6
rs11950716	5p15.2	14,141,834	A	intergenic	TRIO	0.14	0.25	0.51	0.36-0.71	9.45x10-5
rs17638888	7q22	98,335,561	A	intronic	TRRAP	0.03	0.09	0.27	0.14-0.51	5.89x10-5
rs12544799	8q24.2	130,732,692	G	intergenic	MLZE	0.41	0.28	1.87	1.40-2.50	2.53x10-5
rs6985166	8q24.2	130,748,358	G	intergenic	MLZE	0.38	0.26	1.81	1.35-2.43	7.81x10-5
rs13291498	9p21	28,326,185	C	intronic	LINGO2	0.21	0.33	0.55	0.41-0.73	5.44x10-5
rs10761805	10q21	65,294,147	A	intergenic	REEP3	0.49	0.35	1.89	1.42-2.51	1.36x10-5
rs955353	10q21	65,314,593	A	-	-	0.37	0.50	0.57	0.43-0.75	7.20x10-5
rs2111995	10q25	107,497,352	G	intergenic	SORCS3	0.28	0.17	1.97	1.42-2.72	4.10x10-5
rs2647547	11p15	5,359,744	C	intergenic	OR51M1	0.46	0.33	1.75	1.33-2.31	5.85x10-5
rs1532514	11p15	5,373,198	A	intergenic	OR51M1	0.48	0.38	1.71	1.32-2.23	6.37x10-5
rs10838092	11p15	5,400,443	A	exonic	OR51Q1	0.45	0.31	1.73	1.32-2.26	6.73x10-5
rs10838102	11p15	5,414,207	A	intergenic	OR51I1	0.46	0.32	1.78	1.36-2.33	3.06x10-5
rs1498486	11p15	5,418,567	C	exonic	OR51I1	0.48	0.34	1.81	1.38-2.38	2.02x10-5
rs2133235	11p15	5,423,352	A	intergenic	OR51I1	0.44	0.30	1.82	1.28-2.40	2.27x10-5
rs2846186	11q25	134,203,586	G	intergenic	B3GAT1	0.18	0.30	0.50	0.36-0.68	1.18x10-5
rs2661969	11q25	134,223,427	G	intergenic	B3GAT1	0.18	0.29	0.54	0.39-0.73	9.16x10-5
rs1380945	13q13	31,739,619	G	intronic	FRY	0.48	0.35	1.74	1.32-2.30	9.76x10-5
rs4982548	14q11	21,605,911	A	intergenic	OR4E2	0.43	0.57	0.56	0.42-0.73	2.43x10-5
rs2531851	17p13	9,141,140	A	intronic	STX8	0.40	0.27	1.92	1.43-2.59	1.40x10-5
rs5755595	22q12	33,848,839	A	intergenic	RAXLX	0.38	0.26	1.84	1.36-2.48	6.28x10-5
rs5928090	Xp21	32,780,853	G	-	-	0.42	0.27	1.75	1.18-2.60	4.20x10-5

Abbreviations: CI confidence interval; Chr chromosome; OR odds ratio; SNP single nucleotide polymorphism. Intergenic is defined as <500Mb from the gene.

TABLE 3. Results of the genome-wide association study in the two FH replication samples

SNP	Risk allele	MARCH (n=413)		FHFU (N=2,022)		Meta-analysis MARCH+ FHFU		Meta-analysis ARGOS+ MARCH + FHFU	
		OR	p-value	OR	p-value	OR	p-value	OR	p-value
rs12087224	A	1.01	0.96	1.06	0.70	1.04	0.73	1.27	3.1x10-2
rs7581691	A	1.07	0.79	0.86	0.35	0.91	0.51	1.20	1.2x10-1
rs1406333	G	0.98	0.89	0.96	0.71	0.98	0.68	0.84	1.5x10-2
rs2412397	G	NA	NA	0.91	0.36	NA	NA	NA	NA
rs778940	G	0.86	0.53	1.29	0.06	1.16	0.17	1.35	2.9x10-3
rs6835823	C	1.07	0.63	0.94	0.66	1.01	0.93	1.24	8.4x10-3
rs4696207	A	0.90	0.48	1.10	0.33	1.03	0.70	1.19	1.4x10-2
rs7691894	G	1.02	0.99	1.03	0.74	1.02	0.80	1.20	1.2x10-2
rs11950716	A	0.83	0.35	0.94	0.60	0.91	0.34	0.78	5.8x10-3
rs17638888	A	0.52	0.04	0.80	0.36	0.69	0.05	0.54	2.1x10-4
rs12544799	G	1.06	0.70	0.94	0.52	0.98	0.77	1.14	7.6x10-2
rs6985166	G	1.05	0.76	1.09	0.38	1.08	0.36	1.22	6.4x10-3
rs13291498	C	0.92	0.62	1.22	0.06	1.12	0.21	0.92	2.9x10-1
rs10761805	A	1.05	0.76	1.00	0.99	1.01	0.87	1.18	2.2x10-2
rs955353	A	0.88	0.36	1.01	0.91	0.97	0.67	0.85	1.9x10-2
rs2111995	G	0.81	0.22	1.14	0.23	1.03	0.74	1.20	2.3x10-2
rs2647547	C	0.98	0.90	1.04	0.67	1.02	0.79	1.17	2.4x10-2
rs1532514	A	1.04	0.79	1.01	0.88	1.02	0.81	1.16	2.6x10-2
rs10838092	A	1.09	0.59	1.00	0.96	1.02	0.80	1.18	2.4x10-2
rs10838102	A	1.20	0.21	0.96	0.70	1.03	0.71	1.19	1.5x10-2
rs1498486	C	1.11	0.45	1.07	0.51	1.08	0.34	1.23	3.1x10-3
rs2133235	A	1.20	0.22	1.93	0.48	1.01	0.93	1.17	2.5x10-2
rs2846186	G	0.83	0.26	1.06	0.63	0.97	0.76	0.81	1.1x10-2
rs2661969	G	0.83	0.23	0.97	0.97	0.91	0.35	0.79	4.9x10-3
rs1380945	G	1.25	0.13	1.14	0.19	1.17	0.06	1.29	3.0x10-4
rs4982548	A	1.02	0.89	1.07	0.45	1.05	0.48	0.91	1.7x10-1
rs2531851	A	1.07	0.63	0.89	0.23	0.95	0.51	1.11	1.4x10-1
rs5755595	A	0.80	0.15	0.90	0.33	0.86	0.10	1.05	5.4x10-1
rs5928090	G	1.04	0.73	0.95	0.54	0.98	0.73	1.08	2.3x10-1

Combined *p* given for replication samples. Abbreviations FH familial hypercholesterolemia; HR hazard ratio; OR odds ratio.

DISCUSSION

In this study, by using an extreme genetics approach, we aimed to boost our statistical power to identify novel genetic risk variants for CHD. However, none of the suggestive findings were either confirmed in the second stage nor reached the genome-wide significant threshold in meta-analysis of all population combined.

We expected to identify larger effect sizes in our GWA study, compared to traditional GWA studies on CHD, for two reasons. First, we studied genetic risk variants for CHD in

a cohort of FH patients and hypercholesterolemia is one of the most important environmental risk factors for CHD. Based on Rothman's model of causation, one would expect that in presence of similar environmental factors, risk variants in genes will be associated with lager effects.[6] Second, we applied an extreme selection procedure. Therefore, the genetic contrast between cases and controls was expected to increase.[19] This incremental contrast has been shown in simulation studies in quantitative traits.[19-21] Plomin suggested that common disorders could also be considered to be quantitative traits, since risk on a common disease in the population could be regarded as a distribution of 'polygenetic liability' to a disease. The extreme sampling approach should therefore produce larger effect sizes. Thus, the power was expected to improve, despite the reduction in number of individuals as a result of the selection criteria.[19, 20, 22] Our study is the first study to examine this theoretical claim using empirical data. Using Quanto, we estimated that the case-control ARGOS study had more than 80% statistical power to detect effect sizes larger than 1.46 for a conventional p-value threshold of 0.05 and 2.5 for a genome-wide significance level for SNPs with a minor allele frequency > 0.30. So, the study was well-enough powered to identify risk variants with large effect sizes in this high-risk group of subjects. Our findings, however, indicate that one should not expect such an extreme leverage in the effect sizes using this approach. As an example, the well replicated locus at 9p21.3 was only associated with a p-value of 0.05 and had an OR of 1.28, close to 1.25 which was found in the original study of subjects in the general population without an extreme genetics approach. Most other known CHD risk SNPs identified in earlier studies as published by Schunkert et al. were not associated with statistically significant effects in this FH sample.[5] However, out of 18 SNPs that could be tested, 11 ORs were in the same direction while only 6 were not (Supplemental Table 3). Of these 11, most interesting was the SNP in *PCSK9.* This SNP is associated with an 8% higher risk of CHD in the general population, however, the increased risk in ARGOS was more than 50%. The p- value in CARDIoGRAM was not genomewide significant. We believe that this enhanced effect in our study may not be due to chance, but may reflect an interaction between this gene and cholesterol levels. *PCSK9* is a gene encoding proprotein convertase subtilisin/kexin type 9, involved in degradation of LDL receptors. Based on CHD risk reduction of 80% in loss-of-function mutations, PCSK9 inhibitors are tested in phase II trials now, and are expected to be highly effective in FH patients.[23, 24]

One conjecture would be that our definition of extreme groups was too restrictive, since it only constitutes 3.8% of the whole sample of FH patients available (500 out of 13,000).

Our findings may also help to understand why genetic risk prediction models have not yet succeeded. In our approach, we were trying to pick up novel genes by selecting the extreme groups of the CHD risk distribution spectrum. Genetic risk prediction studies, on the other hand, start with genetic information and estimate who will end up in the low and the high risk group.[25] Since we found that the effect sizes in these groups are not as enhanced as we expected, also the contrast needed for prediction might be less than anticipated.

Our approach has a number of limitations. Finding extreme cases is a challenging effort. Although FH is a relatively common genetic disorder, collecting a large sample of subjects

either with early onset CHD or healthy aging is difficult. We had the benefit to have access to registry of our ongoing national screening program for FH that made it possible for us to collect this sample size out of thousands of carriers of LDL receptor mutations. Family members up to second degree were excluded. The low genomic inflation factor (1.01) in the total sample indicated that population admixture was not likely.[26] We realize that from a statistical point of view our sample size was not enough to detect genes with small effect. A last limitation of a GWA study in FH subjects is that the results may not necessarily apply to the general population.[27] Results may be restricted to FH patients or to hypercholesterolemia in general.

We conclude that this extreme genetics approach is not very effective in leveraging the effect sizes and may not lead to significant gains in statistical power. This study might also explain why genetic risk prediction modeling is still disappointing. The results of the SNP rs11206510 in *PCSK9* might point to a more important effect of this variant in FH, and merits further study.

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SUPPLEMENTAL TABLE 1. Frequencies of most common LDL receptor mutations in ARGOS cases and controls. All mutations present more than five times are presented. None of the mutations present in three or four patients were found in cases or controls only. Forty-seven mutations were present in only one individual.

		Cases		Controls	
***LDLR* mutation**	**Null vs defective**	**N**	**%**	**N**	**%**
1359-1 (intron 9)	Null	46	18.5	21	9.7
313+1/2 (intron 3)	Defective	29	11.6	26	12.0
191-2 (intron 2)	Unknown	11	4.4	21	9.7
W23X, exon 2	Null	19	7.6	10	4.6
E207K (exon 4)	Defective	12	4.8	16	7.4
Cape Town-2 (2.5 kb deletion exon 7 en 8)	Defective	15	6.0	6	2.8
S285L (exon 6)	Defective	6	2.4	14	6.5
V408M (exon 9)	Defective	6	2.4	7	3.2
G322S (exon 7)	Defective	5	2.0	7	3.2
R60C (exon 3)	Unknown	1	0.4	8	3.7
L401P (exon 9)	Defective	5	2.0	3	1.4
L590F (exon 12)	Null	6	2.4	2	0.9
P664L (exon 14)	Defective	4	1.6	4	1.8
2390-2 (intron 16)	Defective	3	1.2	4	1.8
R329X (exon 7)	Null	4	1.6	3	1.4
C122X (exon 4)	Null	3	1.2	2	0.9
G571E (exon 12)	Defective	2	0.8	3	1.4
P678L (exon 14)	Unknown	3	1.2	2	0.9
W422C (exon 9)	Defective	1	0.4	4	1.8

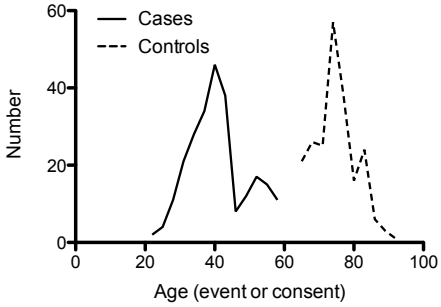
SUPPLEMENTAL TABLE 2. Allele frequencies in the two replication samples

		MARCH			FHFU		
		Frequency			Frequency		
SNP	Reference allele	Overall	Cases	Controls	Overall	Cases	Controls
rs12087224	A	0.11	0.12	0.11	0.11	0.11	0.11
rs7581691	A	0.08	0.09	0.08	0.13	0.12	0.13
rs1406333	G	0.36	0.36	0.37	0.39	0.39	0.40
rs2412397	G	NA	NA	NA	0.35	0.35	0.37
rs778940	G	0.13	0.11	0.13	0.13	0.15	0.12
rs6835823	C	0.44	0.45	0.43	0.38	0.37	0.39
rs4696207	A	0.46	0.45	0.48	0.43	0.45	0.42
rs7691894	G	0.32	0.32	0.32	0.31	0.31	0.31
rs11950716	A	0.19	0.18	0.20	0.18	0.18	0.18
rs17638888	A	0.06	0.04	0.08	0.04	0.04	0.05
rs12544799	G	0.37	0.38	0.37	0.33	0.33	0.33
rs6985166	G	0.32	0.33	0.32	0.29	0.30	0.29
rs13291498	C	0.25	0.25	0.26	0.26	0.29	0.25
rs10761805	A	0.41	0.42	0.41	0.41	0.41	0.41
rs955353	A	0.45	0.44	0.47	0.43	0.43	0.44
rs2111995	G	0.23	0.21	0.24	0.21	0.22	0.21
rs2647547	C	0.44	0.44	0.44	0.42	0.43	0.42
rs1532514	A	0.48	0.48	0.47	0.48	0.49	0.47
rs10838092	A	0.38	0.39	0.37	0.40	0.40	0.40
rs10838102	A	0.42	0.44	0.40	0.41	0.42	0.41
rs1498486	C	0.46	0.47	0.45	0.45	0.47	0.44
rs2133235	A	0.41	0.43	0.39	0.40	0.40	0.40
rs2846186	G	0.26	0.24	0.28	0.23	0.23	0.23
rs2661969	G	0.26	0.24	0.28	0.23	0.23	0.23
rs1380945	G	0.40	0.42	0.37	0.44	0.45	0.43
rs4982548	A	0.48	0.48	0.48	0.50	0.51	0.49
rs2531851	A	0.44	0.45	0.44	0.33	0.30	0.34
rs5755595	A	0.29	0.27	0.31	0.29	0.28	0.30
rs5928090	G	0.44	0.45	0.43	0.40	0.39	0.41

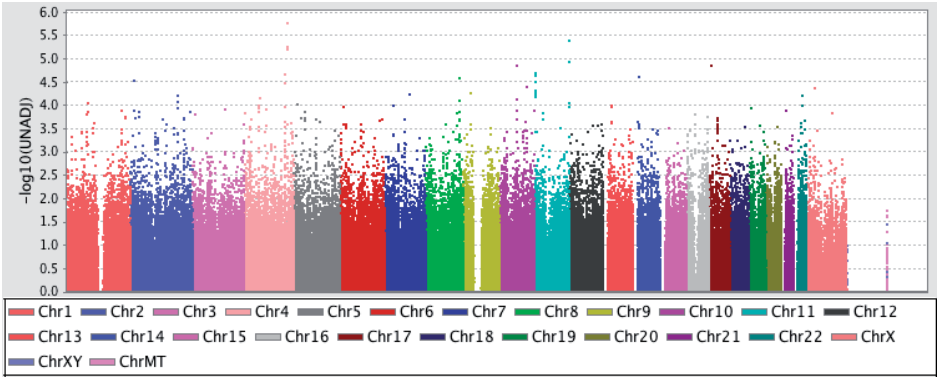
SUPPLEMENTAL TABLE 3. Results of earlier identified genetic risk factors in ARGOS[5]

				CARDIoGRAM					
SNP	**Chromosome**	**Gene(s) in region**	**Risk allele†**	**OR**	**p-value**	**Geno-typed**	**Proxy¶**	**OR§**	**p-value**
rs11206510	1p32.3	*PCSK9*	T (0.82)	1.08	9.1x10-8	Yes		1.52	0.01
rs17114036*	1p32.2	*PPAP2B*	A (0.91)	1.17	2.2x10-6	No	rs9970807	0.87	0.56
rs599839	1p13.3	*SORT1*	A (0.78)	1.11	2.9x10-10	No	rs646776	1.00	0.99
rs17465637	1q41	*MIA3*	C (0.74)	1.14	1.4x10-8	No	No proxy		
rs6725887	2q33.1	*WDR12*	C (0.15)	1.14	1.1x10-9	Yes		1.07	0.76
rs2306374	3q22.3	*MRAS*	C (0.18)	1.12	3.3x10-8	Yes		0.96	0.81
rs12526453	6p24.1	*PHACTR1*	C (0.67)	1.10	1.2x10-9	No	rs4714990	1.03	0.8
rs17609940*	6p21.31	*ANKS1A*	G (0.75)	1.07	2.2x10-6	No	rs820082	0.83	0.25
rs12190287*	6q23.2	*TCF21*	C (0.62)	1.08	4.6x10-11	No	No proxy		
rs3798220	6q25.3	*LPA*	C(0.02)	1.09	3.0x10-11	No	No LD data		
rs11556924*	7q32.2	*ZC3HC1*	C (0.62)	1.09	2.2x10-9	No	No proxy		
rs4977574	9p21.3	*CDKN2A, CDKN2B*	G (0.46)	1.25	1.4x10-22	Yes		1.28	0.05
rs579459*	9q34.2	*ABO*	C (0.21)	1.10	1.2x10-7	No	rs495828	1.35	0.08
rs1746048	10q11.21	*CXCL12*	C (0.87)	1.33	2.9x10-10	Yes		1.41	0.1
rs12413409*	10q24.32	*CYP17A1, CNNM2, NT5C2*	G (0.89)	1.12	1.5x10-6	Yes		1.23	0.36
rs964184*	11q23.3	*ZNF259, APOA5-A4-C3-A1*	G (0.13)	1.13	8.0x10-10	No	No proxy		
rs3184504	12q24.12	*SH2B3*	T (0.44)	1.13	6.4x10-6	Yes		1.01	0.96
rs4773144*	13q34	*COL4A1, COL4A2*	G (0.44)	1.07	4.2x10-7	No	No proxy		
rs2895811*	14q32.2	*HHIPL1*	C (0.43)	1.07	2.7x10-7	Yes		1.05	0.72
rs3825807*	15q25.1	*ADAMTS7*	A (0.57)	1.08	9.6x10-6	No	rs7177699	NA	NA
rs216172*	17p13.3	*SMG6, SRR*	C (0.37)	1.07	6.2x10-7	No	rs2281727	0.85	0.26
rs12936587*	17p11.2	*RASD1, SMCR3, PEMT*	G (0.56)	1.07	4.9x10-7	No	rs11871738	0.83	0.14
rs46522*	17q21.32	*UBE2Z,GIP,ATP5G1,SNF8*	T (0.53)	1.06	3.6x10-6	No	rs962272	1.04	0.73
rs1122608	19p13.2	*LDLR*	G (0.77)	1.14	9.7x10-10	No	rs3786725	0.81	0.17
rs9982601	21q22.11	*MRPS6*	T (0.15)	1.18	4.2x10-10	No	rs7278204	1.28	0.19

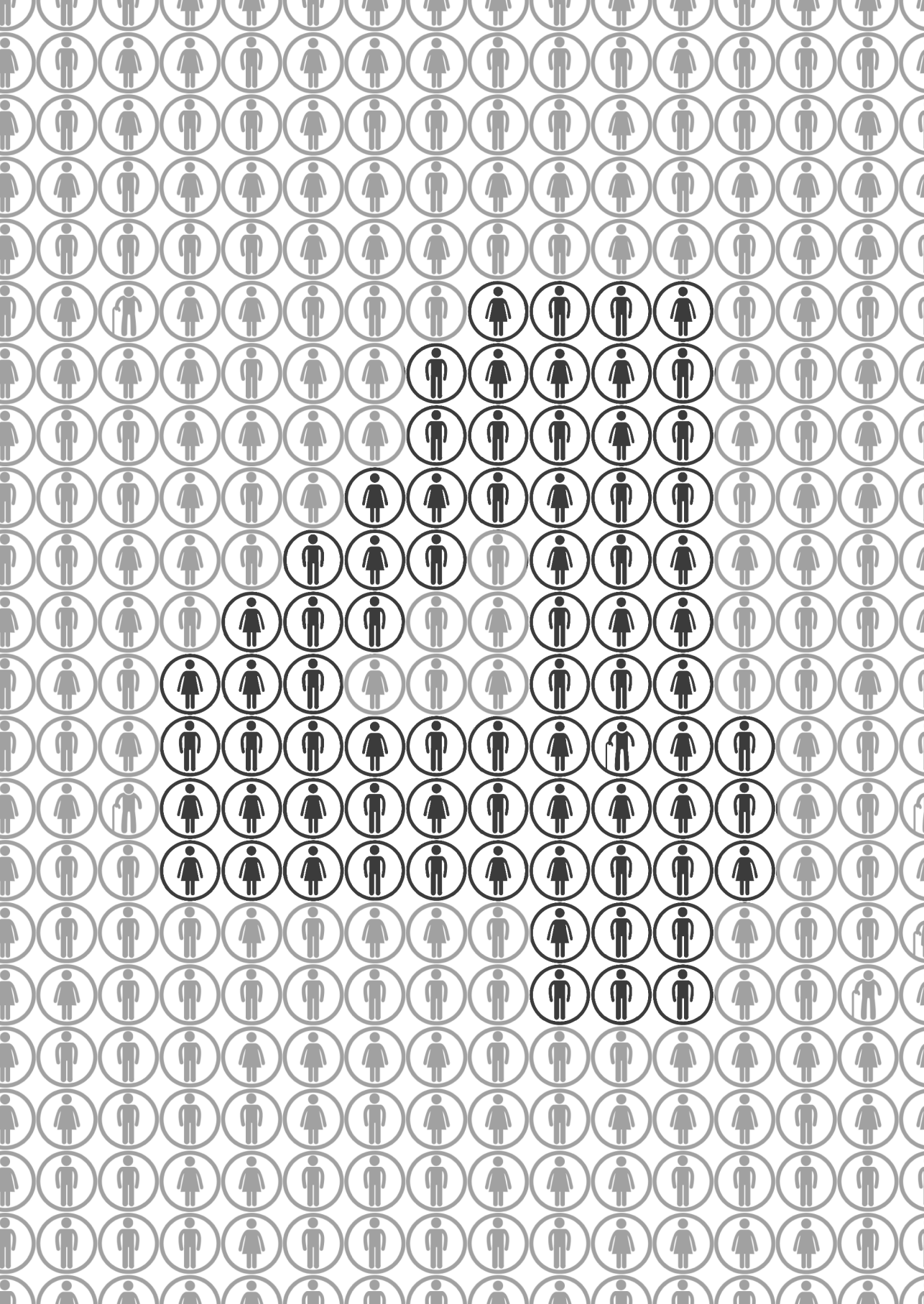
*OR representing combined analysis CARDIoGRAM& replication cohort[5]; †(frequency);
¶ Using SNAP[18] **§** risk allele or representative allele proxy



SUPPLEMENTAL FIGURE 1. Age distribution of cases and controls in ARGOS.



SUPPLEMENTAL FIGURE 2. Signal intensity plot showing the genome-wide association of SNPs with CHD in ARGOS.



4 Low-density lipoprotein receptor mutations generate synthetic genome-wide associations-Signals of common polymorphisms based on rare mutations

Jorie Versmissen†
Daniëlla M. Oosterveer†
Joep C. Defesche†
Suthesh Sivapalaratnam†
Mojgan Yazdanpanah
Monique Mulder
Leonie van der Zee
André G.Uitterlinden
Cornelia M. van Duijn
Albert Hofman
John J.P. Kastelein
Yurii S. Aulchenko
Eric J.G. Sijbrands

†These authors contributed equally to this work.

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ABSTRACT

Objective

Genome-wide association (GWA) studies have discovered multiple common genetic risk variants related to common diseases. It has been proposed that a number of these signals of common polymorphisms are based on synthetic associations that are generated by rare causative variants. We investigated if mutations in the low-density lipoprotein receptor (*LDLR*) gene causing familial hypercholesterolemia (FH, OMIM #143890) produce such signals.

Methods and results

We genotyped 480,254 polymorphisms in 464 FH patients and in 5,945 subjects from the general population. A total of 28 polymorphisms located up to 2.4 Mb from the *LDLR* gene were genome-wide significantly associated with FH ($p<10^{-8}$). We replicated the 10 top signals in 2,189 patients with a clinical diagnosis of FH and in 2,157 subjects of a second sample of the general population ($p< 0.000087$).

Conclusions

Our findings confirm that rare variants are able to cause synthetic genome-wide significant associations, and that they exert this effect at relatively large distances from the causal mutation.

INTRODUCTION

Genome-wide association (GWA) studies comparing hundreds of thousands of common polymorphisms between persons with and without disease have successfully identified genetic risk variants with frequencies > 5%. These genetic variants probably tag frequent causal variants and it is considered unlikely that they are coupled to mutations having frequencies far below 1%.[1, 2] However, Dickson et al. performed intriguing simulation studies suggesting that stochastically occurring coupling between common polymorphisms and rare causal variants can give rise to synthetic association signals in GWA studies.[3] By exploring two autosomal recessive disorders (hearing loss and sickle cell anemia) they showed that GWA signals occur when multiple rare mutations are coupled to a limited number of common polymorphisms. In addition, mutations in the nucleotide-binding oligomerization domain-containing protein 2 (*NOD2*) gene produced synthetic association signals in patients with Crohn's disease and rare variants of hypertriglyceridemia were identified by a GWA.[4, 5] Remarkably, the functional variants were located at a large distances (up to 2.5 Mb) from the synthetic association signal. Nonetheless, the relevance of synthetic signals has been questioned and the examples may be anecdotical.[6-8] The latter is supported by extrapolations from preliminary data of the 1000 Genomes Project showing that associations can be synthetic but that this will hardly occur in case of complex disorders.[9, 10] However, in this phase of the project the power was limited to identify variants with a frequency of >1% and undeniable causal variants were lacking.

Based on our nationwide molecular screening program for familial hypercholesterolemia (FH, OMIM #143890), the rare causal mutation has been identified in a large population displaying extensive heterogeneity at the low-density lipoprotein receptor (*LDLR*) gene.[11] Hence, we had the opportunity to test if well-known rare variants of the *LDLR* gene causing an autosomal dominant disorder create synthetic associations in real data and if such signals can be replicated in an independent sample.

In the present study, we investigated if replicable synthetic associations occur based on mutations in the *LDLR* gene causing FH.

MATERIALS AND METHODS

The 'Association study of coronary heart disease (CHD) Risk factors in the Genome using an Old-versus-young Setting' (ARGOS) population consisted of 500 patients, who were selected from 17000 FH patients with an identified causal mutation in the *LDLR* gene (segregating in the families and not present in controls) from our nationwide molecular screening program for FH. The scope of the original ARGOS study was to identify previously unknown risk factors for CHD. Phenotypic data were acquired from general practitioners and by reviewing medical records at the lipid and cardiology clinics. Stratified for sex, we selected 264 patients with severe premature CHD (defined as myocardial infarction, CBAG and PTCA) and 236 patients without any sign or symptom of CHD. At least all

first, second and third degree relatives were excluded from the study using the pedigree information. After exclusion of patients whose DNA was not available, 464 FH subjects were available as cases for the GWA study. The control group consisted of 5,945 subjects of the general population (Rotterdam study (RS-I) without excluding FH patients).

The Rotterdam Study is a population-based cohort described in detail elsewhere.[12] All cases and controls were of apparent Caucasian descent.

Illumina Infinium HumanHap550K chips (Illumina, San Diego, USA) were used to genotype the subjects of the ARGOS population and the Rotterdam Study. Polymorphisms were excluded if they had *(i)* unsuccessful genotyping in < 98% of all subjects, *(ii)* a minor allele frequency < 5%, or *(iii)* showed deviation from Hardy-Weinberg equilibrium ($p < 0.0001$). For each polymorphism which passed the quality control in both populations we compared allele frequencies between ARGOS and the Rotterdam Study using Plink version 1.06.[13]

Cross tabulation and stepwise forward regression analysis of genome-wide significant polymorphisms in the *LDLR* gene region were performed using SPSS version 15.0. After IBD analysis in Plink version 1.06, a multidimensional scaling (MDS) plot was made with R package 2.8.1. Haplotypes were constructed with haplo.stats in R package 2.8.1.

To replicate our results, we compared the genotype frequencies of the ten most significant polymorphisms between a second cohort of 2,189 patients with clinical FH and another population-based control sample consisting of 2,157 subjects of the Rotterdam Study (RS-II).[12, 14] The above described Illumina chips were used to genotype the subjects of the RS-II. In the second FH cohort, the polymorphisms were genotyped using TaqMan. The replication analyses were performed with SPSS version 15.0.

All subjects gave informed consent and the ethics institutional review board approved the study protocols.

RESULTS

We compared the frequencies of 480,254 polymorphisms between 464 FH patients (ARGOS Study) and 5,945 subjects from the general population (RS-I). Overall, 28 polymorphisms were genome-wide significantly associated with FH (Supplemental Figure). Of these, 13 polymorphisms were located up to 1Mb upstream or downstream from the *LDLR* gene (Table 1 and Supplemental Figure). In addition, a number of signals were even further away from this gene: 15 polymorphisms were located up to 2.4 Mb from the gene (Table 1). Remarkably, the polymorphisms *within* the *LDLR* gene were not associated with FH. Entering the polymorphism in a stepwise forward logistic regression model demonstrated that the rs2304240, rs4804149, rs387865 and rs4804636 polymorphisms were independently associated with FH with *p*-values ranging from 2.21×10^{-34} to 1.61×10^{-10} (Table 1). The genomic inflation factor was 1.20. Correction for up to 20 principal components slightly decreased the genomic inflation factor (1.19). The largest cluster found by identity-by-descent (IBD) analysis existed of five persons and between these persons the maximum shared IBD was 4%. Adjustment for age, which might be considered as an

TABLE 1. Genome-wide significant polymorphisms on chromosome 19 associated with FH

Polymorphism	Position	Allele	Rotterdam Study MAF	ARGOS MAF	OR	*p*-value
rs7259203	8,672,833*	A	0.16	0.24	1.71	$4.32x10^{-11}$
rs2033483	9,115,342*	G	0.11	0.18	1.76	$6.51x10^{-10}$
rs10422772	9,584,423*	A	0.14	0.08	0.49	$1.41x10^{-8}$
rs10405652	9,602,123*	A	0.14	0.08	0.49	$2.03x10^{-8}$
rs10411082	9,602,216*	A	0.21	0.13	0.58	$4.51x10^{-8}$
rs10418705	9,629,925*	G	0.20	0.13	0.57	$3.16x10^{-8}$
rs10417523	9,681,194*	C	0.14	0.07	0.48	$1.87x10^{-8}$
rs10415132	9,724,483*	A	0.21	0.13	0.57	$2.08x10^{-8}$
rs7247038	9,739,695*	G	0.21	0.13	0.58	$4.16x10^{-8}$
rs2431820	9,996,284*	G	0.06	0.11	2.09	$1.05x10^{-10}$
rs10417403	10,033,516*	G	0.26	0.34	1.50	$3.33x10^{-8}$
rs3745264	10,290,566	A	0.15	0.25	1.97	$6.00x10^{-17}$
rs7507634	10,297,562	A	0.11	0.22	2.24	$1.36x10^{-20}$
rs2304240	10,310,392	A	0.16	0.33	2.63	$2.21x10^{-35}$
rs280519	10,333,933	G	0.50	0.40	0.67	$1.78x10^{-8}$
rs8112449	10,381,064	A	0.30	0.22	0.63	$3.63x10^{-8}$
rs7256672	10,440,474	C	0.36	0.45	1.49	$1.14x10^{-8}$
rs1529711	10,884,434	A	0.16	0.25	1.73	$8.58x10^{-12}$
rs4804149	11,145,028	G	0.29	0.40	1.64	$1.83x10^{-12}$
rs387865	11,145,539	A	0.29	0.39	1.57	$1.61x10^{-10}$
rs8111456	11,162,147	G	0.34	0.44	1.54	$3.30x10^{-10}$
rs313624	11,413,910	A	0.48	0.43	1.48	$2.18x10^{-8}$
rs4804636	11,703,323	A	0.34	0.45	1.58	$3.15x10^{-11}$
rs286262	11,708,615	A	0.37	0.48	1.55	$2.43x10^{-10}$
rs11880217	12,279,795*	A	0.16	0.24	1.66	$4.89x10^{-10}$
rs12610507	12,297,410*	C	0.17	0.25	1.62	$1.70x10^{-9}$
rs2967890	12,901,647*	G	0.23	0.31	1.55	$2.91x10^{-9}$
rs16042	13,202,037*	A	0.11	0.17	1.76	$7.05x10^{-10}$

Abbreviations: LDL low-density lipoprotein, MAF minor allele frequency, OR odds ratio. *Located 1-2Mb from the *LDLR* gene (located 11,061,132 – 11,105,490 according to HapMap). The other polymorphisms are located < 1Mb from the *LDLR* gene.

adjustment for group, did lower the inflation factor to 1.03 but the significant association between the polymorphisms on chromosome 19 and FH yielded similar results.

Replication of results

We compared the genotype frequencies of the ten most significant polymorphisms between a second FH cohort of 2,189 patients and 2,157 subjects of the general population (RS-II): all ten polymorphisms on chromosome 19 were significantly associated with

FH (all p< 10^{-4}, Supplemental Table 1). An *LDLR* mutation had been identified in 1,258 patients of this second FH cohort. Analyses restricted to these mutation carriers resulted in higher odds ratios with lower p-values, while analyzing clinical FH patients in whom no *LDLR* mutation was detected (n= 931) did not result in replication with the exception of rs4804149 (Supplemental Table 2).

Associated polymorphisms do not identify patients with (familial) hypercholesterolemia

Because the presence of *LDLR* mutations produced strong signals in our GWA study, we hypothesized that these signals might identify subjects with FH in the general population. The prevalence of FH is approximately 1:500.[11] This indicates that 12 out of 5,945 subjects of the RS-I and 4 out of 2,157 subjects of RS-II were expected to carry an *LDLR* mutation. However, a multidimensional scaling (MDS) plot based on the DNA similarity at the *LDLR* gene ± 1 Mb between subjects from the ARGOS and all controls (RS-I and RS-II) did not show any pattern that distinguished carriers of *LDLR* mutations from non-carriers.

We constructed four haplotypes of rs2304240, rs4804149, rs387865 and rs4804636 polymorphisms that were independently associated with FH. These haplotypes were associated with having an *LDLR* mutation when comparing ARGOS to RS-I and RS-II (p< 2.61×10^{-5}). Within both RS samples the haplotypes were not associated with LDL cholesterol level or possible FH phenotype, defined as total cholesterol level > 7 mmol/l and triglyceride level < 4 mmol/l, or LDL cholesterol level > 6 mmol/l and a personal history of premature coronary heart disease (Suppplemental Table 3).

Because each *LDLR* mutation might co-segregate with different alleles of common polymorphisms, we compared the genotype frequencies of the polymorphisms found by GWA between the subjects of the ARGOS Study and the RS-I, stratified for all mutations that were present in ten or more patients. Different mutations indeed appeared to co-segregate with different alleles of the polymorphisms, as illustrated by the absence of homozygosity for the wild type allele. The results of rs3745264 and rs2304240, the two most significant hits of our study are shown as example in Table 2. The co-segregation of a mutation with specific alleles of common polymorphisms was most pronounced in carriers of the *LDLR* mutation 191-2 in intron 2 (association with the minor allele in seven polymorphisms), S285L in exon 6 (association with the minor allele in nine polymorphisms) and a large deletion of 2.5 kb from exon 7-8 called the Cape Town- 2 mutation (association with the minor alleles in eight polymorphisms). This co-segregation is confirmed by the disappearance of the associations between FH and the two polymorphisms in Table 2 after removing all carriers of these mutations from the analyses: p=0.38 for rs3745264 (191-2, 313+1/2, S285L, G322S and Cape Town-2 removed) and p=0.95 for rs2304240 (191-2, 313+1/2, S285L, Cape Town-2, 1359-1 and L590F removed).

We searched for these particular combinations of minor alleles in persons with a possible FH phenotype in the Rotterdam Study and identified seven possible FH patients.

TABLE 2. Genotype frequencies of two significant polymorphisms close to the *LDLR* gene in the Rotterdam Study, in the whole ARGOS and within ARGOS in carriers of the same *LDLR* mutation

	rs3745264					**rs2304240**				
	AA	AC	CC	*p*-value	Minor allele	AA	AG	GG	*p*-value	Minor allele
Rotterdam Study	131 (2.2)	1506 (25.4)	4283 (72.3)		A	143 (2.4)	1619 (27.4)	4154 (70.2)		A
ARGOS	22 (4.7)	193(41.6)	249 (53.7)	$6.00x10^{-17}$	A	37 (0.8)	228 (49.5)	196 (42.2)	$2.21x10^{-35}$	A
Carriers mutation:										
191-2	6 (18.8)	26 (81.3)	0 (0.0)	$< 1.00x10^{-6}$	C	6 (18.8)	26 (81.3)	0 (0.0)	$< 1.00x10^{-6}$	A
W23X	4 (13.8)	10 (34.5)	15 (51.7)	$5.30x10^{-5}$	A	4 (13.8)	9 (31.0)	16 (55.2)	$3.07x10^{-4}$	A
313+½	5 (9.1)	44 (80.0)	6 (10.9)	$< 1.00x10^{-6}$	A	6 (10.9)	43 (78.2)	6 (10.9)	$<1.00x10^{-6}$	A
E207K	0 (0.0)	5 (17.9)	23 (82.1)	0.45	A	0 (0.0)	4 (14.3)	24 (85.7)	0.19	G
S285L	0 (0.0)	19 (95.0)	0 (0.0)	$< 1.00x10^{-6}$	C	3 (15.0)	17 (85.0)	0 (0.0)	$<1.00x10^{-6}$	A
G322S	2 (16.7)	9 (75.0)	1 (8.3)	$< 1.00x10^{-6}$	C	0 (0.0)	3 (27.3)	8 (72.7)	0.87	G
R329X	0 (0.0)	0 (0.0)	7 (100.0)	0.263	A	0 (0.0)	0 (0.0)	7 (100.0)	0.23	G
Cape Town 2	2 (9.5)	19 (90.5)	0 (0.0)	$< 1.00x10^{-6}$	C	4 (19.0)	17 (81.0)	0 (0.0)	$<1.00x10^{-6}$	A
1359-1	0 (0.0)	12 (17.9)	55 (82.1)	0.15	A	11 (16.4)	49 (73.1)	7 (10.4)	$<1.00x10^{-6}$	A
L401P	0 (0.0)	2 (25.0)	6 (75.0)	0.91	A	1 (12.5)	3 (37.5)	4 (50.0)	0.13	A
V408M	0 (0.0)	2 (15.4)	11 (84.6)	0.59	A	0 (0.0)	3 (23.1)	10 (76.9)	0.79	G
L590F	0 (0.0)	0 (0.0)	8 (100.0)	0.22	A	0 (0.0)	8 (100.0)	0 (0.0)	$2.50x10^{-5}$	A
P664L	0 (0.0)	1 (12.5)	7 (87.5)	0.62	A	0 (0.0)	0 (0.0)	8 (100.0)	0.18	G
P678L	0 (0.0)	2 (40.0)	3 (60.0)	0.73	A	0 (0.0)	2 (40.0)	3 (60.0)	0.78	G

Values are counts (%). *P*-values are result of X^2 test with The Rotterdam Study as reference group.

Sequencing of the *LDLR* gene including the promoter region and all splice sites did not identify FH patients among these subjects.

DISCUSSION

In this study, we demonstrate that *LDLR* mutations give rise to multiple independent synthetic associations and that these signals occur up to 2.4 Mb upstream and downstream of the *LDLR* gene. Using the associated polymorphisms, we were not able to identify FH patients among the general population. Despite abundant and strong synthetic associations of common polymorphisms translation to diagnostic use in the general population was not successful in cohorts of the general population.

The synthetic associations in our study could have been the result of cryptic family relations or population stratification. The genomic inflation factor (1.20) was relatively high. This indicates that there might be population stratification. Correction for up to 20 principal components only slightly decreased the genomic inflation factor (1.19) and did not change the association between the polymorphisms and FH.[15] In fact, our selection procedure of ARGOS aimed at and resulted in genetic heterogeneity at the *LDLR* locus: we took a sample from our nationwide screening program and excluded relatives, which resulted in 96 different *LDLR* mutations in 464 FH patients. The most frequent mutation (1359-1 splice-defect in intron 9) was found in 14%, which is similar to its frequency in the overall Dutch FH population. To further explore cryptic family relations, we performed an IBD analysis. The largest cluster existed of five persons and between these persons the maximum shared IBD was low. Taken together, this confirms that we succeeded in excluding relatives from our study and even distant relationships did not influence our results. Therefore, random inflation by cryptic family relations or population stratification is a very unlikely explanation for the 28 genome-wide association signals at the *LDLR* locus.

Remarkably, the polymorphisms *within* the *LDLR* gene were not associated with FH. Particular mutations associated with specific polymorphism within the *LDLR* gene, but these numbers were too small to produce a genome-wide significant signal. Alternatively, one could argue that they did not tag the mutations due to their location outside the linkage disequilibrium blocks.

We also found a genome-wide significantly associated polymorphism that was not located on chromosome 19 but on chromosome 16, however genotyping of this polymorphism did not result in distinct genotype clusters, indicating a false-positive result.

Because the presence of *LDLR* mutations produced signals in our GWA study, we hypothesized that these signals could identify subjects with FH in the general population. An MDS plot based on the DNA similarity at the *LDLR* gene ± 1 Mb between subjects from the ARGOS and the Rotterdam Study did not show any pattern that distinguished carriers of *LDLR* mutations from non-carriers. Alternatively to this MDS analysis, we tried to identify subjects with FH in the Rotterdam Study by constructing haplotypes of the independently associated polymorphisms. As expected, some of these haplotypes were associated with having

an *LDLR* mutation when comparing ARGOS to the Rotterdam Study. However, within the Rotterdam Study none of the haplotypes were associated with LDL cholesterol level or an FH phenotype. Because both MDS analysis and haplotype analyses were not able to identify FH subjects in the Rotterdam Study, we hypothesized that different mutations might co-segregate with different alleles of the polymorphisms. We therefore compared frequencies of the genotypes of the significantly associated polymorphisms between carriers of the mutations in ARGOS that were present in 10 or more patients. Different mutations indeed appeared to co-segregate with different alleles of the polymorphisms. The significance of these associations was most likely driven by the fact that specific mutations co-segregated with the minor allele of the polymorphism, making it the common allele among carriers of these mutations. For some of the more frequent mutations, co-segregation with the rare allele of a polymorphism at the *LDLR* locus was even more certain, as homozygosity for the common allele of the polymorphism was absent among the carriers of the mutation, however using this information did not lead to identification of FH patients in the general population. Different mutations co-segregated with different alleles, but of course the power to detect specific *LDLR* mutations in the general population was limited.

Our GWA study picked up genetic heterogeneity at a locus of an exemplary Mendelian trait, supporting that multiple rare variants produce synthetic GWA signals. This suggests that we have opportunities to detect rare variants in a GWA. Our study cannot be used to estimate what proportion of heritabilities will be explained by rare variants. We merely show that if rare undeniable causal variants are present strong signals may appear. A very large population (approximately 250,000 persons) is required to identify synthetic signals of *LDLR* mutations with similar power in a population-based setting.

The phenomenon of synthetic associations were first described by Dickson et al.[3] Recently, Anderson et al. suggested that the consistency of GWA results in different populations is an argument against involvement of synthetic associations.[7] We clearly replicated the signals in an independent sample in line with consistency among populations. Both studies were in Caucasians and it would be of great interest to know if our results would be consistent in populations of other ethnicities. The distributions of the LDLR mutations differ between populations and probably the polymorphisms of the synthetic association signals as well. Unfortunately, cohorts of genetically heterogeneous FH patients from other populations are currently not large enough for such an analysis.

Wray et al. argued that the allele frequencies of the signals in GWA's are too high to be explained by rare variants.[6] In the initial and the replication phase, however, we found that a large number of different mutations in the *LDLR* produced a wide range in minor allele frequencies (MAFs) of all found polymorphisms including the four independently associated polymorphism (rs2304240, rs4804149, rs387865, rs4804636).

We confirmed that mutations causing a monogenic disorder give rise to multiple independent synthetic associations and that these signals occur up to 2.4 Mb away from the rare functional variants. Moreover, we found that it is possible to replicate these signals. However, we could not simplify molecular diagnostics of FH with these synthetic associations.

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SUPPLEMENTAL TABLE 1. Replication of the ten most significant polymorphisms in an independent case-control study

Polymorphism	Rotterdam Study II MAF	2nd FH cohort MAF	OR	95% CI	p-value
rs7259203	0.16	0.20	1.36	1.21-1.52	$1.55x10^{-7}$
rs2431820	0.06	0.08	1.42	1.19-1.70	$8.66x10^{-5}$
rs3745264	0.16	0.19	1.26	1.13-1.42	$7.52x10^{-5}$
rs7507634	0.12	0.16	1.44	1.27-1.63	$7.40x10^{-9}$
rs2304240	0.17	0.23	1.41	1.26-1.58	$1.85x10^{-9}$
rs1529711	0.15	0.19	1.34	1.20-1.51	$3.24x10^{-7}$
rs4804149	0.22	0.39	2.44	2.19-2.72	$6.82x10^{-58}$
rs387865	0.29	0.34	1.26	1.15-1.39	$1.52x10^{-6}$
rs4804636	0.36	0.42	1.30	1.19-1.43	$1.26x10^{-8}$
rs286262	0.38	0.45	1.33	1.22-1.46	$5.58x10^{-10}$

Abbreviations: CI confidence interval, FH familial hypercholesterolemia, MAF minor allele frequency, OR odds ratio.

SUPPLEMENTAL TABLE 2. Separate replication analyses of the ten most significant polymorphisms in LDL receptor mutation-carriers and clinical FH patients without mutation relative to a sample of the general population

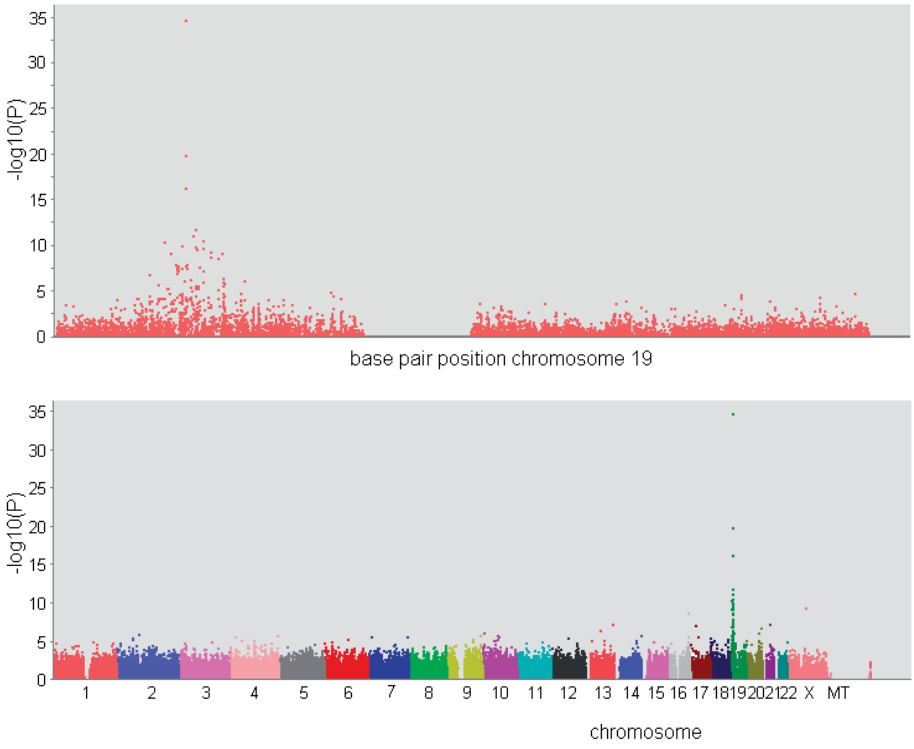
Polymorphism	Rotterdam Study II MAF	FH (mut +) MAF	OR	*p*-value	FH (mut -) MAF	OR	*p*- value
rs7259203	0.16	0.24	1.66	$1.59x10^{-14}$	0.16	1.00	0.95
rs2431820	0.06	0.08	1.57	$1.06x10^{-5}$	0.07	1.24	0.068
rs3745264	0.16	0.22	1.53	$1.25x10^{-10}$	0.15	0.92	0.32
rs7507634	0.12	0.20	1.85	$2.59x10^{-18}$	0.11	0.94	0.49
rs2304240	0.17	0.27	1.79	$2.59x10^{-19}$	0.17	0.96	0.61
rs1529711	0.15	0.23	1.62	$1.52x10^{-13}$	0.15	1.00	0.98
rs4804149	0.22	0.46	3.75	$1.09x10^{-77}$	0.30	1.68	$2.75x10^{-12}$
rs387865	0.29	0.37	1.40	$1.81x10^{-9}$	0.31	1.09	0.18
rs4804636	0.36	0.45	1.53	$1.07x10^{-14}$	0.37	1.05	0.45
rs286262	0.38	0.48	1.52	$1.29x10^{-14}$	0.41	1.11	0.094

Abbreviations: FH familial hypercholesterolemia, LDL low-density lipoprotein, MAF minor allele frequency, (mut +) LDL receptor mutation present, (mut -) LDL receptor mutation absent,OR odds ratio.

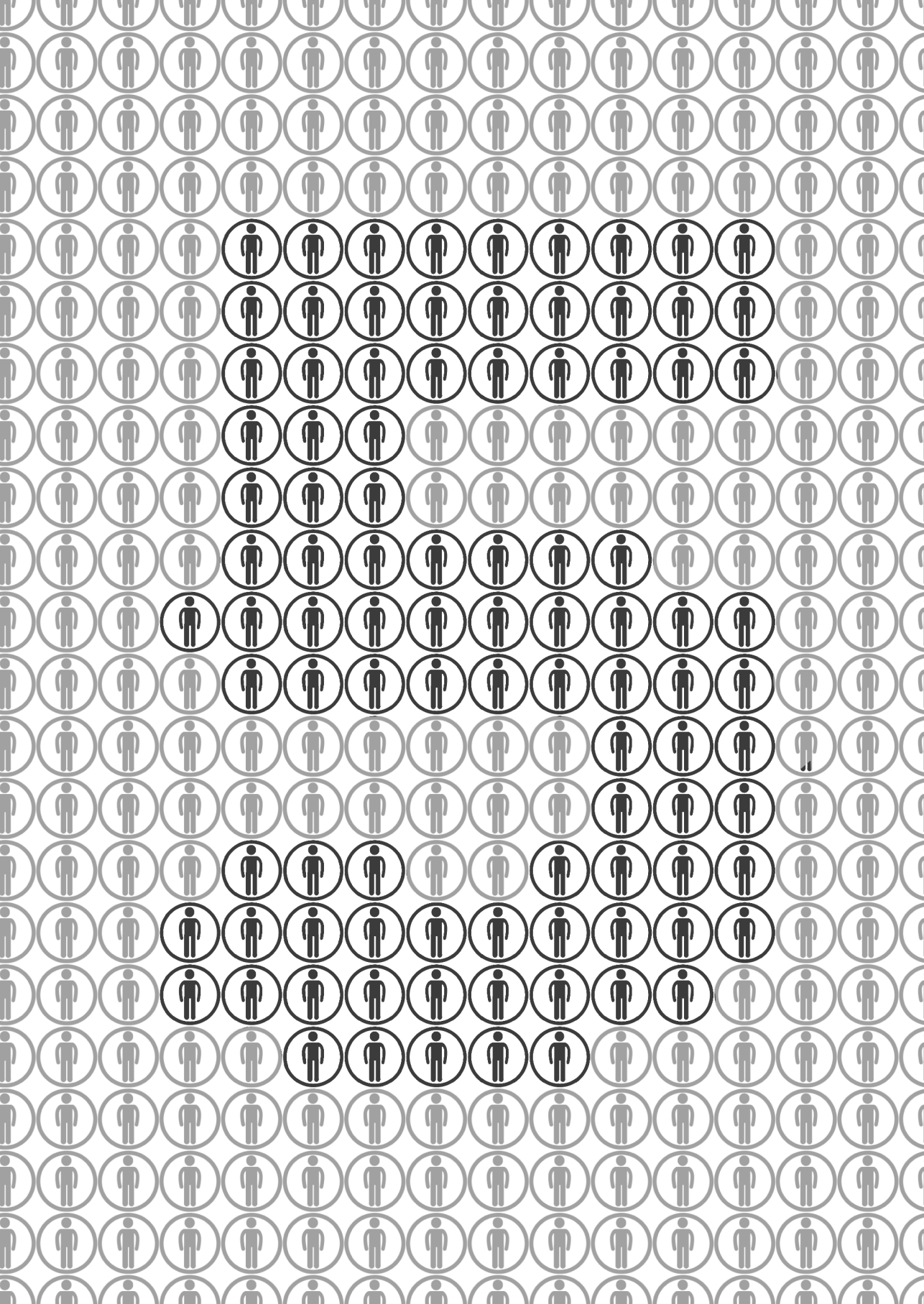
SUPPLEMENTAL TABLE 3. Haplotypes of the four independently-associated polymorphisms and LDL cholesterol in The Rotterdam Study subjects

Haplotype	Haplotype frequentie	LDL cholesterol Beta (SD)	*p*-value	Possible FH Beta (SD)	*p*-value
G-A-G-G	0.27	(ref)		(ref)	
A-A-A-A	0.01	-0.16 (0.20)	0.41	-0.0069 (0.0097)	0.48
A-A-A-G	0.03	0.10 (0.10)	0.29	0.013 (0.0067)	0.054
A-A-G-A	0.03	0.16 (0.12)	0.16	0.0098 (0.0076)	0.20
A-A-G-G	0.05	-0.11 (0.084)	0.18	-0.0036 (0.0053)	0.50
A-G-A-A	<0.01	0.16 (0.23)	0.49	-0.0076 (0.015)	0.61
A-G-A-G	<0.01	-0.064 (0.38)	0.87	-0.0073 (0.014)	0.61
A-G-G-A	0.01	0.11 (0.17)	0.53	-0.0060 (0.011)	0.57
A-G-G-G	0.03	0.021 (0.11)	0.85	-0.0037 (0.0071)	0.61
G-A-A-A	0.08	0.0096 (0.058)	0.87	0.0047 (0.0042)	0.26
G-A-A-G	0.11	0.092 (0.054)	0.091	0.0016 (0.0036)	0.67
G-A-G-A	0.13	-0.013 (0.053)	0.81	-0.0012 (0.0041)	0.76
G-G-A-A	0.02	-0.10 (0.13)	0.43	-0.0075 (0.0073)	0.31
G-G-A-G	0.03	-0.10 (0.10)	0.31	-0.0091 (0.0060)	0.13
G-G-G-A	0.06	0.042 (0.070)	0.55	0.0021 (0.0054)	0.70
G-G-G-G	0.14	0.088 (0.048)	0.068	0.0029 (0.0039)	0.47

Haplotype of rs2304240-rs4804149-rs387865-rs4804636.Haplotype analysis of LDL cholesterol level was adjusted for sex. Possible FH was defined as LDL cholesterol level > 7mmol/l and triglyceride level < 4mmol/l, or LDL cholesterol level > 6mmol/l and a history of premature CHD Abbreviation: FH familial hypercholesterolemia; SD standard deviation.



SUPPLEMENTAL FIGURE. Signal intensity plot of all chromosomes and chromosome 19 seperately.



5 Cholesterol efflux capacity to plasma compromised in familial hypercholesterolemia patients with coronary heart disease but not in those without

Jorie Versmissen
Ranitha Vongpromek
Daniëlla M Oosterveer
Jeroen B van der Net
Leonie van Vark-van der Zee
Jeannette Touw-Blommesteijn
Darcos Wattimena
Trinet Rietveld
Clive R Pullinger
Christina Christoffersen
Björn Dahlbäck
John P Kane
Monique Mulder
Eric JG Sijbrands

MANUSCRIPT IN PREPARATION

ABSTRACT

Objective

Levels of high-density lipoprotein cholesterol (HDL-C) as well as variants in genes involved in HDL metabolism have been associated with coronary heart disease (CHD) risk in familial hypercholesterolemia (FH). However, genetic risk variants are not always coincided with higher HDL-C levels. An important role of HDL is facilitating cholesterol efflux from macrophages. We hypothesized that variation in the ability of plasma to induce cholesterol efflux from macrophages is associated with CHD risk in FH patients and that HDL composition and functionality is more important than absolute HDL-C levels.

Methods and results

Thirteen untreated sib-pairs of which one of the brothers had FH while the other one did not were examined. Seven patients with FH were asymptomatic while the other six had a history of CHD. Plasma of FH patients without CHD induced on average 16% (standard deviation (SD) 22%) higher cholesterol efflux from macrophages, whereas FH patients with CHD had 7% (SD 8%) lower efflux compared to their non-FH brothers (p=0.03, CHD vs non- CHD group). Analyses of the plasma lipoproteins revealed differences particularly in the HDL fractions: FH patients without CHD displayed significantly higher HDL_2 cholesterol while HDL_3 contained higher sphingosine-1-phosphate (S1P). This higher S1P content was coincided with increased levels of apolipoprotein M, the carrier of S1P.

Conclusions

Plasma from FH patients without CHD was more effective in inducing cholesterol efflux from macrophages than that of FH patients with CHD. These functional differences were associated with differences in HDL lipid and protein composition, potentially preventing CHD in these FH patients despite their high LDL cholesterol levels.

BACKGROUND

Familial hypercholesterolemia (FH) is caused by mutations in the low-density lipoprotein (LDL) receptor gene and causes a severe risk of coronary heart disease (CHD): at the age of 60, 52-85% of males and 25-58% of all females with untreated heterozygous FH have developed CHD.[1, 2] CHD risk in FH patients is affected by classical as well as additional genetic risk factors.[3] High levels of high-density lipoprotein cholesterol (HDL-C) were associated with less increased CHD risk in both epidemiological and parent-offspring studies.[3-6] Genetic variants of ABCA1 and CETP, genes involved in HDL metabolism, have been associated with CHD risk in FH as well.[7, 8] However, the mechanisms underlying these genetic effects are unclear, as frequently no effect on HDL-C levels could be established.[7-10] The other way around, genetic risk factors that raise plasma HDL-C levels do not always seem to lower CHD risk.[11] Recently, drug trials studying CETP inhibitors which increased HDL levels were terminated prematurely due to lack of 'clinically meaningful efficacy' and even harmful effects.[12, 13] The role of HDL in the etiology of CHD therefore stays under debate. Most likely, CHD risk is affected by HDL function more than by plasma HDL level.

One of the key roles of HDL is facilitating cholesterol efflux from macrophages and bringing cholesterol to the liver for clearance. Enhanced cholesterol efflux capacity of serum was associated with decreased intima media thickness and less coronary artery disease in a number of studies, although not confirmed in all.[14-17] Moreover, HDL has anti-oxidative and anti-inflammatory properties.[18] Two related components of HDL that have been suggested to be involved in the cardioprotective effects of HDL are sphingosine-1-phosphate (S1P) and its carrier molecule apoliprotein M (apoM).[19, 20] S1P is predominantly stored and released by erythrocytes, but originates to a large extend from the vascular endothelium and is abundantly present in circulating HDL.[21, 22] ApoM levels in HDL correlate with cholesterol efflux.[19, 23] Both HDL S1P levels and polymorphisms in *APOM* were identified to correlate with CHD risk.[24, 25]

We hypothesized that differences in the capacity of plasma to induce cholesterol efflux from macrophages may affect the CHD risk in FH patients. Because cholesterol efflux capacity of HDL *in vitro* is highly variable, we aimed at minimizing environmental and genetic variability by collecting sib-pairs of which one brother had FH while the other one did not. Of the men with FH, 6 out of 13 had developed symptomatic CHD (Figure 1). In effect, we standardized the cholesterol efflux rates on the normocholesterolemic brother and compared cholesterol efflux from cholesterol-loaded macrophages to plasma of FH patients with and without CHD. Using this unique approach we found that plasma of FH subjects without CHD had a higher capacity to induce cholesterol efflux compared to those with CHD. Thereafter, we investigated whether differences in HDL subpopulation distribution and composition might be responsible for this difference, focusing on S1P and ApoM.

METHODS

Study population

Heterozygous male FH patients not participating in an intervention study and having at least one brother without FH were selected for this study. Twenty FH patients were approached, of whom five patients and two brothers refused to participate because of logistic reasons. A total of 13 sib-pairs were collected, of whom seven FH patients had no symptoms of CHD while the other six had developed symptomatic CHD. Therefore, the final selection was as follows: seven sib-pairs consisted of an FH patient without CHD and a brother with neither FH or CHD; the other six pairs consisted of an FH patient with CHD and a brother with neither FH and CHD (Figure 1). To reduce the influence of environmental factors we asked all participants to quit their medication six weeks prior to blood sampling and to refrain from smoking in the week before blood sampling.[26] All blood was sampled after fasting overnight and was placed on ice immediately; during further processing, the temperature was kept at 4° Celsius.

The medical ethical committee approved the protocol and all participants gave written informed consent.

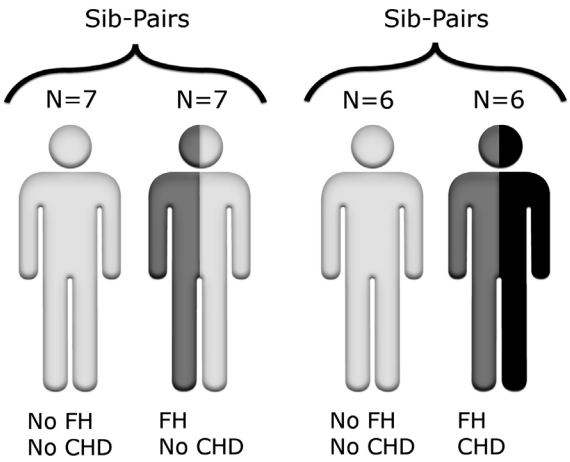


FIGURE 1. Study design.
Seven sib-pairs existed of one brother with FH and one brother without FH, both without CHD. Six sib-pairs consisted of one brother with FH and CHD, and one brother without FH nor CHD.

Cholesterol efflux experiments

Cholesterol efflux experiments were performed as described previously.[27, 28] THP-1 cells, a human monocyte-cell line, were cultured in RPMI 1640 medium supplemented with 10% Fetal Calf Serum, 2 mmol/l glutamine and 100 IU/l penicillin/streptomycin at 37°C

in 5%CO_2. Cells were plated into 24 well plates (250,000 cells/well) and differentiation into macrophages was induced by treatment for 72 hours with 50 ng/ml phorbol 12-myristate 13-acetate. Subsequently, cells were incubated for 24 hours with 0.5 μCi/ml [^{3}H] cholesterol in RPMI supplemented with 0.2% fatty acid free bovine serum albumin (BSA). After 24 hours, cells were washed three times with 0.3 ml phosphate buffered saline supplemented with 0.2% BSA and acceptor medium was added. Acceptor medium was RPMI supplemented with heparin (1.25 units/ml) and 2% plasma. Medium supplemented with 0.2% BSA was used as a control for basal efflux rate. All experiments were performed in quadruplicate and an unrelated control without FH and CHD was used as reference in all experiments. After 4 hours of incubation with different efflux media, medium was collected and cells were dissolved in 0.1 M NaOH. Radioactivity in medium and cells was counted. The ratio between radioactivity in medium and radioactivity in cells plus in medium is the net cholesterol efflux. All experiments were repeated three times.

Cholesterol, triglyceride and pre-β HDL levels

Cholesterol, HDL-C and triglyceride levels were measured using Cobas mira analyzer (Roche). LDL-C was calculated using the Friedewald formula.[29] Pre-β levels were measured using the sandwich enzyme immunoassay technique as described previously, using the commercially available ELISA kit by Daiichi Pure Chemicals, Tokyo, Japan.[30]

ApoAI, ApoAII and ApoM

ApoAI and ApoAII were measured using selectra E, DDS Diagnostic Ssem, Istanbul, Turkey. ApoM was measured by a specific human ApoM ELISA.[31]

Lipoprotein profiles

Lipoprotein profiles were obtained using a modified density gradient ultracentrifuge protocol.[32] Potassium Bromide (KBr) (0.35g/ml plasma) was added to plasma to obtain 1.26 g/ml density. 1 ml plasma was then placed in an ultracentrifuge tube and gradient solutions (1.21 g/ml; 1.10 g/ml; 1.063 g/ml; 1.04 g/ml; 1.02 g/ml; all gradients by adding KBr to physiological NaCl solution) followed by water layered on top. Samples were ultracentrifuged at 40,000 RPM for 18 hours at 4°C using a SW41 rotor in a L-70 Beckman ultracentrifuge (Beckman Instruments, USA). Following ultracentrifuge, the tube was placed in a fraction collector (Frac200 Pharmacia) and subdivided in fractions of 250 μl, starting at the bottom of the tube (i.e. fraction '1' had the highest density).

S1P levels

S1P levels in lipoprotein fractions were quantified by a modified LC-MS/MS method described in detail before.[33] In brief, 0.050 ml Methanol, containing C17-S1P as internal standard, was added to 0.025 ml collected lipoprotein fraction or S1P standards (in KBr) with a density of 1.02 and 1.21 kg/L). The mixture was placed on ice during 30 minutes, and centrifuged during 30 minutes at 18000 g at 4°C. The clear supernatant was transferred to a vial and 0.015 ml was injected onto a Agilent 1200SL system (Agilent Technolgy, Amstelveen, the Netherlands) and separated through a Xterra C18 column (2.1 x 10 mm, 3.5 μm, Waters Chromatography, Etten-Leur, the Netherlands) at 40° C using a gradient, starting from 50 % mobile phase A (water, containing 10 % methanol, 0.25 % formic acid, 2.5 mMol ammoniumformate) for 1 minute, to 90 % mobile phase B (water, containing 90 % methanol, 0.25 % formic acid, 2.5 mMol ammoniumformate) for 6 minutes and thereafter 100 % B during 3 minutes. The effluent was directed to an Agilent 6410 triple quadruple mass spectrometer and analyzed using positive electrospray ionization. The resulting peak areas which represented the areas of the added internal standard C17-1P were used to calculate the concentration of S1P.

Statistical analyses

General characteristics in behalf of Table 1 were compared using ANOVA and chi square. Cholesterol efflux experiments were normalized according to the value in the unrelated healthy control person without FH for all three experiments. The difference between brothers was calculated by subtracting the normalized value of the brother with FH from that of the brother without FH. These paired differences were compared between sib-pairs with CHD and sib-pairs without CHD using an independent t-test. Cholesterol as well as S1P concentrations were compared between FH patients with and without CHD using paired analyses by linear regression modeling adjusted for the difference between the non-FH and the FH sib. Differences were considered statistically significant at $p<0.05$. Lipid profiles were compared by measuring areas under the curve.

RESULTS

All brothers with FH had a confirmed functional LDL-receptor mutation while all brothers without FH had been tested negative for this mutation. The average age of the FH patients without CHD (40.8 years range 23-65; p=0.85) was not significantly different from the mean age at first cardiac event of the FH patients with CHD (39.3 years; range 27-52). The age of the FH patients with CHD at the moment of blood sampling was 50.7 years (range 49-63). Six out of seven FH patients without CHD were current or former smokers; three of them refrained from smoking for at least ten years at the time of sampling. Five

TABLE 1. General Characteristics

	7 Sib-pairs		6 Sib-pairs	
	FH-	**FH+CHD-**	**FH-**	**FH+CHD+**
Age	41±13	41±16	46±8	51±8
Age at event				39±10
Smoking ever	2	6	4	5
Current smoking	1	2	1	1
Total cholesterol [mmol/l]	5.5±0.9†	7.7±2.0†	5.9±0.7*	8.3±1.8*
LDL cholesterol [mmol/l]	3.9±0.8¶	6.0±2.1¶	4.5±0.7*	6.9±1.9*
HDL cholesterol [mmol/l]	1.39±0.33	1.42±0.38	1.190.26	1.15±0.12
Triglycerides [mmol/l]	1.12±0.53	1.24±0.45	1.35±0.32	1.05±0.38
Pre-β HDL [mg/ml]	43±1.9	51±2.1	38±4.5‡	47±4.7‡

*Values are mean ±standard deviation. †p=0.035 ¶p=0.008 *p=0.025 §p=0.017 ‡p=0.006; FH: familial hypercholesterolemia; CHD: coronary heart disease; LDL: low-density lipoprotein; HDL high-density lipoprotein*

out of six FH patients who had a history of CHD smoked at time of event and all but one quitted at least ten years before sampling. Remarkably less individuals without FH had a history of smoking but the number of current smokers was similar in all groups (Table 1). All current smokers admitted to have smoked a few cigarettes in the week before blood sampling even though they were asked not to.

As expected, brothers with FH displayed significantly higher total and LDL cholesterol (LDL-C) levels than their non-FH sibs (Table 1). HDL-C and triglyceride levels were not significantly different between the FH subjects and their respective non-FH sibs (Table 1). Paired analyses did show no significant differences between FH patients with or without CHD when compared to their brothers. Pre-β HDL levels appeared to be higher in FH patients than in non-FH subjects but this was not significant: mean pre- β HDL level in subjects with FH 49 μmol/l (SD 15), without FH 40 μmol/l (SD 13), p=0.13; in FH patients without CHD 12±23 higher than in their sibs, in FH patients with CHD 9±3 (Table 1; paired analysis comparing FH patients with and without CHD standardized on their non-FH sibs p=0.34).

Although not significant, there was a trend towards higher HDL-C levels in FH patients without CHD in comparison with FH patients with CHD (paired analysis p=0.076). The differences between the FH patient and his brother were not significant. Actually, the HDL-C levels showed a strong correlation within the pairs (as clear in Figure 3C).

Efflux experiments

Plasma of six out of seven FH patients without CHD induced a higher cholesterol efflux from cholesterol-loaded macrophages than plasma from their non-FH sibs. In contrast, plasma of only one FH patient with CHD induced a higher cholesterol efflux while plasma of four out of six FH patients with CHD induced a lower efflux than that of their non-FH sibs (Figure 2). The mean paired difference in cholesterol efflux compared to the unaffected brother was 16% (SD 22%) in the FH patients without CHD and -7% (SD 8%) in

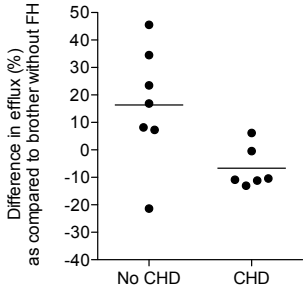


FIGURE 2. Difference in cholesterol efflux within sib-pairs.
The difference in cholesterol efflux between an FH patient and his brother is expressed in percentage.

the pairs with CHD (p=0.03). These efflux percentages were independent of baseline values and within pair differences of HDL-C and LDL-C levels: in a matched multiple linear regression analysis with concomitant inclusion of these co-variables, the difference in efflux was unchanged (data not shown). In contrast to our expectations, pre-β HDL and cholesterol efflux were not correlated (Pearson correlation coefficient -0.4, p=0.2).

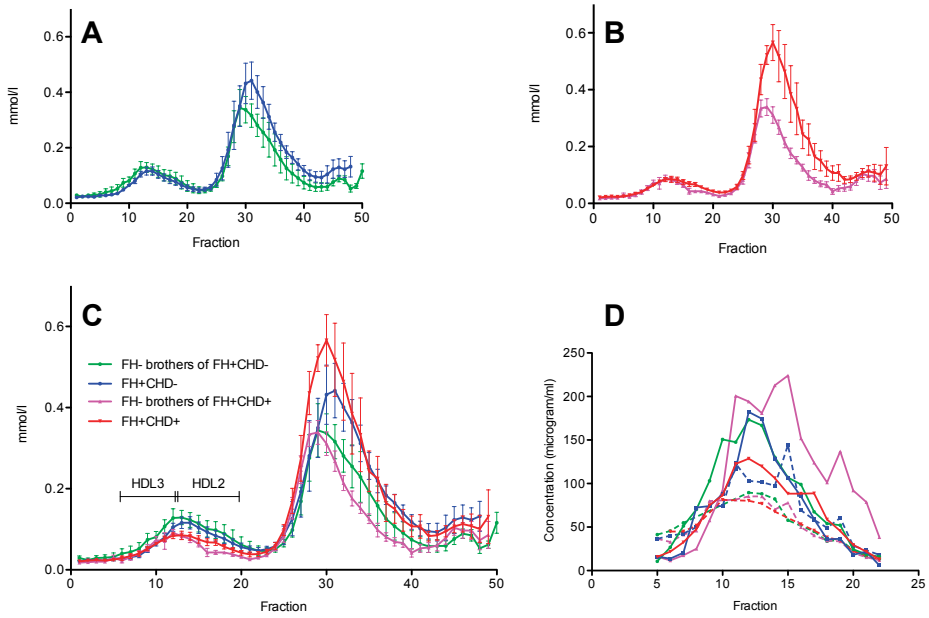


FIGURE 3. Average curves lipoprotein profiles and ApoA1 and ApoA2 content.
On the X axis fraction number, on the Y axis concentration cholesterol in mmol/l (A, B and C) or concentration protein in micrograms/ml (D). Error bars represent standard error (A, B, and C). Legend for different groups indicated in C applies for whole figure. **A** FH patients without CHD and their non-FH sibs. **B** FH patients with CHD and their non-FH sibs. **C** All curves together. **D** Concentration A1 (dashed) and A2 within HDL.

Lipoprotein density profiles and S1P content/composition

Although the total levels of HDL-C and LDL-C did not differ significantly between FH patients without and with CHD, analyses of the full lipoprotein profiles did reveal differences in the distribution of cholesterol within HDL. Subjects with FH without CHD displayed significantly higher HDL_2-cholesterol levels than FH subjects who had a history of CHD (Figure 3: fractions 12-20 representing the density range of 1.16-1.11 g/ml; p=0.05 in paired analysis). However, no correlation between HDL_2 levels and cholesterol efflux capacity could be established. HDL_3-cholesterol levels were not significantly different with respect to their cholesterol content. We measured apoA-I and A-II within the HDL fractions (Figure 3D).[34] Levels of apoA-I were highest in FH patients without CHD, especially in HDL_2. Levels of apoA-II were not different between FH patients without CHD and their non-FH sibs, but much lower in FH patients with CHD than in their non-FH sibs, most prominently in HDL_2.

Next we analysed S1P in the lipoprotein fractions (Figure 4). We confirmed that distribution of S1P within HDL is asymmetric and is mainly found in HDL_3.[35] Visually, S1P levels in HDL_3 appeared higher in the FH subjects without than in the FH subjects with CHD but paired analysis was not significant (p=0.21). However, FH patients without CHD had on average higher S1P levels in HDL_3 than their non-FH sibs while FH patients with CHD

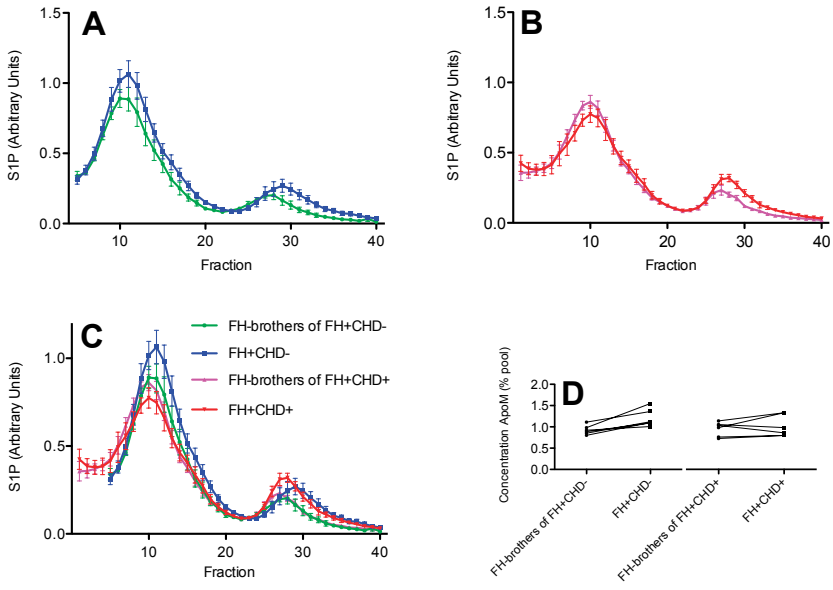


Figure 4. S1P levels and ApoM.
On the X axis fraction number, on the Y axis S1P concentrations given as Arbitrary Units (A, B, C). Error bars represent standard error (A, B, and C). Legend for different groups indicated in C applies for whole figure. **A** FH patients without CHD and their non-FH sibs. **B** FH patients with CHD and their non-FH sibs. **C** all curves together. **D** ApoM levels in HDL of FH patients.

had lower S1P levels in HDL_3 (p for differences FH and non-FH=0.05). LDL S1P levels were not significantly different between FH subjects with and without CHD in paired analyses.

To define whether differences in S1P levels were coincided with differences in apoM levels since ApoM is involved in the trafficking of S1P, we measured ApoM levels in HDL.[34] ApoM levels were significantly higher in HDL from FH patients without CHD than in their non-FH sibs (p=0.015) (Figure 4D). However, comparison between FH patients with and without CHD in paired analysis did not show a significant difference (p=0.7).

DISCUSSION

Our findings support the notion that the functionality of HDL differs between FH patients without and with CHD. The plasma of FH patients without CHD induced the efflux of higher amounts of cholesterol from cholesterol-loaded macrophages than plasma of FH patients with CHD. These findings were independent of absolute levels of LDL, HDL and pre-β HDL cholesterol levels. Density profiles revealed higher HDL_2 cholesterol and ApoA-I in FH subjects without CHD compared to FH subjects with CHD. Although cholesterol levels of HDL_3 were not different, S1P levels were significantly higher in HDL_3 of FH patients without CHD, and these S1P levels appeared to correlate with higher ApoM levels in HDL.

The significance of HDL-C levels and *in vivo* cholesterol efflux in relation to CHD risk has been subject to major discussion. High HDL-C levels have been related to low CHD risk, and genetic risk variants in genes such as ABCA1 and CETP suggest reverse cholesterol transport is a major pathway in reducing CHD risk.[6-10] Increased cholesterol efflux capacity *in vivo* has been associated with decreased CHD risk.[15] However, merely raising HDL levels did not decrease CHD risk.[6, 36] Recently, it was reported that young FH patients display reduced levels of HDL particles that have impaired capacity to induce cholesterol efflux.[37] This study involved only young patients who did not yet experience CHD.

Our current data show that cholesterol efflux capacity is increased in FH subjects without CHD. It has been reported that the LDL receptor is required for sterol mediated induction of ABCA1.[38] Therefore, the enhanced cholesterol efflux in the FH patients without CHD in our study suggest the presence of a compensatory mechanism restoring cholesterol efflux from macrophages with dysfunctional LDL receptors.

In earlier studies, higher pre-β HDL levels have been correlated with higher cholesterol efflux capacity.[39, 40] However, in a recent study, FH patients displayed higher pre-β HDL levels than normocholesterolemic controls but HDL of these FH patients induced lower cholesterol efflux.[37] In line, our FH patients had higher pre-β HDL levels and we did not observe a correlation between pre-β levels and efflux capacity. Moreover, the pre-β HDL levels were similar in FH patients without and with CHD.

The dynamic movements of cholesterol through HDL and the quantities of different HDL populations are most likely more important than static HDL or pre-β HDL levels.[41-44] We determined lipoprotein density profiles and observed significantly increased HDL_2 levels based on their cholesterol content in FH patients without CHD compared to their

non-FH brothers and even more when compared to FH patients with CHD, while HDL_3 cholesterol levels did not differ significantly. This is in line with earlier studies showing a positive relationship between HDL_2 concentrations and cholesterol efflux.[42, 45, 46] Interestingly, in a study comparing FH patients with healthy individuals, HDL_2 from FH patients appeared to be less efficient in inducing cholesterol efflux than HDL_2 from the controls. Possibly, the capacity of HDL_2 to induce cholesterol efflux might define the effectiveness of reverse cholesterol transport in FH patients and as a consequence determine their CHD risk.[37] However, in this study all FH patients were treated, while in our study FH subjects were 'off' statins. Moreover, subfractions of HDL were used for cholesterol efflux capacity analyses while we used total plasma to simulate the *in vivo* situation as close as possible. Recently, it was reported that cholesterol efflux from tissues requires the presence of apoB-containing lipoproteins, which is another reason to use total plasma instead of the HDL fraction exclusively.[48]

Although both efflux capacity, HDL_2 levels and ApoA1 levels within HDL_2 were higher in FH patients without CHD, we could not demonstrate a direct correlation, which might be due to a lack of statistical power.

Since S1P and its carrier ApoM are expected to be major components of HDL explaining its atheroprotective capacities, we measured ApoM and S1P content in HDL.[20, 35, 48] ApoM has been shown to modulate cholesterol efflux in mice.[19, 22, 23] Although ApoM and S1P correlated strongly in an earlier study, we could not replicate this in our study (p=0.07), probably due to small numbers and different kinds of *LDLR* mutations.[34] The total amount of ApoM was increased in FH patients without CHD, indicating a role in protection from CHD in this group. In earlier studies, S1P levels inversely correlated with CHD risk.[24] It affects amongst others endothelial cell survival, proliferation, angiogenesis, migration, permeability and endothelial NO synthase activation.[20, 50] As shown earlier, S1P was mainly located in HDL_3.[35] Interestingly, we found significantly higher S1P levels in the HDL_3 fractions of FH patients without CHD than in that of their non-FH sibs. Since the correlation between cholesterol efflux and S1P levels in HDL_3 was not evident, possibly other roles of S1P such as anti-inflammatory and anti-oxidative actions are increased in FH patients without CHD.[35]

The strength of the current study design is that by including brothers, molecular and genetic heterogeneity were diminished as far as possible. Due to the relatively small study size all samples could be analyzed in a single efflux experiment each time it was performed, eliminating the inter-experiment variability.

A number of limitations of our study merit discussion. Firstly, this study is an exploratory study on a limited number of subjects. This may have led to both type I errors (false positive results) and type II errors (false negative results, e.g. the lack of correlation between HDL_2 levels and cholesterol efflux, and between ApoM and S1P). Differences in HDL_2 levels and apolipoprotein content most likely reflect differences in HDL dynamics. Due to recent developments, we chose to focus on S1P and ApoM, out of all components and apolipoproteins present in HDL. To further unravel mechanisms underlying these differences, it would be of interest to measure activity of for example CETP, ABCA1 and SR-B1 in the

subfractions. We measured CETP levels in HDL but did not find significant differences, but it would be of interest to separate HDL_2 and HDL_3 here as well. Secondly, cholesterol efflux studies are *in vitro* experiments which can only simulate the *in vivo* situation to a limited extend. However, they have been successfully applied as model for reverse cholesterol transport capacity, for instance identifying differences between individuals with low and high HDL levels, and between individuals with and without diabetes, and the effect of anti-diabetic medication.[50-52] These studies are known to have a large variability. By analyzing sib-pairs together in one experiment, we could reduce the influence of inter-experiment variability, but to further unravel differences between FH patients with and without CHD we would need to analyze much larger amounts of FH patients. Finally, the number of negative *LDLR* mutations between the FH patients with and without CHD was not significantly different but residual LDL receptor function was not tested directly.

In conclusion, we show that plasma of FH patients without CHD induced a higher cholesterol efflux from macrophages than plasma of their healthy non-FH brothers, potentially preventing CHD in these FH patients despite their high LDL cholesterol levels. This might be partially explained by increased HDL_2 cholesterol and HDL_2 apoAI levels as well as S1P levels in HDL_3. Increased S1P levels might also point to other differences in HDL functionality. These data strongly suggest that familial factors not related to the LDL receptor locus determine the effectiveness of the reverse cholesterol transport and CHD risk in FH patients.

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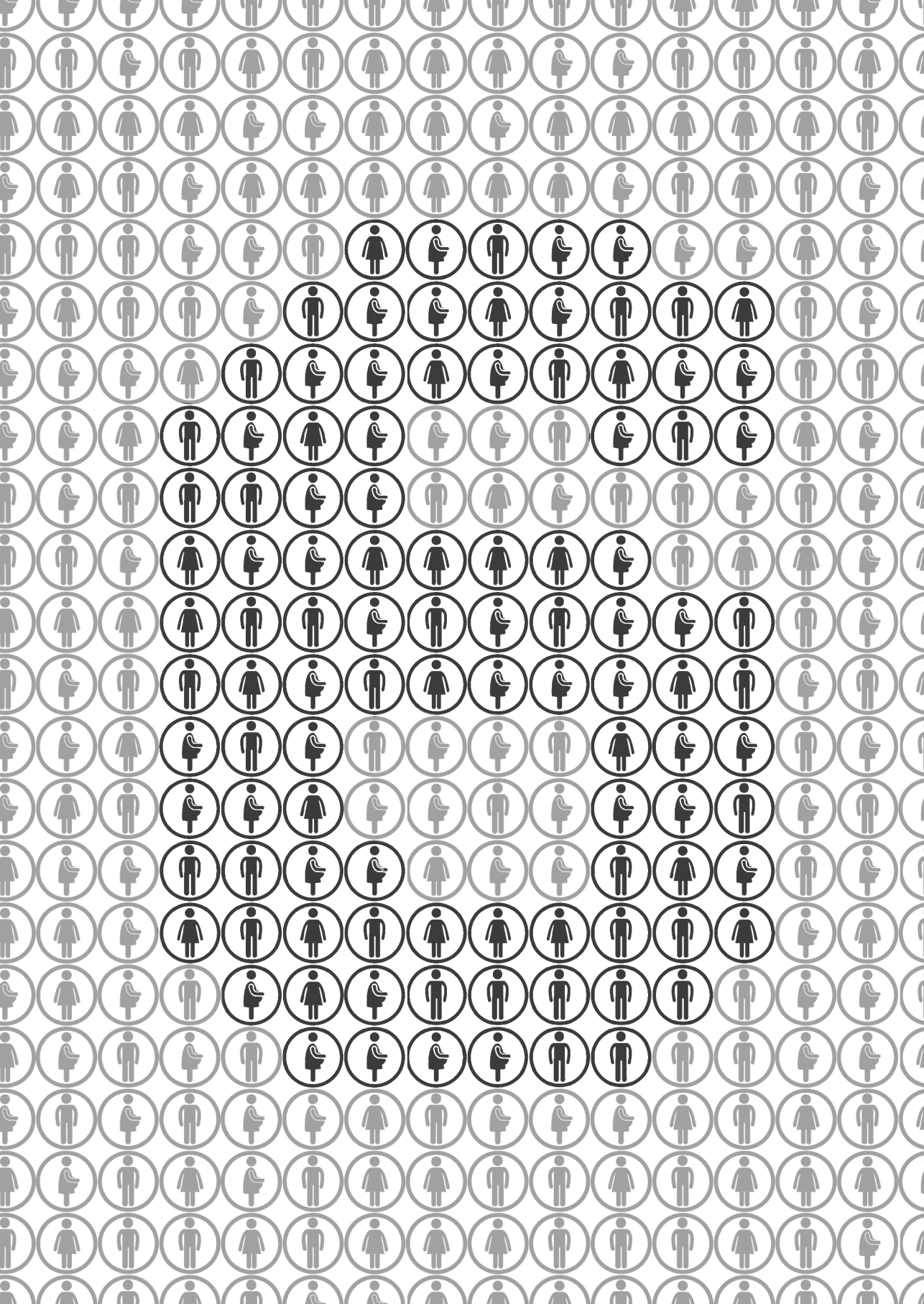

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Maternal inheritance of familial hypercholesterolemia caused by the V408M low-density lipoprotein receptor mutation increases mortality

Jorie Versmissen
Ilse PG Botden
Roeland Huijgen
Daniëlla M Oosterveer
Joep C Defesche
Thea C Heil
Anouk Muntz
Janneke G Langendonk
Arend FL Schinkel
John JP Kastelein
Eric JG Sijbrands[a]

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ABSTRACT

Objective

Fetal exposure to maternal hypercholesterolemia increases the extent of fatty-streak formation in fetal aortas as well as the rate of progression, and may therefore increase coronary heart disease (CHD) risk later in life. We hypothesized that the risk of CHD in untreated individuals with familial hypercholesterolemia (FH) is more extreme when the disease is transmitted maternally.

Methods and results

In a large Dutch pedigree carrying the V408M mutation in the low-density lipoprotein (LDL-) receptor gene, 161 individuals over seven generations were identified for which FH status and parent of origin of FH was known. We calculated standardized mortality ratios (SMR) and compared the consequences of maternal and paternal inheritance of FH by Poisson regression analysis.

Results

Maternally inherited FH was associated with significantly higher excess mortality than FH transmitted by fathers (relative risk 2.2; p=0.048): the SMR of maternal inheritance was 2.49 (95% confidence interval (CI) 1.45-3.99; p=0.001), whereas it was not significantly increased in paternally inherited FH (SMR 1.30, 95% CI 0.65-2.32; p=0.234).

Conclusions

Mortality rates are more increased when FH is inherited through the mother, supporting the fetal origin of adulthood disease hypothesis with all cause death, the most indisputable outcome measure. Future research should explore safe options for cholesterol-lowering therapy of pregnant women with FH in order to prevent unfavourable (epigenetic) consequences leading to atherosclerosis in their children

INTRODUCTION

Familial hypercholesterolemia (FH) is an autosomal dominant disorder characterized by high levels of low-density lipoprotein cholesterol (LDL-C) resulting in severe risk of coronary heart disease (CHD) and premature death when left untreated.[1] The onset of CHD symptoms, however, shows substantial variation amongst individuals with FH. Unfortunately, both classical as well as hereditary risk factors cannot accurately predict who will ultimately develop CHD.

Increasing evidence suggests that the risk of metabolic and cardiovascular disease later in life may be influenced by the environment in utero, the so-called 'fetal origins of adulthood disease hypothesis' as first described by Barker.[2, 3] Prenatal effects on CHD risk later in life have been subject to numerous studies, but so far, most research has focussed on the effects of intrauterine undernutrition, whereas maternal hypercholesterolemia has only recently begun to raise some scientific interest. In fact, elevated LDL-C in the mother has been associated with fatty streak formation in the fetal aorta and a more rapid progression to atherosclerosis later in life.[4, 5] We hypothesized that the parent of origin of FH might therefore in part determine CHD risk: only maternal inheritance of FH exposes a fetus to the consequences of parental hypercholesterolemia. Because of possible teratogenic effects of statins and gastro-intestinal side-effects of bile-acid sequestrants, pregnant women are preferably not treated with lipid-lowering drugs.[6, 7] However, the consequences of maternal hypercholesterolemia for the newborn are unknown and may have been underestimated due to the large time lag between exposure and consequences. To study the consequences of maternally inherited FH, we compared individuals who inherited FH maternally and those who inherited FH through the father in a large FH pedigree, in which the transmission lines of the V408M mutation in the LDL receptor gene were known for a large number of family members over seven generations.[8] The V408M mutation results in more rapid degradation of LDL receptors and unambiguously leads to high cholesterol levels and increased CHD risk.[9-12] We used all cause mortality as primary outcome measure and here we present our results.

METHODS

Subjects

Data were collected from a large pedigree consisting of 412 descendants in eight generations as described in detail previously.[8] Three index cases with the V408M mutation (the new nomenclature according to the human genome variation society recommendations would be p.V429M) in the LDL receptor gene were identified during routine cholesterol screening (n=2) and after a myocardial infarction (n=1).[13] The untreated probands had mean fasting total serum cholesterol concentrations of 10.24, 9.20, and 12.78 mmol/l,

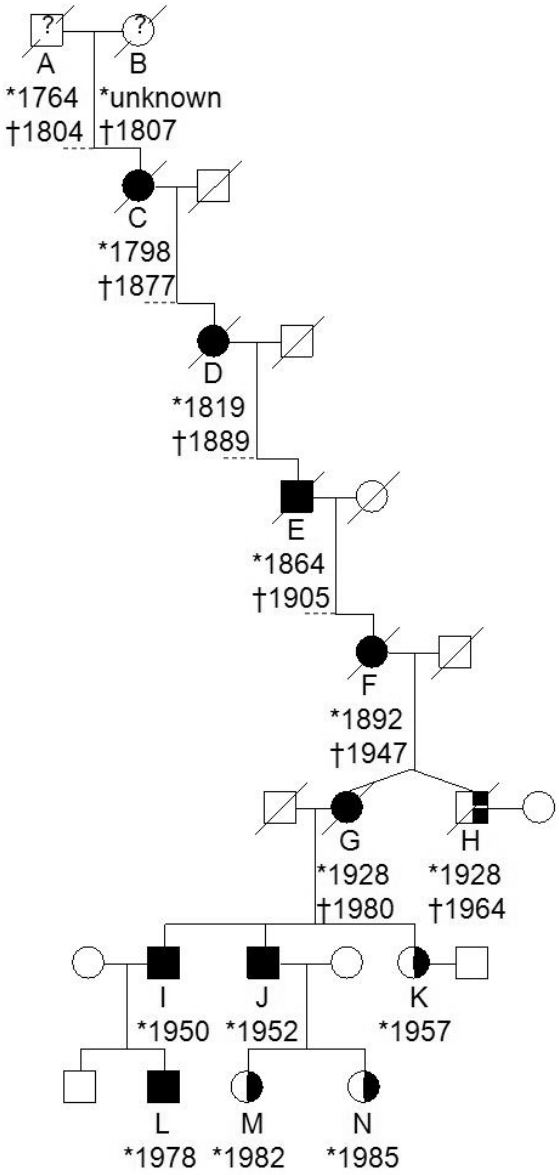

FIGURE. Part of the pedigree as an example of assessment of FH status and parent of origin. Dashed lines indicate a part of the pedigree has been removed for the sake of clarity. Circles represent females, squares represent males. A and B are the most early ancestors, of whom is not known who had FH. C, D, E, F, and G can be assigned to have had FH due to transmission to later generations. H is unknown (50% chance) because he did not have any offspring. I, J, and L have been genetically tested. K, M, and N have not (yet) been tested and therefore still have 50% chance. Of all certain FH carriers, D,E, G, I, and J inherited maternally and L and F paternally. The parent of origin of C`s FH is unknown. L was not included in the analyses because she was too young at the end of follow-up in 1990.

respectively.[8] The molecular diagnosis of familial hypercholesterolemia was based on the presence of the V408M mutation, and since they carried the mutation on an identical haplotype, they were expected to be (distantly) related. Genealogical research and molecular screening were used to collect all carriers of the V408M mutation in the pedigree. One pair of ancestors shared by and connecting the three probands was identified. All living descendants of this pair were screened for the V408M mutation. Only persons for whom diagnosis of FH was certain, i.e. proven carriers of the mutation and individuals on the transmission line of the mutation were included (n=164). For the first generation, FH status could not be assigned. The second generation was excluded (n=3), because we had no information about which parent transmitted the mutation (Figure). The transmission lines through this large pedigree connecting the probands is shown in the original paper describing this pedigree.[8] The molecular and genealogical studies were approved by the hospitals' review boards, and all family members studied gave informed consent.

Statistical analysis

The standardized mortality ratio (SMR) was calculated by comparing mortality in the pedigree with the mortality in the general Dutch population.[8] The SMR is the ratio of observed to expected number of deaths. The expected number of deaths is calculated by multiplying the total number of years lived by the people in the pedigree in each calendar period in each age and sex category by the age and sex specific mortality rates of the Dutch population for each calendar period. We ignored the first two decades of life for all people in the pedigree since descendants had to live until reproductive age before they could pass on the gene mutation and data on juvenile mortality might have been incomplete in the 19th century; also, mortality before this age is unlikely to be a consequence of heterozygous FH. We compared mortality risk between people who inherited FH paternally and those who inherited it maternally using Poisson regression (relative risk) adjusted for the differences in distribution of sex, calendar period and age category. The observation period was censored in 1990 when statins became available.

RESULTS

A schematic drawing of a part of the pedigree is shown in Figure: this represents one of the transmission lines of the V408M mutation originating from one pair of distinct ancestors. Of all 161 persons with FH and knowledge of parent of origin of FH, 48 were younger than 20 at the end of follow up on January 31st, 1990. They were excluded from the analyses. All carriers of the V408M mutation in our pedigree whose untreated cholesterol levels were available invariably had cholesterol levels above the 95th percentile.[11]

During 3,023 person years, 28 deaths occurred among the 113 patients over 20 years of age. Overall, the SMR was 1.83 (95% confidence interval (95% CI) 1.22-2.64; p=0.002);

1.62 in females (95% CI 0.86-2.77; p=0.062) and 2.06 in males (95% CI 1.15-3.39; p=0.008). A total of 58 patients inherited FH from their mother while 55 patients inherited FH from their father. The SMR of maternally inherited FH was 2.49 (95% CI 1.45-3.99; p=0.001) while in paternally inherited FH patients the risk was not significantly increased (SMR1.30, 95% CI 0.65-2.32; p=0.23; Table). This difference in mortality between paternally and maternally inherited FH was statistically significant (Poisson regression: relative risk (RR) 2.2; p=0.048). The difference between the two types of inheritance was more pronounced in female offspring: maternally inherited FH associated with a 2.30 times increased mortality ratio (95% CI 0.99-4.53; p=0.026) while in women who inherited FH paternally no excess mortality was observed (SMR 0.86, 95% CI 0.23-2.18; p=0.68; Table). Due to small numbers, this difference was not significantly different (RR 3.5, 95% CI 0.81-15.2; p=0.09). In males, the SMR of maternally inherited FH was 2.69 (95% CI 1.23-5.10; p=0.008) while in paternally inheritance it was 1.84 (95% CI 0.74-3.79; p=0.092; Table).

TABLE. SMR according to maternally and paternally inherited FH

	N	Person years	Observed deaths	Expected deaths	SMR	95% CI	p-value
Maternal inheritance							
Overall	58	1539	17	6.83	2.49	1.45-3.99	0.001
Males	27	733	9	3.35	2.69	1.23-5.10	0.008
Females	31	805	8	3.48	2.30	0.99-4.53	0.026
Paternal inheritance							
Overall	55	1484	11	8.5	1.30	0.65-2.32	0.23
Males	26	714	7	3.8	1.84	0.74-3.79	0.092
Females	29	770	4	4.7	0.86	0.23-2.18	0.68

N=number of persons at risk; SMR=Standardized mortality ratio; 95% CI= 95% confidence interval
Patients who inherited FH maternally had 2.2 times higher mortality risk relative than those who inherited it paternally (95% CI 1.01-4.08; p=0.048).

DISCUSSION

We demonstrate that all cause mortality in individuals with FH is more extremely increased when the disorder is inherited maternally compared with paternal inheritance. This seems more pronounced in females: mortality in females who inherited FH paternally was not significantly different from that in the general population, but much higher when females inherited the disorder from their mothers. This is to our knowledge the first study proving the 'fetal origins of adulthood disease' hypothesis with all cause death.

Strong methodological aspects of our study include first, that we used an undisputed outcome measure, all cause mortality, and second, that follow-up took place before patients were treated with statins, and last, that patients were not selected on the presence of CHD; in fact, the majority of individuals were unaware of their condition. This avoids important biases of studies that have addressed the consequences of maternal hypercholesterolemia during pregnancy. Most previous human studies were surrogate marker

studies in children from families who sought medical attention.[14, 15] At our own Lipid Clinic, 74% (95% CI 62-83%) of the children below the age of 18 years have inherited FH paternally. This illustrates the clear selection bias that occurs due to a higher CHD risk in males. Such a selection phenomenon might in fact explain why Tonstad et al. found that carotid intima media thickness was not significantly different between children who inherited FH maternally or paternally.[14] In our study, this potential selection bias was avoided by studying multiple generations of one particular mutation, identified on the basis of only three index cases.

Most studies investigating the consequences of hypercholesterolemia during pregnancy were *in vivo* and *in vitro* animal studies.[16-21] In a study using mice lacking the ldl receptor (*ldlr-/-* mice), *ldlr+/-* offspring of *ldlr-/-* mothers had abnormal vascular responses later in life, as well as increased aortic lesions and altered aortic gene expression.[16, 17] The ldlr+/- offspring of ldlr+/+ mothers without fetal exposure to hypercholesterolemia did not show any of these signs of enhanced early atherogenesis. Also, adult mice lacking one allele of apolipoprotein E (*apoE* +/-) descending from hypercholesterolemic *apoE-/-* mothers had more severe atherosclerotic lesions than the genetically identical offspring of normocholesterolemic wild type (*apoE*+/+) mothers.[18] This *apoE+/-* offspring of *apoE-/-* mothers was also more prone to the effects of hypercholesterolemia on progression of vascular disease later in life, a situation very similar to that of an FH child from an FH mother.[19]

Intra-uterine conditions may lead to permanent non-genetic DNA changes, so-called epigenetic effects. In mice exposed to prenatal hypercholesterolemia chromatin modifications such as histone methylation, acetylation, and ubiquination were identified. [19, 20] These changes might alter the regulation of genes that influence risk of CHD and mortality later in life. First studies in offspring of hypercholesterolemic mice indeed showed differences in gene expression in the offspring up to three months after birth that might have been caused by these epigenetic changes.[17] Since LDL-particles at physiological levels only pass the placental barrier in modest quantities, it is not clear whether chromatin modifications result from hypercholesterolemia itself or from the inflammation and oxidative stress caused by the hypercholesterolemia in the mother.[21] The presence of these chromatin modifications could further confirm our findings. This has not yet been studied in offspring of mothers with FH.

During the last trimester of pregnancy in healthy mothers, LDL-cholesterol is expected to rise by 25-50%.[22] This secondary hypercholesterolemia is thought to have no effect on the mothers` lifelong CHD risk.[23] In pregnant FH women, the absolute increase of LDL-C levels is considerably greater than in women without FH, but the consequences have not been extensively studied.[24, 25] Nevertheless, fatty streak formation in the fetal aorta is impressively increased when maternal hypercholesterolemia is present.[4] In addition, the extent and severity of lesions progress at a steeper rate in the children who have been exposed to maternal hypercholesterolemia in utero.[5]

Statins are the first choice of treatment for FH. These potent cholesterol-lowering drugs are also reported to be anti-inflammatory and anti-oxidative.[26] However, as reported in animal studies and birth defect case reports of women that have coincidentally used statins

during pregnancy, most statins are potentially teratogenic, especially the lipophilic compounds that can pass the placenta.[6, 7] In general, hypercholesterolemia during pregnancy is accepted and statin treatment interrupted.[25] We recommend thorough examination of teratogenic effects of statins to exclude the possibility of a recall bias in retrospective studies, as seen for other drugs.[27] To date, treatment of FH during pregnancy has been reserved for patients with extreme increases of LDL-C levels and is mainly restricted to bile-acid sequestrants.[25] However, current policy does not take the effects of maternal hypercholesterolemia on the newborn's CHD risk later in life into account. The current finding suggests that an important unmet medical need exists for cholesterol-lowering medication that is safe for both mother and child.

Our study has a number of limitations. First, we studied a relatively small number of individuals with FH caused by a specific mutation. We need replication with other LDL receptor mutations before our findings can be generalized. However, we discovered strong consistency of results with an undisputed outcome: all cause mortality. For example, our finding was consistent in males and females, although the latter have a lower basal CHD risk.[8] It may be counterintuitive that the SMR of untreated but paternally inherited FH was not significantly increased, while overall excess mortality in FH carriers was severe in this pedigree. The point estimate of the SMR of males, who inherited FH paternally, was in line with the expected excess mortality but it was not statistically significant (SMR 1.84; p=0.09). The point estimate of the SMR of females did not reveal an increased risk. Probably the power in these subgroup analyses was too limited to observe statistically significant excess mortality in case of paternal inheritance.

Second, we did not know the cause of death; however, by comparing a relatively rare disorder with the population at large and adjustment for the differences in the distribution of sex, calendar period and age category, we could determine the excess mortality resulting from FH. Specific causes of death are less reliable endpoints than all cause mortality due to low accuracy of death certificates, even in current times and especially for cardiovascular causes of death.[28, 29] The mortality rates in this pedigree were in line with the expected burden from untreated FH: CHD risk is approximately seven times increased by heterozygosity for the V408M mutation.[12] Third, in the present study, effects of maternal hypercholesterolemia on cholesterol levels could not be calculated, due to small numbers of known untreated cholesterol levels. Recently, in a study consisting of patients largely identified by our genetic cascade screening, it was shown that children who inherited FH from their mothers had higher LDL cholesterol levels.[30]

Fourth, we did not study the effects of hypercholesterolemic mothers on the non-FH offspring since it is impossible to assign 'non-FH' with 100% certainty in non-tested individuals in this pedigree. This is unfortunate, because it might be hypothesized that this offspring has a higher mortality risk as well. Finally, it could be argued that paternal FH might have other (postnatal) consequences for the offspring: 'today, extreme premature CHD of the father may have led to a healthier lifestyle of the offspring.' However, the majority of persons in this pedigree lived in times during which cholesterol was unknown and lifestyle interventions had not been developed.

In conclusion, we demonstrate that mortality rates are more increased when FH is inherited through the mother, supporting the fetal origin of adulthood disease hypothesis with all cause death, the most indisputable outcome measure. Future research should further explore this interaction and eventually safe options for cholesterol-lowering therapy of pregnant women with FH in order to prevent unfavourable epigenetic consequences leading to atherosclerosis in their children.

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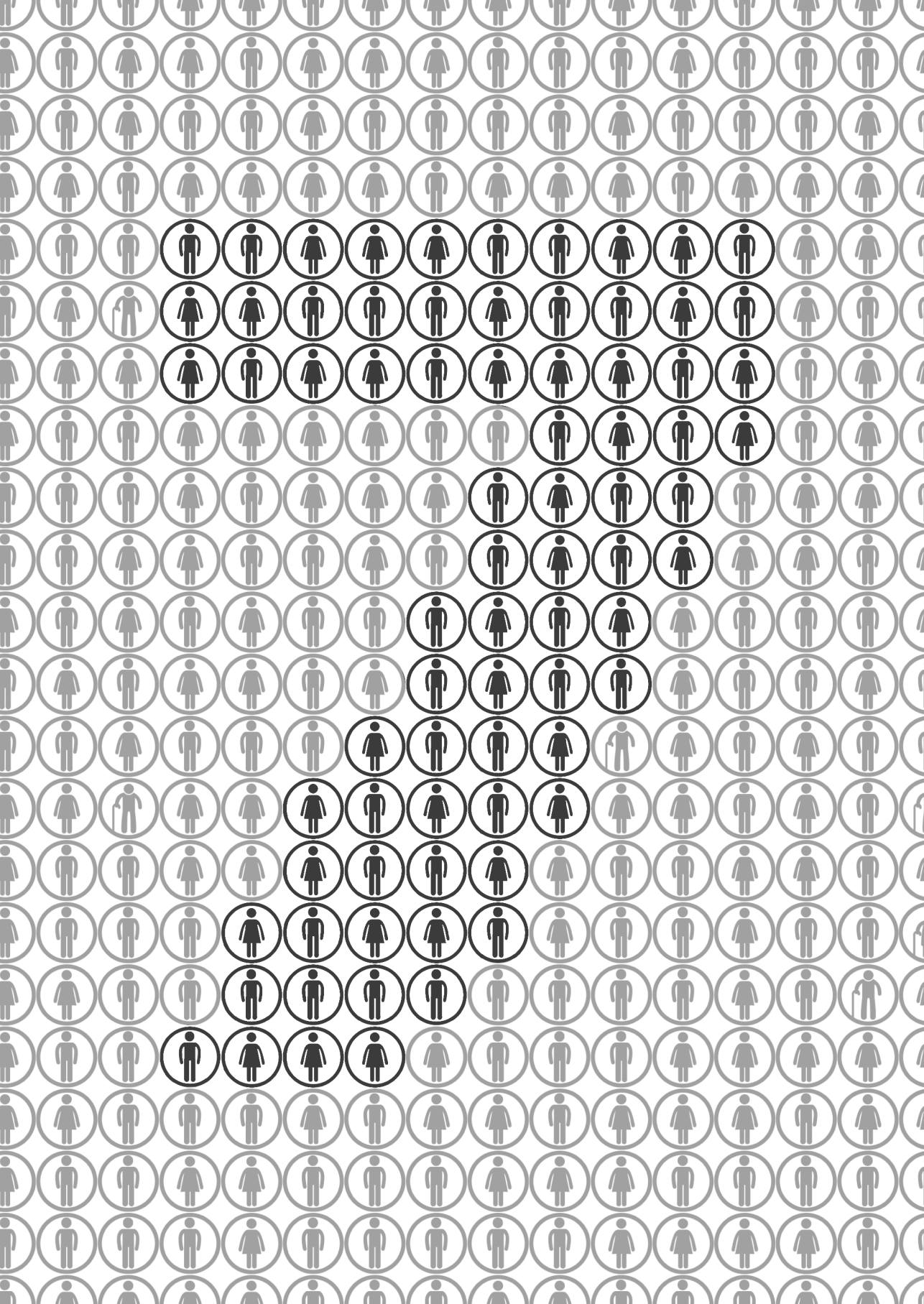
PART II

TREATMENT OF FH



7

Efficacy of statins in familial hypercholesterolemia: a long term cohort study

Jorie Versmissen
Daniëlla M Oosterveer
Mojgan Yazdanpanah
Joep C Defesche
Dick CG Basart
Anho H Liem
Jan Heeringa
Jacqueline C Witteman
Peter J Lansberg
John J P Kastelein
Eric J G Sijbrands

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ABSTRACT

Objective

To determine the efficacy of statin treatment on risk of coronary heart disease in patients with familial hypercholesterolemia.

Design

Cohort study with a mean follow-up of 8.5 years.

Setting

27 outpatient lipid clinics.

Subjects

2146 patients with familiar hypercholesterolemia without prevalent coronary heart disease before 1 January 1990.

Main outcome measures

Risk of coronary heart disease risk in treated and 'untreated' (delay in starting statin treatment) patients compared with a Cox regression model in which statin use was a time dependent variable.

Results

In January 1990, 413 (21%) of the patients had started statin treatment, and during follow-up another 1294 patients (66%) started after a mean delay of 4.3 years. Most patients received simvastatin (n=1167, 33 mg daily) or atorvastatin (n=211, 49 mg daily). We observed an overall risk reduction of 76% (hazard ratio 0.24 (95% confidence interval 0.18 to 0.30), p<0.001). In fact, the risk of myocardial infarction in these statin treated patients was not significantly greater than that in an age-matched sample from the general population (hazard ration 1.44, 95% confidence interval 0.80 to 2.60; p=0.23).

Conclusion

Lower statin doses than those currently advised reduced the risk of coronary heart disease to a greater extent than anticipated in patients with familial hypercholesterolemia. With statin treatment, such patients no longer have a risk of myocardial infarction significantly different from that of the general population.

INTRODUCTION

Familial hypercholesterolemia is a monogenic disorder associated with a greatly increased risk of coronary heart disease.[1] Statins are first choice treatment for all patients with the condition.[2] Placebo controlled trials were not carried out in these patients when statins were introduced, as it was considered unethical to withhold causal treatment from patients with an inborn error of lipid metabolism.[3] We therefore lack estimates of the true efficacy of statin treatment in such patients. The results of the atorvastatin versus simvastatin on atherosclerotic progression (ASAP) study have suggested that we should treat such patients preferably with potent statins to maximally decrease low density lipoprotein (LDL) cholesterol concentrations and induce regression of atherosclerosis.[4, 5] In the recently published ENHANCE study, adding ezetimibe to the highest dose of simvastatin resulted in substantial further reductions of LDL cholesterol concentrations and high sensitivity C reactive protein levels but did not further reduce the carotid intima-media thickness.[6] One possible explanation for these results is that patients with familial hypercholesterolemia are currently treated optimally with statins, resulting in delipidated arterial walls of normal thickness, and, therefore, further lowering of LDL cholesterol levels has no beneficial effect. In two observational studies, secular trends suggest that statins have roughly halved the risk of coronary heart disease in patients with familial hypercholesterolemia.[7, 8] However, the exact prognosis of treated asymptomatic patients remains unknown, and this lack of hard endpoint data has, for example, limited access to life insurance.[9]

In this study we investigated the effect of statins on the risk of incident coronary heart disease in patients with familial hypercholesterolemia, using the variation in the time of starting statin treatment to mimic a clinical trial.

METHODS

Study population

During 1989-2002, we recruited a cohort of 2400 patients with familial hypercholesterolemia from 27 lipid clinics as described previously.[10] We reviewed medical records to establish extensive phenotypic data including anthropometric measures, cardiovascular

events, and use of lipid lowering drugs.[11] The ethics institutional review board of each participating hospital approved the study protocol. All patients gave their informed consent.

In the present study, we chose 1 January 1990, just after the first statin (simvastatin) became available in the Netherlands, as the start point. We excluded patients who already had coronary heart disease by 1990 to mimic a controlled primary prevention trial starting at the introduction of the first statin. We determined serum lipid concentrations in fasting patients who had not used lipid lowering drugs for at least six weeks, and calculated LDL cholesterol concentrations using the Friedewald formula.[12]

In addition, we compared the risk of incident myocardial infarction in patients with familial hypercholesterolemia with that in the general population. For this analysis, we selected patients with familial hypercholesterolemia who were older than 55 years at 1 January 1990 and a selection of the population in the Rotterdam study matched for age and sex. The Rotterdam study is a large population based, prospective, follow-up study starting in 1990 assessing the disease burden in elderly people.[13]

Outcome measures

We defined coronary heart disease in our study cohort as at least one of the following:

- Myocardial infarction, proved by at least two of the following:
  - Classic symptoms (>15 minutes)
  - Specific electrocardiographic abnormalities
  - Elevated cardiac enzymes (>2xupper limit of normal)
- Percutaneous coronary intervention or other invasive procedures
- Coronary artery bypass grafting
- Angina pectoris, diagnosed as classic symptoms in combination with at least one unequivocal result of one of the following:
  - Exercise test
  - Nuclear scintigram
  - Dobutamine stress ultrasound scan
  - >70% stenosis on a coronary angiogram.

In the Rotterdam study, no data on angina were available. We therefore chose to study myocardial infarction as the end point in this analysis. Patients with percutaneous transluminal coronary angioplasty, coronary bypass grafting, or angina pectoris were excluded from the sample of patients from our cohort and as from the selected population from the Rotterdam study.

Statistical analysis

We compared patients' general characteristics using analysis of variance for continuous variables and χ^2 test for categorical variables. We analysed lipid concentrations, after adjustment for age and sex, with multiple linear regression analyses. We calculated absolute risk by taking into account whether statin use was started before the onset of coronary heart disease. The number of coronary events among statin treated and untreated patients was divided by the total event-free survival. Event-free survival time was defined as the period from 1 January 1990 to the date of first coronary event, death, or censoring at the end of our observation.

We also compared survival among treated and untreated patients using the Kaplan-Meier survival method. In this analysis, we considered people to be untreated when they had not taken a statin for longer than one month before a coronary event or censoring. All patients who received a statin during follow-up for more than a month before an event or censoring were considered as treated.

We performed separate analyses for patients older than 55 years to compare them with the selected population from the Rotterdam study. Since the maximum follow-up in our cohort was 12 years, we chose 2002 as the end point in the Rotterdam study as well.

We used the Cox proportional hazard model to estimate the risk of coronary heart disease among statin treated patients compared with untreated patients. Since both groups had variable periods without statin treatment, we analysed statin treatment as a time dependent variable. This variable was equal to zero for the time statins were not used, and 1 for the time from start of statin treatment to the date of first coronary event or censoring. In the primary model, we adjusted the analyses for sex and year of birth.

In additional models we adjusted for other classic risk factors (Table 2). We performed subgroup analyses stratified by previously identified risk factors to define effect differences. For LDL cholesterol concentration, we used the median value to split the total population in two subpopulations. We classified high density lipoprotein (HDL) cholesterol concentration according to our normal laboratory values: <0.9 mmol/l for males, <1.1 mmol/l for females. To test for lifestyle improvements occurring at the start of statin treatment, we adjusted the Cox proportional hazard model for smoking cessation within six months after the start of statin treatment. We tested whether deselection of worst cases occurred by adding the patients who were never treated with statins to the treated group as if they had started treatment on 1 January 1990 and repeated the time dependent Cox proportional hazard analysis.

RESULTS

Of the 2400 patients recruited, we excluded 254 who already had coronary heart disease by 1990. We excluded a further 188 patients because the type of lipid lowering treatment or the date of starting statin treatment was unknown, leaving 1950 patients with sufficient

data on drug use (Figure 1). In January 1990, 413 patients were treated with a statin, and a further 1294 patients were prescribed statins during follow-up. The mean delay in starting statin use was 4.3 years (SD 3.3 years). The 196 patients who were excluded because of missing data or loss to follow-up had serum LDL cholesterol concentrations 1.14 mmol/l higher than those of the included patients. We did not find other differences between these groups.

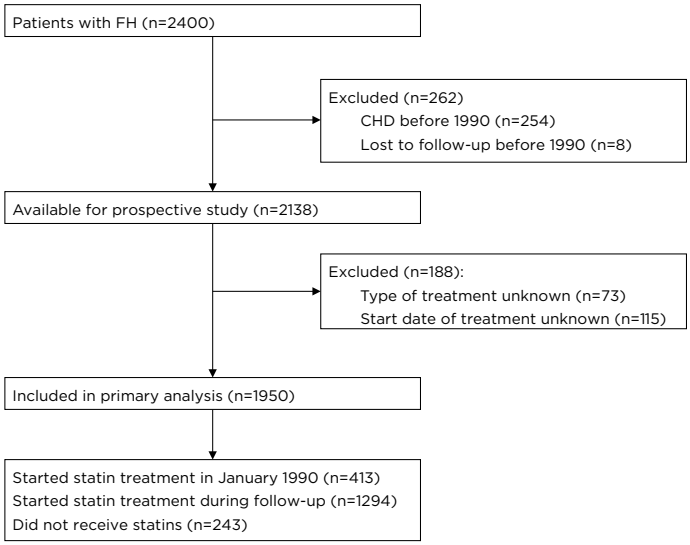


FIGURE 1. Flow diagram to show included and excluded patients.

TABLE 1. General characteristics of patients with familial hypercholesterolemia by their receipt of statin treatment in January 1990. Values are numbers (percentages) of patients unless stated otherwise

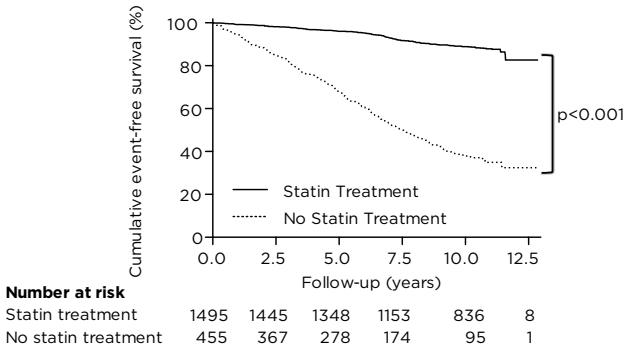
	Taking statins (n=413)	Not taking statins (n=1537)	p-value
Men	197 (48)	727 (47)	0.89
Ever smoked	281(77)	1007 (72)	0.09
Hypertension	47(11)	48 (3)	<0.01
Diabetes	6 (1)	5 (0.3)	0.06
Mean (SD) age at first visit to lipid clinic (years)	43.3 (13.3)	43.8 (12.3)	0.36
Mean (SD) age at 1 January 1990 (years)	41.7 (13.2)	38.2 (12.5)	<0.001
Mean (SD) body mass index (kg/m^2)	24.8 (3.5)	25.1 (3.5)	0.18
Mean (SD) total cholesterol concentration (mmol/l)	10.1 (1.9)	9.0 (1.9)	<0.001
Mean (SD) LDL cholesterol concentration (mmol/l)	8.0 (1.9)	6.9 (1.8)	<0.001
Mean (SD) HDL cholesterol concentration (mmol/l)	1.13 (0.34)	1.19 (0.37)	0.02
Mean (SD) triglyceride concentration (mmol/l)	1.45 (0.71)	1.58 (0.48)	0.007

LDL=low density lipoprotein. HDL=high density lipoprotein.
All lipid concentrations are before statin treatment and adjusted for sex and year of birth.

The patients who immediately received statin treatment in 1990 were on average 3.5 years older than the patients who started statin treatment later, had higher total and LDL cholesterol concentrations (both p<0.001), had lower HDL cholesterol levels (p=0.02), and were significantly more likely to be hypertensive than the other patients (Table 1). Twenty eight patients stopped taking statins for unreported reasons.

The mean follow-up time was 8.5 years (SD 3.1 years). During 7473 person years for statin treated patients and 9319 person years for untreated patients, 408 patients had an incident coronary event, of whom 161 patients had been using statins for an average 3.4 years (median 2.7 years, range 1 month to 11.6 years). Most patients (n=1167) used simvastatin with a mean dose of 33 mg (SD 20 mg), leading to 44% (SD 16%) lower LDL cholesterol compared with before they started statin treatment. A further 211 patients used atorvastatin with a mean daily dose of 49 mg providing a reduction in LDL cholesterol level of 49% (SD 15%). Less commonly used statins were pravastatin and fluvastatin. During statin treatment the mean total cholesterol concentration was 5.9 mmol/l (SD 1.2 mmol/l), mean LDL cholesterol was 4.0 mmol/l (SD 1.2), and mean HDL cholesterol was 1.28 mmol/l (SD 0.41).

In familial hypercholesterolemia, the absolute risk of first onset of coronary heart disease was 11/1000 person years in statin treated patients compared with 119/1000 person years in untreated patients. Incident coronary heart disease occurred at younger age in untreated patients (48.6 *vs* 50.9 years, p=0.05). The treated group had a significantly better event-free survival (p<0.001, Figure 2). After adjustment for year of birth and sex, statin treated patients had a 76% reduction in risk of coronary heart disease compared with untreated patients (hazard ratio 0.24 (95% confidence interval 0.18 to 0.30), p<0.001). Further adjustment for baseline characteristics such as smoking, HDL and LDL cholesterol concentrations, diabetes, and hypertension resulted in an overall 82% risk reduction (hazard ratio 0.18 (0.13 to 0.25), p<0.001) (Table 2).



Number at risk						
Statin treatment	1495	1445	1348	1153	836	8
No statin treatment	455	367	278	174	95	1

FIGURE 2. Kaplan-Meier curve estimates of cumulative coronary heart disease-free survival of familial hypercholesterolemia patients on and off statin treatment.
Follow-up starts at 1-1-1990.

TABLE 2. Time dependent risk reduction (95% confidence intervals) with statin use

		Model I		Model II		Model III		Model IV	
	N	Hazard ratio (95% CI)	p-value	Hazard ratio (95% CI)	p-value	Hazard ratio (95% CI)	p-value	Hazard ratio (95% CI)	p-value
All	1950	0.24 (0.18 - 0.30)	<0.001	0.22 (0.17 - 0.28)	<0.001	0.20 (0.15 - 0.27)	<0.001	0.18 (0.13 - 0.25)	<0.001
Males	924	0.20 (0.15 - 0.28)	<0.001	0.20 (0.14 - 0.28)	<0.001	0.16 (0.11 - 0.25)	<0.001	0.17 (0.11 - 0.26)	<0.001
Females	1026	0.30 (0.20 - 0.43)	<0.001	0.25 (0.17 - 0.37)	<0.001	0.28 (0.18 - 0.43)	<0.001	0.21 (0.13 - 0.34)	<0.001

Model I = adjusted for sex and year of birth. Model II = adjusted for sex, year of birth and smoking. Model III = adjusted for sex, year of birth and low-density lipoprotein cholesterol level without medication; model IV=adjusted for sex, year of birth, smoking, high- density lipoprotein cholesterol level, low-density lipoprotein cholesterol level, diabetes and hypertension.

As expected, men had a 2.5 times greater risk of coronary heart disease than women (95% confidence interval 2.1 to 3.1, P<0.001). Whether women with familial hypercholesterolemia benefit from statins as men do is still under debate, and we therefore studied them separately.[14] The women taking statins had a 79% reduction in risk of coronary heart disease compared with women not taking statins(hazard ratio 0.21 (0.13 to 0.34), p<0.001); the men had an 83% risk reduction (hazard ratio 0.17 (0.11 to 0.26), P<0.001) (Table 2).

Patients who developed coronary heart disease had higher serum LDL cholesterol concentrations before treatment than did those without coronary heart disease (7.5 mmol/l *vs* 7.2 mmol/l, p=0.03). During statin treatment, however, LDL cholesterol levels were identical among patients with and without coronary heart disease (4.1 mmol/l *vs* 4.0

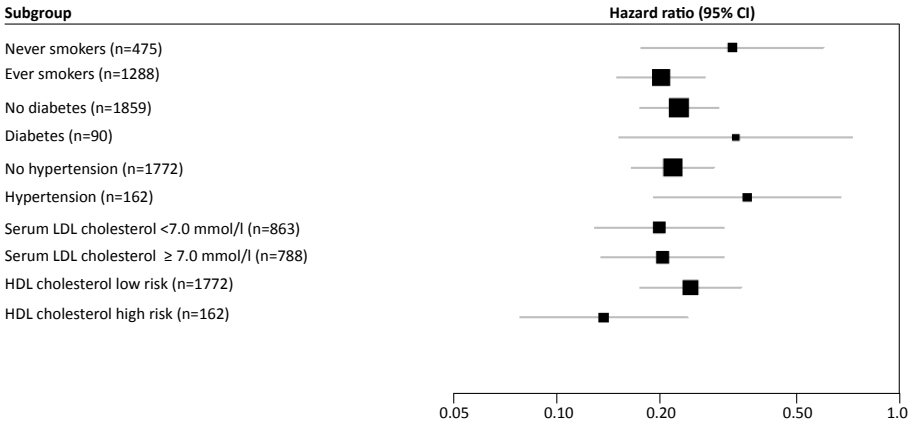


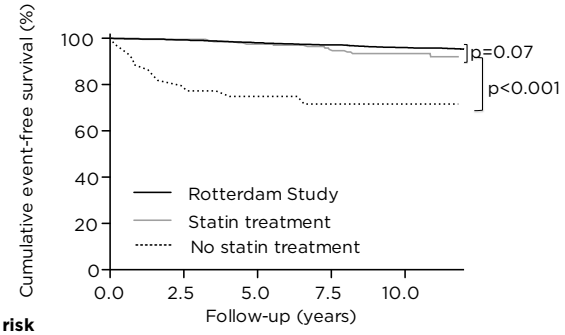
FIGURE 3. Subgroup analyses for risk groups based on classical risk factors.
Smoking, diabetes and hypertension were compared based on ever/never present. Low-density lipoprotein cholesterol level was split using the median. High-density lipoprotein cholesterol level was classified according to our normal laboratory values as indicated. The risk reduction by statin use in a time dependent way is shown on a logarithmic scale. LDL=low density lipoprotein. HDL= high density lipoprotein. Low risk HDL serum concentrations: women >1.1 mmol/l, men >0.9 mmol/l. High risk HDL serum concentrations: women <1.1 mmol/l, men <0.9 mmol/l.

mmol/l, P=0.38). Classic risk factors were, as expected, more common in patients with coronary heart disease. The subgroup analyses in Figure 3 suggest that the efficacy of statin treatment did not substantially differ between smokers and non-smokers, between patients with or without diabetes, and between patients with or without hypertension. However, the size of the subgroups were too small for meaningful analyses.

Of all 1288 patients who had ever smoked, 333 had stopped before 1990. A total of 407 statin users never smoked, and 388 had stopped before statin treatment was started. A further 105 patients (10% of all those for whom smoking cessation date was known) quitted within six months of starting statin treatment. To test if lifestyle improvement related to the start of statin treatment could explain why smokers showed a larger risk reduction with statins, we adjusted for smoking cessation within six months of the start of statin treatment, but this did not materially change the effect of statin treatment on coronary heart disease risk (hazard ratio 0.20 (0.15 to 0.26), p<0.001). Unfortunately, body mass index and waist circumference were not measured during follow-up.

A total of 243 patients in our cohort were never treated with statins. We performed an additional analysis adding those patients to the treatment group, as if statin treatment had started on 1 January 1990, to estimate the effect of an intention to treat analysis. The hazard ratio was even lower under these assumptions (data not shown).

We finally compared the risk of myocardial infarction in patients with familial hypercholesterolemia who were older than 55 years (n=261, 64 men) with that in 1975 people in a subgroup of the participants in the Rotterdam study. The mean age in both subgroups was 61.6 years and both had 24.5% men as a result of stratified selection from the Rotterdam study. The absolute risk of myocardial infarction was 6.7/1000 person years in our statin treated patients, 60.5/1000 person years in our untreated patients, and 4.1/1000 person years in the sample from the Rotterdam study. Event-free survival of our statin



Numbers at risk

Rotterdam Study	1975	1922	1851	1775	1668
Statin treatment	215	208	191	162	115
No statin treatment	46	36	26	19	12

FIGURE 4. Kaplan-Meier curve estimates of cumulative myocardial infarction-free survival of familial hypercholesterolemia patients older than 55 years on and off statin treatment compared to a comparable subgroup of the Rotterdam Study representing the general population.

treated patients was not significantly different from that of the Rotterdam study sample (log rank test p=0.07), whereas our untreated patients clearly had a higher risk of coronary heart disease (log rank test p<0.001) (fig 4). After adjustment for year of birth and sex, the point estimate of risk of myocardial infarction in our treated patients with familial hypercholesterolemia was higher than the risk in the subgroup of the Rotterdam study but this was not significant (hazard ratio 1.44 (0.80 to 2.60), p=0.23), whereas the risk in our untreated patients was 8.7 times higher (hazard ratio 8.69 (4.77 to 15.82), p<0.001).

DISCUSSION

We report here that relatively modest doses of statins reduced the risk of coronary heart disease by about 80% in patients with familial hypercholesterolemia. This is a much more pronounced reduction than was anticipated based on secular trends in earlier studies.[7, 8] We also observed that statin treated patients older than 55 years had a risk of myocardial infarction approaching that of the general population. Finally, men and women experienced similar risk reductions in our study.

Strengths and weaknesses of our study

Our follow-up study has a number of limitations. Firstly, it was observational and not a randomised study. Therefore, the patients who started treatment immediately in 1990 may have represented a selected subgroup with more severe risk: they were selected first because they had further progressed in their disease process. If this were so, it would have resulted in a higher risk for the treated group compared with a randomised trial that distributed the risks equally. However, it seems unlikely that we have underestimated the risk reduction as the effect was unexpectedly large.

Secondly, it could be argued that our approach exaggerates the effect of the treatment, because our study was not placebo-controlled. Patients might have improved their lifestyle in conjunction with starting statin treatment. However, adjustment for smoking cessation within six months after the start of treatment (as a proxy for lifestyle improvement) did not change the effect of statin treatment.

Thirdly, we analysed statin treatment as a time dependent variable, whereas an intention to treat analysis might have yielded smaller risk reductions. We analysed the 28 patients who stopped statin treatment as if they had stayed on treatment, in line with an intention to treat analysis. We also made an analysis in which all patients who were never treated with statins were added to the treated group as if they had started treatment on 1 January 1990, to estimate the effect that an intention to treat analysis could have had: this showed an even larger effect. Although this value is a rough estimation sensitive to choice of date of start of treatment, the decrease in hazard ratio indicates that our results are not overestimating the effect as a result of deselection of worst cases.

Although some of the weaknesses associated with lack of randomisation have been addressed, there is always the danger that unrecognised confounding factors, for which we made no adjustment, might have affected our results.

Comparison with other studies

The large risk reduction and the overlap of the event-free survival between the treated patients and a sample of the general population (from Rotterdam study) suggest that statin treatment has profoundly improved the prognosis for familial hypercholesterolemia.

Two previous studies have investigated this issue. A study in the United Kingdom compared mortality in patients with familial hypercholesterolemia with that in the general population before and after the introduction of statins.[7] Their results suggested that statins did indeed reduce mortality, but that mortality was still higher than in the general population. Exact information about the start date of statin treatment was not available, however, suggesting there might have been an unrecognised delay in statin initiation similar to what we found in the Netherlands.

In a much smaller study, 214 statin treated patients with familial hypercholesterolemia still had increased risk of cardiovascular disease.[8] As suggested by the high frequency of premature cardiovascular disease (45%) in first degree relatives, the patients of this study might have been selected preferentially for severe risk of coronary heart disease. Moreover, statin treatment was not assessed against untreated familial hypercholesterolemia and not in a time dependent fashion. The latter may have resulted in underestimation of the statin effect because of misclassification of periods without treatment. In contrast, in our study, we prospectively compared treated and untreated patients with familial hypercholesterolemia as well as the general population in a direct manner and in the same calendar period.

Implications of findings

The standard treatment used currently is more aggressive than that used in our study: current regimens use simvastatin and atorvastatin doses up to 80 mg daily.[2, 4] It should be emphasized, however, that in our study we excluded all prevalent cases, thereby restricting our study to primary prevention. Our results cannot be extrapolated to secondary prevention, which may require more aggressive treatment.

Support for optimal treatment with statins can also be deduced from the results of the ENHANCE study: patients with familial hypercholesterolemia had normal carotid intima-media thickness at baseline, and even simvastatin 80 mg alone prevented thickening of the arterial wall.[6]

In previous studies we tested statin treatment of children with familial hypercholesterolemia and showed attenuation of progression of carotid intima-media thickness.[15, 16]

Our present study suggests that starting aggressive treatment during early childhood, as currently done and advised by the American Academy of Pediatrics, is probably not necessary to reduce coronary heart disease risk.[17] Although atherosclerosis is present in children, this process is to a certain extent reversible, as shown by the close to normal intima-media thicknesses at baseline in the ENHANCE trial and our findings in the present study. In both studies, statin treatment was initiated after childhood. It is probably safe to limit statin treatment of children with heterozygous familial hypercholesterolemia to those whose first degree relatives have severe premature coronary heart disease.

In conclusion, our data show that lower statin doses than currently advised result in impressive reductions of coronary heart disease risk in patients with familial hypercholesterolemia. These findings warrant an immediate start of statin treatment after familial hypercholesterolemia has been diagnosed since such treatment leads to near normalisation of coronary heart disease risk.

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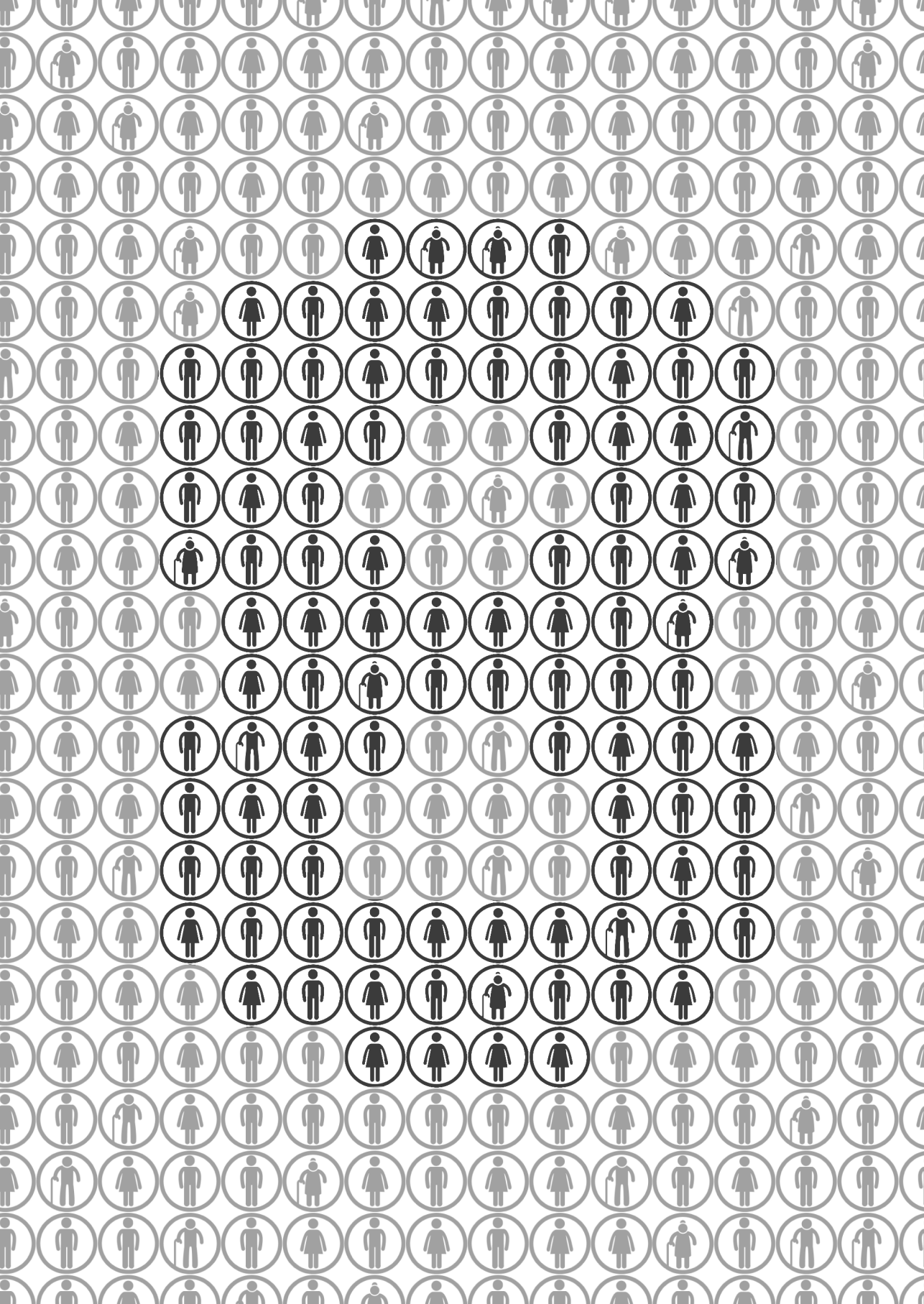

8 Cascade Screening for Familial Hypercholesterolemia (FH): Prevention of coronary heart disease in a large cohort of Dutch FH heterozygotes

Roeland Huijgen
Jorie Versmissen
Daniëlla M. Oosterveer
Iris Kindt
Joep C Defesche
Aeilko H Zwinderman
John JP Kastelein
Eric JG Sijbrands

SUBMITTED

ABSTRACT

Aims

Familial hypercholesterolemia (FH) is associated with a severely increased risk of coronary heart disease (CHD). Active screening for FH is ongoing in the Netherlands since 1994 and we previously showed that in this programme 85% of FH patients were initiated on statin treatment. It is well known that statin treatment yields an impressive reduction of the risk of a first CHD event in asymptomatic FH patients referred to Lipid Clinics. Using modelling based on these previous results, we estimated the number of CHD events prevented by active cascade screening.

Methods and results

In a retrospective cohort study, we used a Cox proportional hazard model to compare CHD event-free survival in 9,620 relatives with FH identified by active screening to that of 1,333 referred patients, clinically diagnosed with FH, and 18,440 relatives without FH. Subsequently, we estimated the number of CHD events after the FH diagnosis was established until the expected age of death in order to calculate avoidable events in the patients identified by active screening. In those patients, untreated and free of CHD at diagnosis, 65% of CHD events might be prevented over a lifetime.

Conclusions

Genetic testing for FH is highly effective in the prevention of CHD with an overall efficiency of three untreated individuals to be diagnosed to avert one CHD event in a single patient.

INTRODUCTION

Familial hypercholesterolemia (FH) is a common autosomal dominant disorder, caused by mutations in the low-density lipoprotein (LDL) receptor (*LDLR)* gene. These molecular variants result in defective uptake of plasma LDL cholesterol by the liver cells. As a consequence, patients with FH have high plasma LDL cholesterol levels and an increased risk of coronary heart disease (CHD).[1-4]

In the vast majority of patients fulfilling the clinical criteria for FH, the causal *LDLR* mutation can be identified by DNA analysis.[5] Knowledge of the causal mutation enables testing of family members for the presence of the same *LDLR* mutation. In fact, recent guidelines recommend such genetic cascade screening of family members of a patient in which a molecular FH diagnosis has been established.[6] Since 1994, such a screening programme for FH is ongoing in the Netherlands.[7] At the present time approximately half of the Dutch FH patients have been identified, i.e. 21,000 individuals.

So far, systematic follow-up of individuals diagnosed with FH through active cascade screening is lacking and the overall effectiveness of such an FH screening programme in terms of CHD prevention is not exactly known. This efficacy was estimated previously on the basis of assumptions from older cohorts from other countries[8]. However, two recent publications on the percentage of statin use and efficacy of statins allow a more accurate assessment.[9, 10]

We aimed to predict the consequences of active FH screening for the prevention of CHD. Here, we present our results.

METHODS

In order to estimate the number of CHD events prevented after molecular FH diagnosis through genetic cascade testing, we used a stepwise approach (Figure 1)

Study cohorts

The study population consisted of individuals that were actively recruited by cascade screening for FH between January 1994 and January 2010. We refer to the clinically diagnosed FH patients that served as the starting point for cascade screening as 'index patients', family members identified with the *LDLR* mutation were referred to as 'family members with FH' and those without that mutation were referred to as 'family members without FH' or 'controls'. We excluded subjects tested for mutations in the Apolipoprotein B gene that cause Familial Defective Apolipoprotein B or *LDLR* mutations that were later proven to be non-pathogenic.[11, 12] Subjects with angina pectoris only were also excluded from the analyses.

For analysis 1 (assessment of CHD risk in family members as compared to index patients), we selected all persons born before 1990 and compared them with a large cohort of index patients as described in detail previously.[13]

For analysis 2 (assessment of CHD risk reduction by cascade screening), we selected

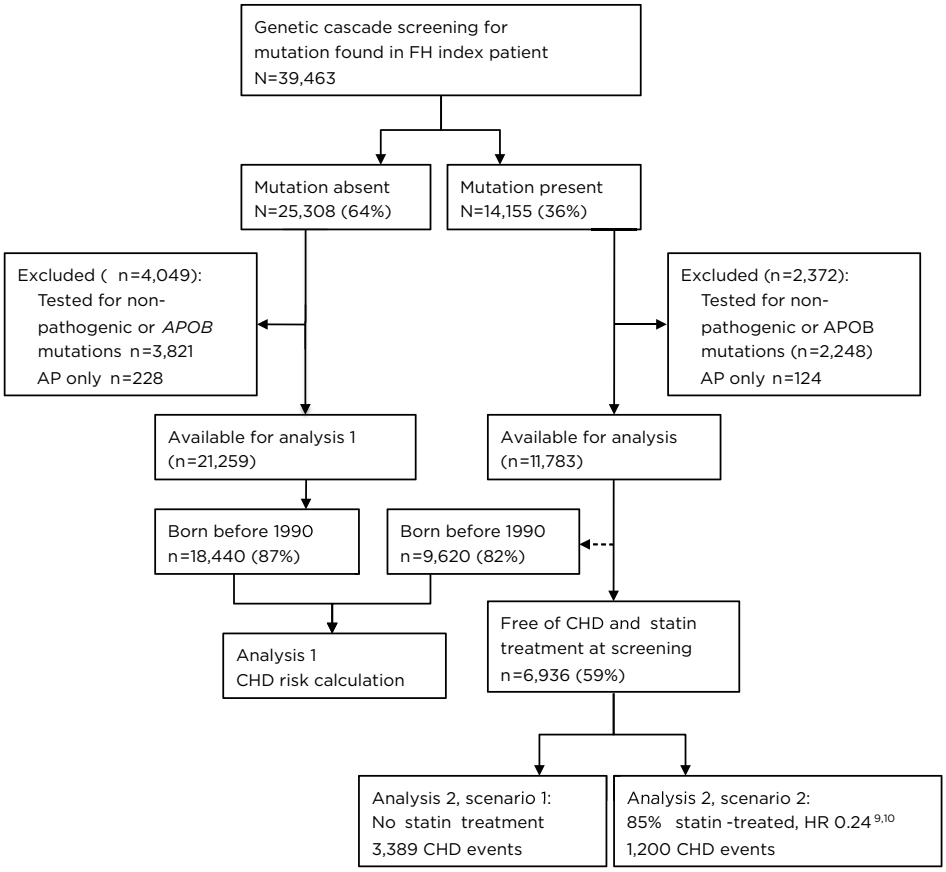

FIGURE 1. Flow diagram to show included and excluded patients for analysis 1 and analysis 2. Abbreviations: *APOB*=Apolipoprotein B gene, CHD=coronary heart disease, FH=familial hypercholesterolemia, HR=hazard ratio, *LDLR* = Low Density Lipoprotein Receptor gene.

all family members with FH who were untreated and free of CHD at diagnosis. (Figure 1)

Clinical information was obtained by specialised nurses at the time of blood sampling for mutation analysis. In particular, an extensive history of cardiovascular disease, risk factors and medication use was recorded. DNA was isolated from 10 ml of freshly collected blood. Mutation analysis of *LDLR* was performed as described previously.[14]

Primary outcome measures

The primary outcome measure was the predicted number of a first CHD event after genetic FH diagnosis until the expected age of death. CHD was defined as the occurrence of one of the following non-fatal cardiac outcomes:

a) sudden cardiac arrest
b) myocardial infarction
c) coronary artery bypass graft
d) percutaneous transluminal coronary angioplasty

Analysis 1: assessment of CHD risk in family members and comparison to index patients

CHD incidence was assessed in the period before statin treatment became available (1990) in order to better reflect the natural course of FH. Since FH is present from conception, event-free survival was defined as the period from birth until the year of the first CHD event or censoringat January 1st 1990, whichever came first.

First, we determined whether this CHD risk was similar between those clinically diagnosed and those identified by active genetic screening. We compared survival of clinically diagnosed FH patients (index patients) and FH family members and family members without FH using Kaplan-Meier survival analysis and the logrank test, stratified for sex. Subsequently, we compared CHD risk between clinically diagnosed FH patients and FH family members and family members without FH using a Cox-proportional hazard model, adjusted for sex, year of birth, hypertension, diabetes, smoking and body mass index and these variables included were fixed over time. The Cox-proportional hazard analyses were repeated in R, taking family ties into account. We also calculated the CHD incidences for 10 year age periods.

Analysis 2: assessment of CHD risk reduction by cascade screening

The incidences as calculated in the pre-statin era were applied to the years after FH diagnosis for family members that were untreated and free of CHD at genetic testing to estimate the number of expected CHD events. For each individual, we estimated the number of years until the expected age of death, which was based on the average life expectancy for individuals of the same age and sex in the general population in the Netherlands (Central Bureau for Statistics in the Netherlands: www.cbs.nl). At the beginning of each decade we entered all subjects at risk for a first CHD event, and for each subsequent decade, we removed the calculated number of individuals suffering from a first CHD event in the previous decade. The numbers of events that were expected over the decades were

added to estimate the overall incidence of first non-fatal CHD events when no treatment was available (scenario 1).

Subsequently, we applied assumptions on long-term statin treatment use after FH diagnosis and efficacy of such treatment on CHD risk reduction derived from previous publications in clinically diagnosed FH patients.[9-12] In a survey on medication use after the diagnosis of FH in 711 subjects, 85% used statin therapy two years after FH diagnosis.[9] Versmissen et al. determined in 2008 that CHD risk reduction by statins in FH patients was 76% (95% confidence interval 70-82%).[10]

Based on the results of analysis 1 and these assumptions, we calculated the number of subjects that need to be identified with FH to prevent one CHD event during the average expected number of years of life after FH diagnosis.

Statistical analysis

General characteristics between the cohorts were compared using ANOVA and chi-square tests. We compared survival of FH patients and controls using Kaplan-Meier survival analysis and the logrank test, stratified for sex. All data were analysed using SPSS software (version 16.0.2, SPSS, Chicago, IL, USA). A two-sided *p*-value of 0.05 was considered as significant.

RESULTS

Study cohorts

39,463 family members of genetically confirmed FH patients were actively screened for the specific *LDLR* mutation between January 1994 and December 2010. Of these, 6,069 subjects were screened for *APOB* or non-pathogenic *LDLR* mutations and therefore excluded from analysis. From the remaining 33,394 subjects, 11,907 (35.7%) family members were diagnosed with FH ('FH patients'), whereas 21,487 (64.3%) carried no mutation ('controls'). 352 subject with angina pectoris only were excluded from the analyses. The median year of testing for family members was 2005. FH patients had higher mean total cholesterol levels than controls (9.72 ± 2.62 mmol/L vs. 6.15 ± 1.91 mmol/L; p <0.001). Other clinical characteristics at the time of testing are shown in Table 1. Among the 11,783 FH patients with a pathogenic *LDLR* mutation, 6,936 (59%) did not receive any medication and had not experienced CHD at the moment of molecular testing (Figure 1).

TABLE 1. General characteristics of the study populations

	Index patients (clinically diagnosed FH)	Family members with FH	Family members without FH	p-value Index vs family members with FH	p-value Index vs family members without FH	p-value Family members with FH vs without FH
At screening (n=34,375)						
N	1,333	11,783	21,259			
Median year of screening	1995	2005	2005			
Age (years)	42±12	37±20	43±19	<0.001	0.001	<0.001
Male gender (%)	614 (46)	5,636 (48)	10,001(47)	0.224	0.497	0.171
Body mass index (kg/m²)	24.7±3.5	23.7±4.8	24.5±4.5	<0.001	0.127	<0.001
Hypertension (%)	99(7.5)	937(8.5)	2,461(12.3)	0.248	<0.001	<0.001
Diabetes (%)	65(4.9)	234(2.1)	643(3.3)	<0.001	0.002	<0.001
Smoking (%)	395(31)	3,275(29)	7,047(35)	0.109	0.016	<0.001
Total cholesterol (mmol/l)*	10.2±2.1	9.7±2.6	6.2±1.9	<0.001	<0.001	<0.001
Cholesterol-lowering medication (%)	622(47)	4,794(41)	1,953(9)	<0.001	<0.001	<0.001
Coronary heart disease (%)	175(13.1)	829(7.0)	765(3.6)	<0.001	<0.001	<0.001
At January 1st 1990 (n=29,393)						
N	1,333	9,620	18,440			
Age (years)	38±13	28±17	33±16	<0.001	<0.001	<0.001
Coronary heart disease (%)	90(6.8)	227(2.9)	175(0.9)	<0.001	<0.001	<0.001

Continuous traits are given as mean ±standard deviation. Abbreviations: FH= familial hypercholesterolemia.
*Highest cholesterol level without treatment.

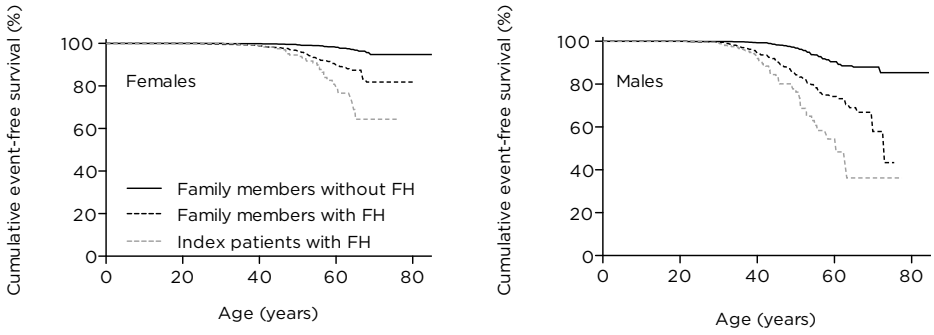

FIGURE 2. Cumulative coronary heart disease (CHD) event-free survival in familial hypercholesterolemia (FH) before 1990.
Kaplan-Meier curves for cumulative CHD-event free survival for males and females.
Log-rank tests: differences between controls and FH patients for both sexes: $p<0.001$. Abbreviation: CHD=coronary heart disease; FH=familial hypercholesterolemia.

CHD risk before the introduction of statins

In total 401 CHD events occurred before 1990: 277 (2.9%) in 9,620 family members with FH (269,932 person years), and 175 (0.9%) in the 18,440 control subjects (599,446 person years). The CHD-event-free survival was shorter for FH patients than for controls with a 90% event-free survival of 51 years and 71 years, respectively. The incidence of CHD was higher in males than in females, as shown by the Kaplan-Meier curves (Figure 2). The incidence of CHD per age decade in FH patients ranged from 0.6% between 20 and 30 years of age to 18% between 60 and 70 years of age.

CHD risk attributable to FH per se was comparable between referred FH index patients and family members with FH identified by cascade testing. In the age range with the highest relative risk of CHD and sufficient numbers of observations, i.e. between 20 and 50 years, both clinically diagnosed FH patients and FH family members had an increased CHD risk compared to that in non-carriers (9.5, 95%CI: 6.5 - 13.8; $p<0.001$ and HR 7.3, 95%CI: 5.6 - 9.7; $p<0.001$ respectively). The risk difference between the two FH cohorts was small and did not reach statistical significance in this age period: HR 1.3, 95%CI: 0.9-1.8; $p=0.14$.

The estimated effect of treatment on incidence of CHD after genetic FH diagnosis

To calculate the effect of DNA testing and statin prescription on CHD risk, we first calculated what the consequences of untreated FH would be for the cumulative incidence of CHD. For this, we focussed on the 6,936 FH patients, identified by genetic testing, who did not receive medication and who had not experienced a CHD event yet (Figure 1). The average age at testing was 29 years and we calculated that those 6,936 subjects would be alive another 360,880 person years until estimated age of death. We calculated that 3,389 events would occur if all would remain untreated, based on the incidence of CHD in the pre-statin era. Subsequently, we calculated the expected CHD incidence with the assumption that 85% would be treated after FH diagnosis (Figure 1, assumption C) and that this treatment would be associated with a hazard ratio of 0.24 (95% CI; 0.18-0.30). In that scenario, only 1,200 CHD events would occur. Thus, 2,189 of the 3,389 CHD events (65%) would be prevented (95% CI; 2,016-2,362; Figure 1).

To calculate the number of subjects needed to be identified with FH to prevent one from experiencing CHD during the remaining years of life, we assumed that without genetic testing all would remain untreated, whereas in contrast, the proportion of treated patients would increase to 85% after genetic diagnosis. With this sharp increase in treatment, twelve family members need to be screened (26,035/2,189 =11.9) and three FH patients need to be identified (6,936/2,189=3.2) to prevent one FH patient from experiencing a CHD event.

DISCUSSION

We first demonstrated again that the relative risk of CHD is severely increased in patients with FH compared to their unaffected relatives. Earlier we showed that 85% of actively screened patients were treated with statin treatment after genetic FH diagnosis and that such cholesterol lowering intervention would yield a relative risk reduction of 76%.[9, 10] Based on all of the above, we calculated that 65% of the untreated FH patients, who were free of CHD at diagnosis, would be protected against a first CHD event as a consequence of the genetic testing and subsequent statin prescription.

In fact, we demonstrated in a prediction model that three untreated individuals free from CHD would need to be diagnosed with FH in order to protect one individual against a CHD event. Wonderling and colleagues estimated in 2004 that 100 FH patients would need to be identified and treated with statins between the ages of 18 and 60 to avoid 26 myocardial infarctions (approximately 4 FH patients identified to prevent 1 myocardial infarction).[8] These authors based their findings on extrapolations from data of the Dutch screening programme as well as from the Simon Broome Register in the United Kingdom.[1, 8, 15] Based on those numbers, they concluded that the Dutch cascade testing programme was cost-effective.[8] Notably, our findings in a much larger set of individuals clearly outperform assumptions made previously.[8, 16]

A number of limitations of our study merit discussion. First, the testing organisation does not perform systematic follow-up on medication use and clinical outcomes of subjects diagnosed with FH. Therefore, we could only estimate use of medication in a random sample rather than making a direct observation. Similarly, the numbers of CHD events prevented were calculated using assumptions on CHD risk and medication use and compliance after FH diagnosis. Nonetheless, we feel that our estimates of this retrospective analysis are modest and accurate, based on the large number of patients both in this study as well as in the original studies that provided the data for our assumptions.[9, 10] The comparison of CHD risk between index patients and FH patients identified by cascade screening might be most precarious due to selection on outcome, but we showed that the difference was small and not significant in the group in which comparison was best possible.

Second, two important issues that could have biased our findings are selective survival and exclusion of angina pectoris. Standardised mortality was calculated during 10 years of follow up in the Simon and Broome Register, a cohort of 526 clinically diagnosed FH patients from the United Kingdom. The adult patients showed a 3.9 (95% CI 2.1-6.4) excess mortality due to coronary heart disease.[1] In the current analysis we did not have data available on the family members who died before FH testing was carried out, because data on cardiovascular history were collected at the time of testing. Besides, we excluded the subjects who only experienced angina pectoris from our event-free survival analysis. Angina was excluded, because such a symptom reported by tested family members proved unreliable when checked against the official diagnoses from medical records when available (data not shown). We have no reason to assume that the hazard ratio for

angina pectoris free survival for unaffected family members and FH patients would be any different from the more severe CHD events. Thus, both inclusion of living individuals and exclusion of subjects with angina pectoris has probably resulted in a considerable underestimation of the CHD risk conferred by FH and therefore in a underestimation of the efficacy of active identification for the prevention of cardiovascular events.

Third, the estimate of the number of CHD events was restricted to subjects untreated at genetic diagnosis. The substantial proportion of FH patients that received some form of treatment before testing (41%) may limit the overall benefit of the programme. However, we have previously shown that subjects who used cholesterol-lowering medication at testing received a significantly more intensive treatment regimen after DNA diagnosis of FH.[9, 17] Such intensification of statin treatment has been reported to be cost-effective for CHD reduction in FH patients.[18] However, clinical outcome studies in FH comparing the efficacy of intensive versus moderate statin therapy are lacking. Therefore, we were unable to quantify the effect of intensified therapy on CHD risk.

In conclusion, genetic cascade testing of family members of index patients with FH can identify subjects with a severely increased risk of CHD. Genetic FH diagnosis and subsequent initiation or more intensive treatment contributes to the prevention of the majority of CHD events in these patients. Accordingly, genetic cascade testing for FH, as is currently being performed in the Netherlands, might be also considered in other countries.

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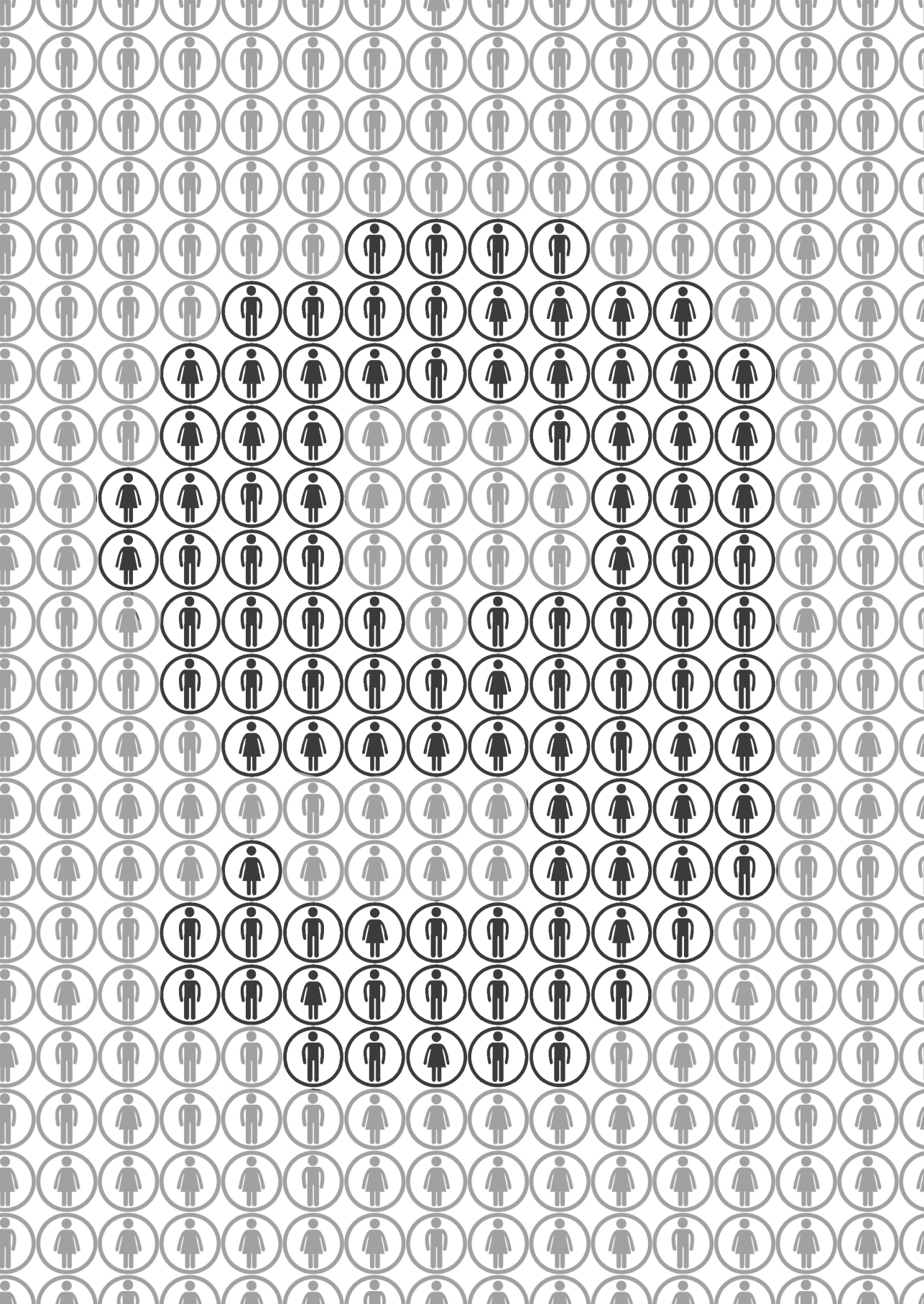

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A frequent variant in the *ABCA1* gene is associated with increased coronary heart disease risk and a better response to statin treatment in familial hypercholesterolemia patients

Jorie Versmissen
Daniëlla M Oosterveer
Mojgan Yazdanpanah
Monique Mulder
Abbas Dehghan
Joep C Defesche
John JP Kastelein
Eric JG Sijbrands

EUR HEART J 2010; 32:469-475

ABSTRACT

Aims

Statins are essential for the reduction of risk of coronary heart disease (CHD) in familial hypercholesterolemia (FH). One of many genes influenced by statin treatment is the ATP-binding cassette transporter A1 (ABCA1) gene which plays an important role in metabolism of high-density lipoprotein (HDL). The present aim was to test if the ABCA1 C69T polymorphism influences CHD risk and response to statin treatment.

Methods and results

In a large cohort of 1,686 FH patients without a history of CHD before 1 January 1990, we analyzed statin-ABCA1 C69T polymophism interaction by comparing treated and untreated patients. We used Cox proportional hazard modeling adjusted for sex, birth year and smoking. In analyses restricted to untreated patients, the TT genotype was associated with 1.7 times higher CHD risk than the CC genotype (hazard ratio (HR) =1.65; 95% confidence interval (95% CI) 1.08-2.53; p=0.02). Conversely, in statin-treated FH patients, CHD risk in TT individuals was not increased (HR 0.65, 95% CI 0.35-1.24; p=0.2). Formal testing confirmed this interaction (p=0.02). HDL-cholesterol levels were significantly more raised in statin-treated patients with the TT than with the CC genotype (two-way ANOVA p=0.045).

Conclusions

In untreated FH patients, the TT genotype of the ABCA1 C69T polymorphism was associated with increased CHD risk. However, in statin-treated patients, CHD risk was no longer significantly different between genotypes, at least partially explained by a higher rise in HDL-cholesterol levels during statin treatment in TT individuals.

INTRODUCTION

Familial hypercholesterolemia (FH) is a hereditary disorder of lipoprotein metabolism associated with a severely increased risk of coronary heart disease (CHD).[1] In heterozygous FH, statin treatment reduces CHD risk substantially.[2-4] Statins effectively inhibit endogenous cholesterol synthesis, which results in upregulation of LDL receptor expression on the cell surface of hepatocytes. Next to this, statins have a number of additional beneficial effects. These so-called pleitropic effects, such as raising high-density lipoprotein cholesterol (HDL-C) levels, increasing cholesterol efflux from cholesterol loaded macrophages and inhibiting inflammation may explain that CHD risk reduction by statins is greater than expected from LDL-cholesterol (LDL-C) lowering alone.[5-7]

The genetic background of an individual may influence the response to statin treatment of lipid levels as well as CHD risk. However, not many convincing genetic modifiers of statin response have been identified so far.[8-10] Since most FH patients receive statin treatment upon diagnosis, this situation provides a good opportunity to study gene-treatment interactions in these patients.

An interesting candidate gene is the ATP-binding cassette transporter A1 (ABCA1) gene. Hepatic ABCA1 mediates HDL synthesis and ABCA1 is an efficient exporter of cholesterol from macrophages and other cells to HDL in plasma. It is therefore a major factor in CHD risk protection.[11-15] A number of polymorphisms in this gene associate with HDL-C levels and CHD risk.[16-24]

Statins inhibit HMG CoA reductase, the rate limiting enzym in the biosynthesis of cholesterol. By inhibiting this early step in the mevalonate pathway, production of isoprenoids and oxysterols is inhibited as well, which are small molecules that influence ABCA1 levels via signaling pathways involving nuclear receptors such as liver-X receptor (LXR) and persoxisome proliferator-activated receptor-γ (PPAR-γ).[25-27] Therefore, statin treatment might influence effects of genetic variation in ABCA1.[12] The most promising polymorphism to study with regard to response to statin treatment is C69T, since the T allele of this polymorphism was associated with increased CHD risk compared to the C allele in the placebo arm of a pravastatin trial.[16] As earlier shown for a polymorphism in the gene encoding kinesin-like protein 6 (KIF6), an association restricted to placebo-treated individuals may indicate an interaction between genotype and treatment response.[28] Therefore, we hypothesized that ABCA1 C69T TT individuals have an increased risk of CHD, but statin therapy can reduce CHD risk more effectively in TT individuals than in wild type individuals. The best setting to study gene-statin interaction is a placebo-controlled clinical trial. For ethical reasons these are, however, not allowed in FH patients which prompted us earlier to simulate such a trial in a large cohort of FH patients to study the efficacy of statins in primary prevention.[4] In the current study, we used the variation in delay of starting statin treatment after the introduction in 1990 and the fact that a considerable number of patients refrained from therapy to study gene-statin interaction.

In this large cohort study, we investigated the interaction between the ABCA1 C69T polymorphism and statin treatment on CHD risk in three ways: first, we analyzed the

association after stratification by statin treatment; secondly, we compared the efficacy of statin treatment between the different genotypes with a Cox regression model in which statin treatment was a time dependent variable; and finally, we calculated the relative excess risk due to interaction (RERI) as a formal test of interaction.[29]

METHODS

Study population

During 1989-2002, we recruited a cohort of 2,400 FH patients from 27 lipid clinics in The Netherlands as described in detail previously.[30] In short, 4,000 patients were randomly selected from the Dutch DNA database containing over 9,300 samples from hypercholesterolaemic patients from more than 60 lipid clinics. From these 4,000 patients, 2,400 fulfilled the diagnostic criteria for FH . Medical records were reviewed to establish extensive phenotypic data.[31] All DNA samples were screened for the presence of LDL-receptor mutations. All patients gave informed consent, and the ethics institutional review board of each participating hospital approved the protocol.

In the present study, to simulate a clinical trial, we chose 1 January 1990, as a starting point, just after the first statin (simvastatin) became available in The Netherlands. We excluded 254 patients, who had prevalent CHD before 1990 to mimic a primary preven-

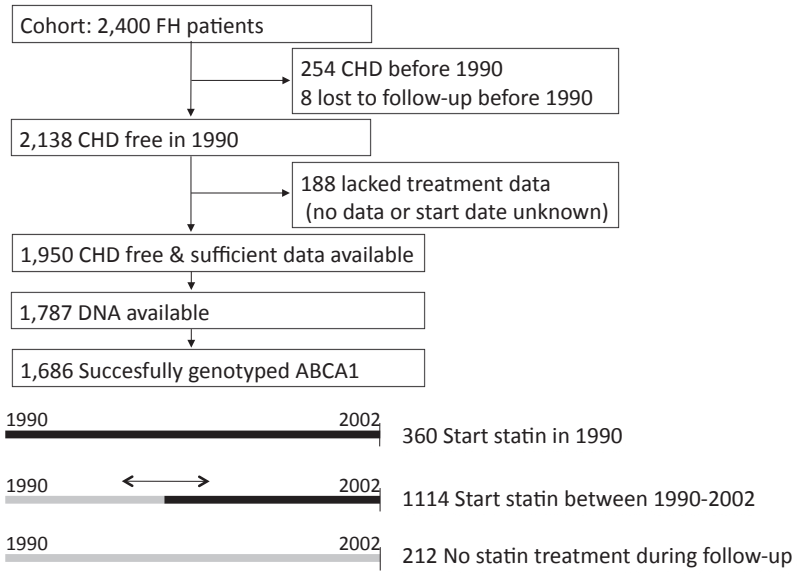

FIGURE 1. Study design: flow diagram to show included and excluded patients, and the different possibilities for starting point of statin treatment.

tion trial (Figure 1).[4] Another 188 patients were excluded from the analyses because the type of treatment or the date of starting was unknown. Sufficient data on statin use were available of 1,950 event-free FH patients. Of these, DNA was available for 1,787 patients. CHD was defined as myocardial infarction, percutaneous coronary intervention, coronary artery bypass grafting or angina according to earlier described criteria.[30] To determine untreated lipid levels, patients were asked to quit cholesterol lowering medication for six weeks prior to blood sampling.

Genetic analyses

Genotyping of the C69T polymorphism (rs1800977) was performed by Roche Molecular Systems, CA, USA.[21]

Statistical analyses

General characteristics were compared using analysis of variance for continuous variables and X^2 test for categorical variables. Because the residuals for the lipid measures were not normally distributed plasma total cholesterol, HDL-C and LDL-C and triglyceride levels were log transformed. To analyze differences in treatment response of lipid levels, we performed two-way ANOVA analysis. Deviation from Hardy Weinberg Equilibrium was tested with a X^2 test using 1 degree of freedom.

Cox-proportional hazard model was used to model the association between the ABCA1 polymorphism and CHD. Event-free survival time was defined as the period from 1 January 1990 to the date of first CHD or death or censoring at the end of follow-up. Homozygosity for the common allele was assigned as reference.

To compare influence of statin treatment on association between the polymorphism and CHD, we first calculated hazard ratios separately in two strata defined by statin treatment. We considered persons who started statin treatment less than one month before an event occurred as untreated.

Next, we stratified by genotype and analyzed the influence of statin treatment using statin treatment as a time dependent variable since most patients experienced a variable period without statin treatment. This variable was equal to zero for the time no statin treatment was used, and 1 for the time from start of statin treatment to the date of first CHD or censoring. Therefore, treated and untreated person-years rather than persons were compared. All analyses were adjusted for year of birth, sex, and smoking.

As an additional formal test for interaction we determined the relative excess risk due to interaction (RERI).[29] RERI is determined as

$$\frac{(HR_{11}-HR_{00})-[(HR_{10}-HR_{00})+(HR_{01}-HR_{00})]}{HR_{00}} = HR_{11}-HR_{10}-HR_{01}+1$$

in which HR_{00} denotes persons without both risk factors and is set as reference group (HR=1). In our study, the risk factors studied were treatment and risk genotype. Therefore, the reference represents untreated CC individuals. HR_{10} and HR_{01} represent persons with either treatment or the risk genotype (TT) compared to untreated persons with the CC genotype and HR_{11} denotes the hazard ratio of treated persons with the risk genotype compared to untreated persons without the risk genotype.

All analyses were performed using SPSS statistical software (Version 14.0, SPSS Inc, Chicago, Ill), except the additional test for interaction which was performed using SAS software (Version 9.1.3 SAS institute Inc., Cary, NC).

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

RESULTS

We successfully genotyped 1,686 patients for the ABCA1 C69T polymorphism. ABCA1 C69T was in Hardy Weinberg equilibrium (p=0.86).The genotype frequencies were CC 46.8%, CT 43.4% and TT 12.0%. This is comparable to the genotype frequencies found in the general population: CC 46.9%, CT 40.7 % and TT 12.4% (HapMap Phase 3). The general characteristics including lipid levels were not significantly different between genotypes (Table 1) except diabetes mellitus, which was most common in CC individuals (p=0.03). In a total of 926 patients an LDL receptor mutation was identified, the remaining 760 had FH based on clinical criteria.[30] The LDL receptor mutations were equally distributed amongst the three ABCA1 C69T genotypes and the mutation frequencies were similar to those of our nation-wide FH screening program.[32]

In January 1990, 360 of these patients were treated with a statin, whereas during follow-up 1113 patients were prescribed statin treatment as well (Figure 1). Of the statin-treated patients, 79% was treated with simvastatin (mean dosage 33 mg), 13% with atorvastatin (mean dosage 48 mg), 6% pravastatin (mean dosage 30 mg) and 2% with other statins. The age of starting statin treatment was not significantly different between genotypes (Table 1).Twenty-one patients discontinued statin treatment for unknown reasons.

During a total follow-up of 14,085 person-years, 360 CHD events occurred. Of these, 139 patients had been receiving statins before their events during a median period of 2.3 years; the other 221 events occurred in patients who had not yet been treated with statins. Eleven patients had DM prior to their events. As we showed earlier, statin treatment reduced CHD risk by approximately 80% in a Cox regression model using statin treatment as a time dependent variable.[4] In the complete population, including treated as well as untreated patients, the ABCA1 variant was not associated with CHD risk (TT versus CC hazard ratio (HR) 1.01, 95% confidence interval (95% CI) 0.72-1.41 p= 0.97).

CHD risk according to ABCA1 C69T genotypes in statin-treated and untreated patients is shown in table 2. Untreated TT individuals had an increased CHD risk compared to untreated CC individuals (HR 1.65; 95% CI 1.08-2.53; p=0.02) but this was not the case

TABLE 1: General characteristics and treatment parameters per genotype

	ABCA1 C69T genotype			
	CC	**CT**	**TT**	**p-value**
Number	752	732	202	
Male (%)	352 (46.8)	351 (48.0)	88 (43.6)	0.54
Age January 1990	39.6±12.8	38.9±12.9	38.1±12.4	0.26
Smoking ever (%)	511 (75.5)	484 (73.0)	127 (70.6)	0.34
Hypertension ever (%)	70 (9.4)	58 (8)	16 (8)	0.60
Diabetes Mellitus ever (%)	46 (6.1)	25 (3.4)	7 (3.5)	0.03
Cardiac event (%)	167 (22.2)	151 (20.6)	42 (20.8)	0.74
BMI (kg/m²)	25.0±3.4	25.1±3.7	24.6±3	0.30
Statin January 1990 (%)	164 (28.7)	160 (28.6)	31 (20.5)	0.11
Simvastatin (%)	436 (76.4)	457 (81.6)	118 (78.1)	0.68
Dosage (mg)	31±20	32±19	34±20	0.40
Age of starting statin	43.2±125	42.5±12.8	42.6±12.1	0.60
Lipid levels				
Without treatment				
Total cholesterol (mmol/l)	9.21±1.80	9.17±1.82	9.09±1.86	0.69
LDL-C (mmol/l)	7.04±1.72	7.02±1.78	6.92±1.78	0.72
HDL-C(mmol/l)	1.17±0.33	1.18±0.33	1.18±0.34	0.88
Triglycerides (mmol/l)	1.56±0.79	1.56±0.79	1.60±0.79	0.78
During statin treatment				
Total cholesterol (mmol/l)	5.93±1.24	5.97±1.25	5.89±1.25	0.60
LDL-C (mmol/l)	3.93±1.17	4.0±1.17	3.82±1.34	0.14
HDL-C (mmol/l)	1.27±0.35	1.29±0.35	1.34±0.38	0.41
Triglycerids (mmol/l)	1.23±0.60	1.1±0.55	1.19±0.66	0.30

BMI: Body mass index Value is mean±standard deviation

TABLE 2: Association of ABCA1 C69T genotype and CHD risk without or during statin treatment

	ABCA-1 C69T			
	Genotype	**n**	**HR (95% CI)**	**p-value**
Not using statins	**CC**	181	(ref)	
	CT	172	1.11 (0.83-1.50)	0.48
	TT	51	1.65 (1.08-2.53)	0.02
During statin treatment	**CC**	571	(ref)	
	CT	560	0.92 (0.64-1.33)	0.66
	TT	151	0.65 (0.34-1.24)	0.19

HR: hazard ratio; 95% CI: 95% confidence interval
Adjusted for sex, smoking, year of birth; CC as reference

in treated patients (HR 0.65 95% CI 0.35-1.24 p=0.2). CT individuals had CHD risks in between CC and TT individuals; the p for trend was 0.045 in untreated individuals but not significant in treated individuals (Table 2).

TABLE 3: Interaction between genotype and statin treatment on CHD risk

		ABCA-1 C69T		
Statin treatment	**Genotype**	**n**	**HR (95% CI)**	**p-value**
No	**CC**	181	(ref)	
Current	**CC**	571	0.15 (0.11-0.21)	<0.001
No	**CT**	172	1.09 (0.81-1.47)	0.56
Current	**CT**	560	0.14 (0.10-0.19)	<0.001
No	**TT**	51	1.65 (1.08-2.53)	0.02
Current	**TT**	151	0.10 (0.05-0.18)	<0.001

Adjusted for sex, smoking, year of birth; CC without treatment as reference

We further analyzed the gene-drug interaction comparing both treated and untreated carriers of the different genotypes using untreated CC individuals as reference (Table 3). Relative to CC individuals without treatment, statin treatment reduced CHD risk in CC individuals by 85% (HR 0.15 95% CI 0.11-0.21 p<0.001) and in TT individuals by 90% (HR 0.097 95% CI 0.05-0.181 p<0.001). This indicates a larger CHD risk reduction by statin treatment of TT individuals as 75% (HR 0.25) instead of 90% (HR 0.097) was expected if there had been no interaction between genotype and treatment. We calculated the expected HR by multiplying the HR of CHD risk reduction by statin treatment of CC individuals by CHD risk in untreated TT individuals (0.15x1.65=0.25).

Comparison of risk reduction by directly comparing untreated and statin-treated person-years using statin use as a time-dependent variable also showed that the use of statins is associated with a higher risk reduction in TT individuals (HR 0.12, 95% CI 0.04-0.36; p-value <0.001), although 95% confidence intervals overlapped between different genotypes. Risk reduction in CC individuals was 74% (HR 0.26, 95% CI 0.18-0.37, p<0.001).

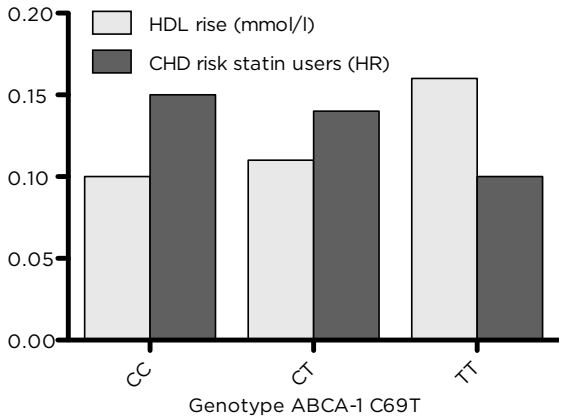

FIGURE 2. Effect of statin treatment on HDL-C rise and CHD risk in per genotype. CHD risk statin users relative to CC non-users (compare table 3). Numbers on ordinate either hazard ratio (HR) for CHD risk or mmol/liter (rise in HDL-C by statin) .

Finally, we confirmed this interaction formally by determination of the relative excess risk due to interaction (RERI) (-0.29, 95% CI -0.03 to -0.55; p=0.03).

To define whether HDL-C increase in response to statin treatment could explain this difference between untreated and statin-treated patients, we performed a two-way ANOVA according to genotype comparing HDL-C levels with and without statin treatment in all persons who started statin treatment during follow up (Table 1). TT individuals appeared to experience a larger rise of HDL-C level than CC individuals during statin treatment (13.6% vs 8.5%; p= 0.045). In Figure 2, HDL-C response to statin treatment and CHD risk in statin-treated patients are shown per genotype. These observations seem to be correlated since CHD risk decreases while HDL-C levels increase. In line with partially explaining the effect on CHD risk, adding HDL-C response to the Cox regression model moved the point estimate towards 1 in treated TT individuals and annihilated the significance (HR 0.78, 95% CI 0.38-1.61; p=0.51). For the model without HDL-C response we refer to Table 2 (HR 0.65, 95% CI 0.34-1.24; p=0.19).

DISCUSSION

In this large cohort study of FH patients, we discovered an interaction between a genetic variant in the ABCA1 gene, statin treatment and CHD risk. In an untreated situation, the TT genotype of the ABCA1 C69T polymorphism was significantly associated with higher CHD risk in FH patients. Conversely, in statin-treated patients, CHD risk in TT individuals was not increased. HDL-C levels increased to a significantly higher extend in the TT individuals during statin treatment. Taken together, these results suggest that the interaction between the ABCA1 C69T polymorphism and statin treatment changes the impact of the ABCA1 C69T polymorphism on CHD risk, at least partially due to a greater increase of HDL-C in TT individuals. DM was significantly different between genotypes. We do not think this has influenced our results with regard to CHD risk reduction because of the low numbers and the fact that most DM occurred only after a CHD event had taken place. It is however an intriguing finding, reassuring recent studies that found a role for ABCA1 in beta cell function.[33] Unfortunately, our study was not sufficiently powered to perform in-depth analyses or interaction studies with treatment.

ABCA1 has an essential role in the regulation of HDL metabolism. The liver is the major source of HDL and hepatocytes require ABCA1 for HDL generation.[11] Increased production of HDL may result in an accelerated overall reverse cholesterol transport and thereby be protective against the development of CVD. Moreover, ABCA1 is involved in reverse cholesterol transport itself, controlling the rate-limiting step in cellular cholesterol efflux from macrophages to HDL. Cholesterol efflux from macrophages protects these cells from accumulating cholesterol, thereby delaying the formation of foam cells, one of the early steps in the atherosclerotic process.[12-15]

The association between the C69T polymorphism, also known as -14T or rs1800977, and CHD risk has been studied before but the results were inconsistent.[16-20] A significantly

higher number of TT individuals was found amongst Indian CHD patients than amongst Indian controls, but in this study no differences were found in other ethnicities.[17] Tregouet et al. concluded that there was no association between the polymorphism and CHD.[18] In another small study, Ergen et al. found no significant differences in genotype distribution between persons with or without a myocardial infarction (MI), but in line with our findings a trend was observed towards a higher percentage of TT individuals in the MI patients than in the controls.[19] In contrast, in patients with diabetes, a significantly lower number of TT individuals was present in the group with prevalent MI than in the group without MI.[20] However, one third of patients without and 39% of patients with MI were on lipid-lowering drugs, which might have influenced the association between the genotypes and MI. The potential interaction with lipid-lowering medication or more specifically statin treatment was not investigated in these studies. The only study that covered statin treatment was a placebo-controlled randomized trial by Zwarts et al: TT individuals had higher CHD risk, but the differences between statin-treated and placebo-treated patients were not specifically reported.[16] In line with our results, CC individuals in the placebo group had lower CHD rates (12.4%) than CT (26.9%) and TT individuals (35.3%). The statin-treated TT group had the fewest events, but the numbers were too small to perform a meaningful statistical analysis.

The association between ABCA1 C69T genotype and HDL-C levels has been studied more extensively but the results are inconsistent as well. In most studies, no relationship was found.[16, 19, 20, 24] In both a Turkish and a Chinese population, the TT genotype was associated with higher HDL-C levels in men only, while in a large French cohort the this was only observed in men with BMI below 25 kg/m^2.[17, 22, 23] Remarkably, in another study the TT genotype was associated with lower HDL-C.[24] So far, the response of HDL-C levels to statin treatment has not been reported. In our study, baseline HDL-C levels were not affected by the genotypes. Nonetheless, HDL-C was raised to a significantly higher extent by statin treatment in TT individuals than in CC individuals.

Notably, these contradicting effects in literature about the effect of this polymorphism on CHD risk and HDL-C levels are present at the molecular level of ABCA1 transcription and translation as well. The ABCA1 gene uses a number of transcriptional start sites and the predominant transcript differs between cells and tissues.[34] As a consequence, ABCA1 transcription rate may differ according to cell type. Also ABCA1 levels might in some but not all tissues may be regulated post-transcriptionally.[35] Even more puzzling, the effects of higher ABCA1 expression may differ between cells and tissues and in presence or absence of hypercholesterolemia.[36] Joyce and Van Eck found opposite effects of ABCA1 expression in the liver and macrophages: while selective transgenic overexpression of ABCA1 in the liver was atherogenic in LDL receptor knockout mice due to accumulation of cholesterol-enriched apoB-lipoproteins, overexpression in macrophages exerts an atheroprotective effect.[37, 38] However, a recent study demonstrated the opposite: Brunham et al. showed that deletion of Abca1 in macrophages had no effects on atherosclerosis, but liver-specific deletion of Abca1 in Ldlr-/- mice led to increased atherosclerosis.[39]

The location of the polymorphism in the promoter region between a TATA box and one of the transcriptional start sites suggests that the C69T sequence variant might change transcriptional activity but this effect might also be dependent on factors such as cell type and other factors as described above. In two earlier studies, the TT genotype showed higher transcriptional activity than the CC genotype in different cell lines and in peripheral blood mononuclear cells of hypercholesterolemic patients.[22, 40] In normocholesterolemic patients no differences were observed. The effect of the C69T polymorphism on ABCA1 mRNA and protein levels in the liver remains to be established.

The location of the polymorphism might also explain the sensitivity to molecules influencing transcription such as statins, although this may vary between cell types as well. In peripheral blood mononuclear cells of statin-treated patients, no differences in transcriptional activity in monocytes were observed between CC and TT individuals in contrast to observations in hypercholesterolemic patients.[40] Interestingly, statin treatment had different effects on ABCA1 in hepatocytes compared to macrophages: Tamehiro et al. found that compactin treatment resulted in upregulation of ABCA1 in the liver, while it downregulated ABCA1 mRNA levels in peripheral cells in rats.[13]

Alternatively, instead of changing gene transcription, the C69T polymorphism may tag a functional polymorphism in the ABCA1 gene. It would be of great interest to study the exact consequences of the C69T polymorphism in more detail, since prediction of treatment response will facilitate personalized medicine. As an example, Celera is now preparing to launch a test for KIF6 to 'identify those patients at additional risk for an event and those whose incremental risk could be ameliorated by statin therapy'. [28](www.celera.com) However, not many examples of genes influencing lipid levels and CHD risk reduction in response to statins are known yet.[8-10]

The strength of the current study lies in the fact that statin treatment is recommended for all FH patients independently of other risk factors for coronary heart disease, which diminishes the risk of an indication bias. However, treatment was neither randomized nor placebo-controlled which may introduce other kinds of bias. In our earlier study, we showed that the patients who started statin treatment immediately in 1990 had more severe CHD risk profiles: they had more often hypertension, were older and had higher cholesterol levels than the patients who started later. However, this selection bias would have resulted in an underestimation of effect of statin treatment since the most at risk were treated first. In addition, patients might have improved their lifestyle in conjunction with starting statin treatment. Therefore, we analyzed the effect of smoking cessation within six months after starting statin treatment but this did not change the effect of statin treatment.[4] Although these or unrecognized confounding factors may have influenced the determination of efficacy of statin treatment, in the present study we analyzed statin-genotype interaction and as a result of 'Mendelian randomization' the classical risk factors such as age, smoking, LDL-C,and HDL-C were evenly distributed amongst genotypes. Moreover, the variation in delay of starting statin treatment and the percentage of patients refraining from therapy was similar between genotypes: the genotypes were not related to the decision to start a statin. In summary, we conclude that the TT genotype

of the ABCA1 C69Tvariant is associated with a higher CHD risk in untreated FH patients. Statin-treated TT individuals, however, have CHD risk similar to the other genotypes. This effect is most likely at least partially caused by a greater increase of HDL-C levels in TT individuals. Our results suggest that statins reduce CHD risk partly through HDL-related mechanisms.

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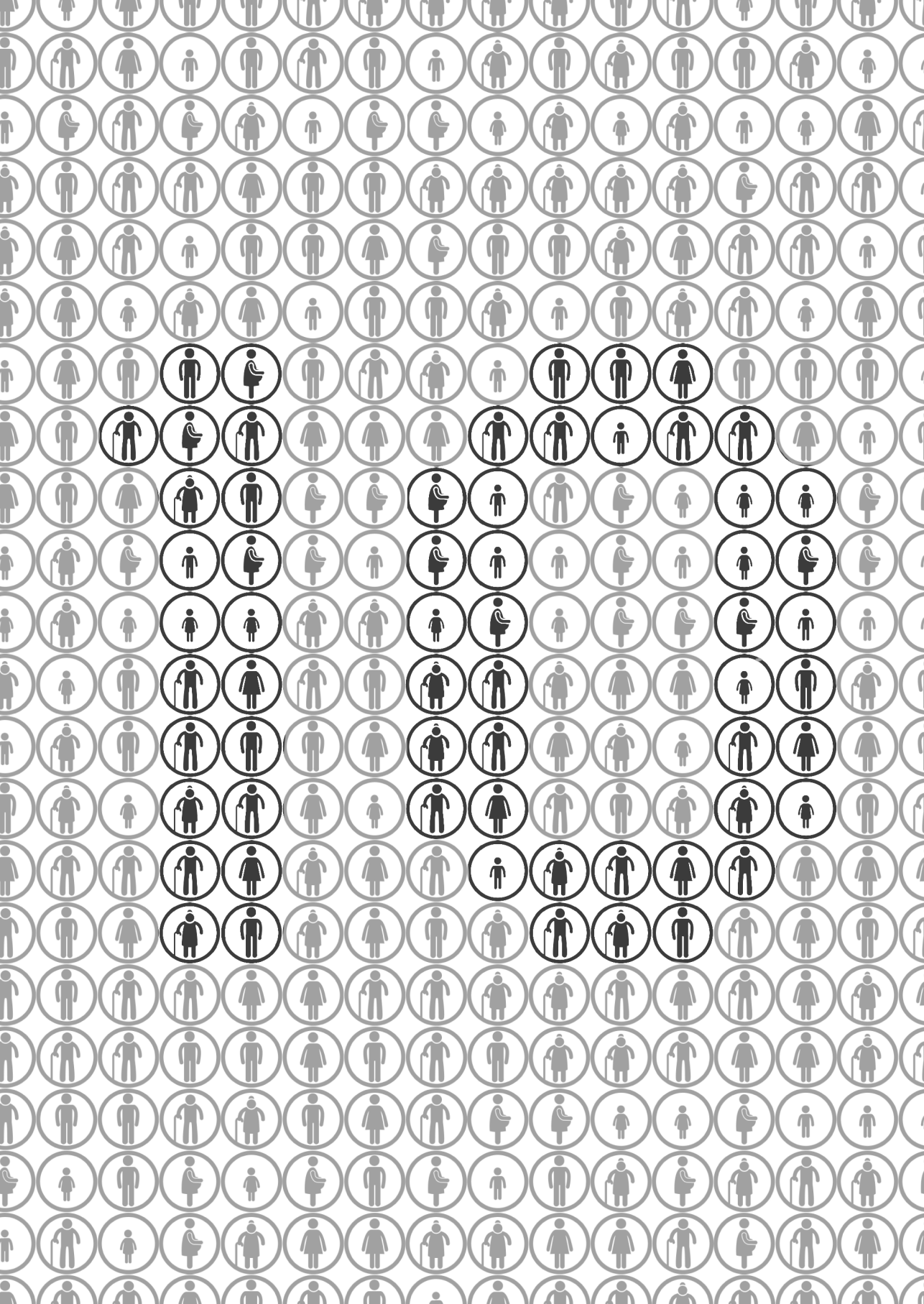

10 Summary and General Discussion

SUMMARY

Mutations in the low-density lipoprotein (LDL) receptor gene (*LDLR*) cause a monogenetic disease: familial hypercholesterolemia (FH). The *LDLR* is mainly expressed in the liver where it takes up the LDL-cholesterol (LDL-C) from the blood. Because of the genetic defect, LDL-C uptake is less efficient, and as a consequence the endogenous cholesterol production by the liver increases. FH is therefore characterized by high levels of LDL-C, leading to increased risk of cardiovascular disease at young age, mainly coronary heart disease (CHD). In this thesis, we studied several CHD risk modifying factors.

In the first part of this thesis, we studied different genetic and other biological CHD risk modifiers in FH. In **chapter 2**, we showed that the *APOE4/E4* genotype, a well-known CHD risk factor in the general population, did not increase risk in FH patients with an *LDLR* mutation. In **chapter 3**, we aimed to identify previously unknown genetic CHD risk variants in a genome wide association (GWA) study comparing the youngest and eldest FH patients with CHD, respectively. In this GWA study, we also showed that common single nucleotide polymorphisms (SNPs) can significantly associate with rare mutations, the so-called phenomenon of synthetic associations (**chapter 4**). In **chapter 5**, we identified differences in plasma cholesterol efflux capacity between FH patients with and without CHD and hypothesized that this difference might at least partially explain the difference in CHD burden. The first part ends with an analysis supporting that maternal inheritance of FH might increase CHD risk (**chapter 6**).

In the second part, we studied the present-day main risk modifier in FH, namely statin treatment: we showed that statin treatment normalized CHD risk in asymptomatic FH patients, both in FH patients who came to our clinical attention (**chapter 7)** as their family members identified by cascade screening (**chapter 8**). We extended this work with a pharmacogenetic study described in **chapter 9**. A genetic risk variant in the ATP-binding cassette transporter A1 (*ABCA1*) gene encoding an important cholesterol transporter associated significantly with CHD. This association disappeared when the FH patients were treated with statins, which could at least partially be explained by a higher rise in high-density lipoprotein cholesterol (HDL-C) levels during statin treatment of the patients with the *ABCA1* risk genotype.

Here, we will critically discuss all our findings, including clinical implications and future perspectives.

GENERAL DISCUSSION

Limitations of the work presented in this thesis

For most studies presented here, replication in another FH population or pedigree would be ideal. However, the population in which we studied efficacy of statin treatment in FH and the genetic risk factor in *ABCA1* is rather unique. It consists of 2,400 FH patients and

it is unlikely that data collection of such a large cohort with both untreated and treated patients will soon be reproduced, especially since this study was very successful in gathering information on genetic and classical risk factors of CHD in FH.[1-3] Also, a pedigree as complete as presented in chapter 6 will be hard to find.

Another limitation is that we do not know whether our results in FH can be extrapolated to the general population since we did not study persons without FH.

Part I: CHD risk modifiers in FH

ApoE

LDL receptors have two major ligands: Apolipoprotein B100 and ApoE. Together they regulate the uptake of VLDL and LDL by the liver. Mutations in ApoB100-receptor result in hypercholesterolemia to a bit milder extent as LDL receptor mutations (familial dysbetalipoproteinemia (FDB)). *APOE* has three major haplotypes, called *APOE2*, *APOE3* and *APOE4*. APOE4 is mainly located on VLDL particles, while ApoE2 and ApoE3 are mainly located on HDL. In *APOE4* carriers, this leads to an increase in conversion of VLDL to LDL and consequent down-regulation of LDL receptors. ApoE2 has the lowest affinity to the LDL receptor leading to up-regulation of LDL receptors, while ApoE4 has the highest.[4] As a consequence, *APOE2* carriers paradoxically display lower LDL-cholesterol (LDL-C) levels and *APOE4* carriers higher LDL-C levels.[5, 6]

ApoE4 is notorious for its strong relationship with Alzheimer's disease: in a meta-analysis, the OR appeared to be 3.2 for *E3/E4* genotyped persons and 14.9 for *APOE4/E4* genotyped persons.[7 8]

ApoE4 also increases CHD risk.[9] However, in earlier studies, *APOE4* was never a CHD risk factor in FH patients heterozygous for an *LDLR* mutation. [9-11] Besides, mice expressing human ApoE4 developed substantial atherosclerosis only when the human LDL receptor was also expressed.[12, 13]

We hypothesized that *LDLR* mutations might protect against the atherosclerotic effects of ApoE4. Indeed, CHD risk was not increased in *APOE4*/E4 genotyped FH patients with an *LDLR* mutation. On the contrary, CHD risk even seemed to be smaller in FH patients with an *LDLR* mutation and the *APOE4/E4* genotype than in FH patients with an *LDLR* mutation and the wildtype *APOE3/E3* genotype. This effect was independent of LDL-C levels.

The first possible explanation of this decreased CHD risk is that the strong LDL receptor-ApoE4 binding results into 'trapping' of ApoE. This may enhance sequestration of VLDL at the hepatocyte surface. Consequently, the delayed internalization may lead to an increased conversion to atherogenic remnant particles and consequently small dense LDL particles.[12, 14, 15] A recent study supports this theory: diabetic ldlr-/- mice had higher levels of triglycerides when expressing human apoE4 instead of ApoE3. [16] However, this study was in STD-induced diabetic mice, a situation not comparable to FH patients with low VLDL and triglyceride levels. 'Trapping' of ApoE might also occur at the outer membrane

of macrophages. [12, 14, 15, 13, 17] We explored this option in a small study: we compared the cholesterol efflux capacity of macrophages from individuals with and without an *LDLR* mutation with the *APOE3/E3* and *APOE4/E4* genotype but did not find any differences (data not shown in this thesis).

Alternatively, increased binding to other receptors, such as related *LDLR* family members or proteoglycans, could also explain why the presence of an LDLR mutation might be beneficial for *APOE4/E4* genotyped persons.[38-42] Many of these receptors display favourable effects on atherosclerosis, which might be more pronounced if more ApoE is able to bind to these receptors.[38-43] A promising candidate is Megalin, which is important for clearance of Lp(a), but nothing is known yet about differences in affinity to ApoE3 and ApoE4. [18, 19]

For both CHD and Alzheimer's disease risk further research should focus on this ApoE4-LDL receptor interaction. The focus should be on HDL including cholesterol efflux, VLDL kinetics or alternate receptors binding ApoE.

The ultimate consequence of our finding in chapter 2 would be that statin treament in *APOE4/E4* genotyped FH patients with an *LDLR* mutation would be contraindicated, since statins increase the number of functional LDL-receptors in heterozygous FH patients.[20, 21] However, negative effects of statin treatment cannot be confirmed based on our findings and since statins are believed to reduce CHD risk to a larger extent than can be expected on LDL-lowering alone due to so-called pleiotropic effects, statin use should remain the treatment of first choice also in FH patients with *APOE4/E4* genotype.[22-24]

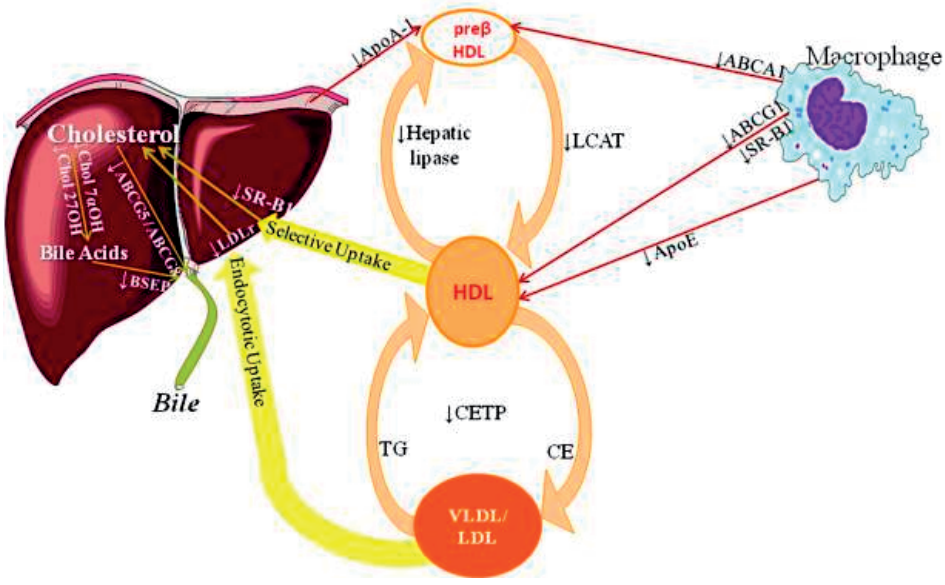

FIGURE. Reverse cholesterol transport. (From: Feingold and Grunfeld, JLR 2010[25])

Reverse Cholesterol Transport

This thesis once again demonstrates the importance of reverse cholesterol transport (RCT; Figure[25]). Reverse cholesterol transport (RCT) is an anti-atherogenic pathway by which cholesterol from macrophages is returned to the liver by HDL particles. The main proteins that enhance cholesterol efflux from macrophages to HDL are the ATP-binding cassette A1 transporter (ABCA1) and ABCG1. A role for the LDL receptor in enabling ABCA1 for cholesterol efflux has been suggested.[26] Cholesteryl ester transfer protein (CETP) transfers cholesterol from HDL to VLDL and LDL, which can be cleared by LDL receptors in the liver.

High HDL-C levels associated with low CHD risk in FH.[1, 27] However, merely increasing HDL-C levels in FH patients did not decrease CHD risk.[28] Genetic variants of *ABCA1* and *CETP* also have been associated with CHD risk in FH.[1, 29-33] Nonetheless, these genetic CHD risk variants often did not associate with plasma HDL-C levels. [29-32, 34] Most likely, a functional difference above and beyond a quantitative difference in HDL-C levels determines CHD risk. In line with this, plasma from FH patients without CHD induced higher cholesterol efflux from macrophages than plasma from FH patients with CHD, independently of HDL-C levels (chapter 5).[35, 36] This might explain why not all FH patients develop CHD despite their high LDL-C levels.

Since we expected differences in the composition of HDL to explain differences in cholesterol efflux capacity, we analyzed pre-β HDL levels, density profiles as well as sphingosine-1-phosphate, ApoA1 and ApoM levels within HDL.[37-41] Pre-β HDL levels were higher in FH patients than in their brothers, but similar in FH patients without and with CHD and they did not associate with efflux capacity. However, we found higher cholesterol as well as apolipoprotein A1 (apoA1) concentrations in HDL_2 from FH patients without CHD compared to FH patients with CHD. This was in line with earlier studies showing a positive relationship between HDL_2 cholesterol concentrations and cholesterol efflux.[42, 43]

In addition to these differences at the level of HDL_2, S1P levels in HDL_3 were increased. S1P is gaining interest as the major component in HDL defininig its atheroprotective function.[44, 45] S1P levels inversely related with CHD risk.[46] Besides increasing cholesterol efflux, S1P levels also affects endothelial cell survival, angiogenesis and endothelial NO synthase.[44, 45] In our study, S1P did not directly correlate with cholesterol efflux, but levels were highest in FH patients without CHD. So either no correlation with cholesterol efflux was found due to a power problem, or one of the other functions of S1P explains this finding. Together with increased ApoM levels, our data strongly support further studies on the role of S1P and other sphingolipids, especially since other CHD risk factors such as hypertension and diabetes mellitus (DM) seem to influence its homeostasis as well.[47, 48]

Since all FH patients are preferentially treated with statins, it is important to know its effects on RCT. Overall, statins are expected to increase RCT.[49] Statins increase HDL-C levels.[50, 51] However, effects of statins on ABCA1 are still unclear and differ between studies using different cell types; overall, *ABCA1* in hepatocytes seems to be up-regulated but not by all statins, while most studies show a decrease in macrophages but cholesterol load and differentiation status seem important.[52-58] In chapter 9, untreated FH patients with the

TT genotype of the C69T polymorphism in the promoter region of *ABCA1* had increased CHD risk independent of HDL-C levels. During statin treatment, however, the risk genotype TT was associated with a high increase of HDL-C and TT was no longer a risk variant. Apparently, this genotype is more sensitive to up-regulation of HDL-C by statins. Since this increase translates into a decrease in CHD risk, most likely RCT is increased as well; we did not test this directly.

Future experiments have to show whether statins influence differences in HDL density profiles and S1P content as shown in chapter 5. One earlier study showed that S1P receptors are upregulated by statins, enabling its effects on endothelial cells. It is tempting to speculate that favourable effects including the ones on top of LDL-C lowering take place at least partly via the S1P pathway.

GWA studies: extreme genetics and synthetic associations

In addition to candidate gene studies, focusing on well-known genes of interest, the last decade hypothesis-free genome-wide association studies have been performed. Two hypotheses regarding susceptibility to complex diseases have been postulated: the 'common disease, rare variant' hypothesis and the 'common disease, common variant' hypothesis.[59]

The 'common disease, rare variant' hypothesis suggests that many rare variants are responsible for the same common disease. Actually, the monogenetic background of FH is an example of this hypothesis, each mutation being rare, leading to the same phenotype which is rather common (prevalence 1:400).

GWA studies are not designed to identify rare variants. The 'common disease, common variant' hypothesis implies that complex diseases are caused by a number of genetic variants, each of which has a very small effect. A striking example is the best replicated risk allele in the *TCF7L2* gene that consistently associates with diabetes mellitus. This risk allele increases risk by 20-50% and has an allele frequency of 25-28%.[60, 61] Persons with more risk variants for a particular disease have an increased risk, as shown in several gene load studies.[62]

GWA studies usually consist of huge cohorts, clustered into even larger consortia.[63] In the study described in chapter 3, we expected to identify larger effect sizes in our relatively small GWA study, compared to traditional GWA studies on CHD, for two reasons. First, we studied genetic risk variants for CHD in an FH population. Based on Rothman's model of causation, one would expect that in presence of similar environmental factors, genes will render larger effects.[64] Second, we applied an 'extreme genetics' approach by genotyping the phenotypic extremes of FH.[65] Thus, the power was expected to improve, despite the reduction in number of individuals.[65-67] Our study is the first study to examine this theoretical claim using empirical data. Our findings, however, indicate that one should not expect such an extreme leverage in the effect sizes using this approach. As an example, the well replicated locus at 9p21.3 that rendered a close to significance p-value (0.05) had an OR of 1.28, close to 1.25 which was found in the original study without extreme genetics approach. As a result of this lack of gain in statistical power,

we did not find genome-wide significant hits in our GWA study, and the selection of top SNPs with a p-value < 10^{-4} could not be replicated in two independent FH cohorts. This study might be seen as a conceptual proof of principle why prediction modeling is still disappointing. While we were trying to pick up novel genes by selecting extreme groups across the CHD risk distribution spectrum, genetic risk prediction studies try to start with genetic information and estimate who will end up in the low and the high risk group. [68] Since we show the effect sizes in these groups are not as enhanced as we expected, also the contrast needed for prediction might be less than anticipated.

Probably, for CHD the SNPs with strongest effects have been identified, and further genotyping and even sequencing of large populations will only reveal more common variants with small effect size.[69] Focus should be on interaction studies, although for complex diseases this is intricate due to large numbers of risk factors with small effect sizes and interaction at different levels, and statistical challenges: should one search for interactions, or for association while allowing for interaction.[70]

Besides rare variants directly causing disease, it might also be that common variants identified in GWA studies link to rare causal variants hundreds of kilobases upstream or downstream as we described in chapter 4. This finding, earlier described by Dickson et al., may have implications for the interpretation of earlier GWA studies: until recently, only the genes nearby a GWA signal were sequenced looking for the causal variant, but it might be that a much larger area has to be sequenced.[59, 71]

Maternally versus paternally inherited FH

In addition to genetic risk modification, epigenetic risk modification might play an important role in defining susceptibility to CHD. Ever since Barker postulated his 'fetal origin of adulthood disease hypothesis', prenatal effects on CHD risk later in life have been subject to numerous studies, but most were complicated by the large time lag between exposure and outcome.[72, 73] First studies were showing increased risk of cardiovascular disease and DM at adult age in babies with low birth weight.[72, 74, 75] Later studies also showed unfavorable effects of increased birth weight, for example, increased risk of atrial fibrillation and diabetes.[76, 77] However, a bias might have occurred since high birth weights might have been caused by maternal dietary factors during pregnancy, a trend the mothers probably continue in the postnatal lifes of these children.[78, 79] As a consequence, these studies cannot differentiate between an effect in utero or in early childhood. The limitations of these studies render the Barker hypothesis still controversial.[73, 80] The advantage of studying persons with FH is that their high cholesterol levels are not necessarily related to unhealthy lifestyles. In chapter 6, the difference between paternal and maternal inheritance of FH was most clear in females: the standardized mortality ratio (SMR) was not increased in paternally inherited FH in females. However, the statistical power became smaller after splitting the population by gender. However, it might be that epigenetic factors have a stronger effect in females. This is not supported by observations in *ldlr*$^{+/-}$ mice in which male instead of female offspring of *ldlr*$^{-/-}$ mothers were more affected.[81, 82] Research in mice suggested that irreversible epigenetic effects occur in offspring of hypercholesterolemic

mothers.[83, 84] It would be of interest to study these effects in offspring of human mothers with FH. Methylation and CpG essays could be performed on DNA of this offspring, for example in umbilical blood cells.

Part II: Treatment of FH

Treatment studies

Before the widespread use of statins, the mean age of CHD in men with FH was between 40 and 45 years and in women about ten years later.[85] As summarized in a HuGE review, CHD burden in men aged 60 differed among studies between 52 and 85% and in females between 32 and 70%.[85] However, about 40% of the untreated FH patients had a normal lifespan even without treatment.[86] Until now, CHD risk prediction using classical risk factors, gene risk scores and/or biomarkers does not accurately discriminate between patients, who will and will not develop CHD. Therefore, all FH patients need to be treated, preferably with statins.

Statins have many pleiotropic effects in addition to their cholesterol-lowering effect: for example, they attenuate inflammation, reduce thrombotic risk and may even reduce blood pressure.[22, 87, 88, 89]

In chapter 7 and 8 of this thesis, we show that statin treatment is effective, both in patients who have come to clinical attention as in their family members identified by cascade screening. The effective treatment of FH by statins was confirmed for primary prevention in large observational studies in the United Kingdom and Spain and a small Japanese study.[90, 91] In addition, the carotid intima media thickness (IMT), which is a surrogate marker for atherosclerosis risk, of FH patients undergoing long-term aggressive treatment with high doses of statins was in the normal range.[92-94]

Statins are generally considered very safe drugs, but the effects of lifelong treatment are still unknown. Besides, statins are not tolerated by all patients, myopathie being the most common side-effect. Myopathie may be due to reduction of co-enzyme Q10, a product of the mevalonate pathway.[95-97] Also vitamin D deficiency might lead to statin-induced myopathie, but this was only shown in two small studies.[98]

There are several reasons for need of additional therapies on top of or instead of statins. In comparison with primary prevention, the results of secondary prevention are less satisfactory: CHD risk remains much higher in FH patients after their first event compared to patients without FH in large secondary prevention trials.[90, 99] Even the efficacy of primary prevention is under debate considering the recent results of multi-sclice CT imaging studies in asymptomatic heterozygous FH patients. Imaging of coronaries of asymptomatic FH patients showed dramatic increases in coronary plaques and calcium scores, even in treated patients.[100] The predictive value of increased plaques and calcium scores in treated asymptomatic patients needs, however, to be established: the study mentioned represents the baseline characteristics of an ongoing long-term follow-up study. However, first results advocate more aggressive lipid-modifying therapy. In addition to these issues in heterozygous FH patients, homozygous and compound heterozygous FH patients can

definitely not be optimally treated with statins, although life expectancy has improved by decades.[101] An undervalued request for new therapies is for pregnant FH mothers. As described in chapter 6, LDL-C lowering treatment during pregnancy might need to be reconsidered since accepting high cholesterol levels during pregnancy seems to be safe for the mother but epigenetic effects for the fetus may have been overlooked. In pregnancy, the safety of statins cannot be guaranteed.[102-104]

Further improving prognosis of FH

It would be of great value to predict who will benefit best from treatment in general, and statin treatment in FH patients in particular. In chapter 9, we identified a variant in ABCA1 that associates with a better response to statin treatment. One could argue that the ideal pharmacogenetic study would be a randomized placebo-controlled trial. Nonetheless, treatment choices are independent of genotypes. This so-called Mendelian randomization approach is a helpful tool in pharmacogenetic studies.[105] Although a number of variants predict the response to statin treatment, their effect sizes are small and therefore these variants have no consequences for clinical decision-making.[106-109]

A combination of different targets may more effectively reduce LDL-C levels and CHD risk. First cholesterol-lowering drugs inhibited uptake in the intestine. These uptake-inhibitors, so-called bile-acid sequestrants, resulted in relatively moderate cholesterol lowering and CHD risk reduction.[110, 111] They are, however, considered safe in pregnancy.[104] Unfortunately, due to gastrointestinal side effects compliance with this treatment is low. Ezetimibe, a more recently developed inhibitor of cholesterol uptake, appeared promising but did not improve carotid IMT of FH patients when added to statins.[94]

Statins inhibit HMG-CoA-Reductase, the rate-limiting enzyme in cholesterol synthesis. Inhibition of a later step in cholesterol synthesis, squalene synthase, was tested but it had hepatic side effects.[112] An important consequence of inhibition of cholesterol synthesis by statins is the upregulation of LDL-receptors. A promising strategy in increasing LDL receptors is by inhibition of Proprotein convertase subtilisin/kexin type 9 (PCSK9). PCSK9 is involved in degradation of LDL receptors and natural loss-of-function mutants of the gene encoding PCSK9 have decreased cholesterol levels and CHD risk.[113] Early phase trials on PCSK9 inhibitors are promising.[114-119] Inhibition of PCSK9 is particularly promising on top of statins since statins undesirably induce *PCSK9* expression.[119-121]

Other ways to lower LDL-C levels is by inhibition of VLDL and LDL formation. Inhibition of microsomal triglyceride transfer protein (MTP) decreases formation of VLDL and has shown to decrease total cholesterol and LDL-C levels in heterozygous and homozygous FH patients. Phase II and III trials using MTP inhibitors are on the way.[114, 122-124] Inhibition of LDL formation via inhibition of the *APOB* gene expression by antisense apoB mRNA Mipomersen shows promising results in a number of phase 2 and 3 trials.[125-127] The sub-cutaneous administration of these drugs may be a limitation for large scale use, and the use is restricted to FH patients with extremely high LDL levels such as homozygote and compound heterozygote patients, and FH patients needing secondary prevention who do not reach LDL-C targets with upper limit statin treatment.

Besides lowering LDL-C, raising HDL is still a major treatment target, even despite the disappointing results with CETP inhibitor Torcetraptib and ApoA1 Milano [28, 128, 129] The ILLUMINATE trial studying Torcetrapib had to be terminated prematurely due to increased cardiovascular morbidity and mortality in the treated group, despite impressive increases in HDL-C.[130] It appeared that the agent itself was toxic, leading to hypertension, electrolyte imbalance and impaired endothelial function. Other CETP inhibitors such as dalcetrapib are being tested now. [131, 132 133] Positive results on IMT have been published but the DalOUTCOMES trial has to be awaited for results on CHD risk reduction. [132] At least, so far no serious side effects and effects on blood pressure have been reported, suggesting CETP inhibition itself did not cause these effects.[133]

The initially very promising development of supplementing the ApoA1 function by administration of apoA1-Milano or D-4F peptides does not seem to be followed by recent clinical data.[128, 129] One reason might be that HDL function rather than quantity might determine the atheroprotective effect, as shown in our and earlier studies.

Niacin is an example of an HDL increasing drug targeting outside the statin pathway. In a recent secondary prevention trial niacin caused significantly more IMT decrease on top of statins than Ezetimibe.[134] However, in the AIM-HIGH study, patients who reached target LDL-C levels by statin use experienced no additional CHD risk reduction by niacin over a follow-up period of 36 months, despite significant increases in HDL cholesterol levels and decreases in triglyceride levels.[135] LDL-C levels in this study were as low (<1.8 mmo/l) as

TABLE 1. Medication aimed to reduce CHD risk in FH.

LDL lowering	Comment
Uptake inhibitors	
Bile-acid sequestrants	GI Side effects Modest LDL-C lowering Modest CHD risk reduction
Ezetimibe	Well-tolerated CHD risk reduction not proven
Inhibitions of synthesis of cholesterol, LDL or VLDL	
Statins	Well-tolerated Proven CHD risk reduction
PCSK9 inhibition	Particularly efficient in combination with statin CHD risk reduction not proven
MTP inhibitors	Inhibit formation of VLDL (and chylomicrons by enterocytes) CHD risk reduction not proven
HDL increasing	
CETP inhibitors	Dalcetrapib probably safe CHD risk reduction not proven
ApoA1 mimetics	CHD risk reduction not proven
Niacin	Flushes, to be prevented by NSAID CHD risk reduction not proven

Others: relevance not clear
Squalene synthase inhibitors, MTP inhibitors, squalene synthase inhibitors, thyromimetics.

usually not reached in FH patients.[136, 137] Whether niacin can decrease IMT and CHD risk in FH patients remains to be established, but the effects on lipid levels are promising.[134] The HPS-Thrive trial, a secondary prevention trial without preselection on LDL-C levels will give way on expectations in FH patients. Unfortunately, no trial in FH patients has been or will be performed. A last exciting new development in increasing HDL are the gold nanoparticles that mimic the HDL function, but clinical testing is not likely in the near future.[138]

Thyroid upregulates both LDL-receptors and Scavenger Receptor B1 (SR-B1).[139] SR-B1 is the major HDL receptor and is important for reverse cholesterol transport. Since administration of thyroid hormone has several unwanted effects, thyromimetics specifically acting on metabolic targets have been developed.[140] In animal studies, increase in RCT and decrease in LDL, and most importantly decrease in atherosclerosis were reported.[140, 141] In the first published phase II trials LDL-C decreased and HDL-C increased; however, all further trials have recently been stopped because of cartilage problems observed in toxicology studies in dogs.[142-144]

Future

The genes identified by GWA studies and replicated in FH populations are interesting targets for new drugs; however, little is known about most of them and extensive research with respect to their function and development of novel concepts is needed.

Overall conclusion of this thesis

In conclusion, we identified several CHD risk factors in FH, but since CHD prediction is still difficult, all FH patients need treatment. Statin treatment is efficient and safe, but for specific high-risk groups and secondary prevention alternative or additional therapy is required. Research on a lot of promising novel approaches is ongoing; genetic association studies might give directions for unknown pathogenetic pathways and therefore new therapies.

What was already known on CHD risk reduction in FH
FH increases CHD risk tremendously
Statin treatment reduces LDL-C, high dosages improve carotid IMT
Both environmental factors and genetic risk factors define CHD risk in the individual with FH
APOE4/E4 is a risk genotype for both CHD as Alzheimer's disease
Reverse cholesterol transport is an important atheroprotective pathway

What this thesis adds
Statin treatment nearly normalizes CHD risk of asymptomatic FH patients identified in a screening program
Rare variants can cause synthetic associations in genome wide association studies
CHD risk in FH is partly determined by the parent of origin
APOE4/E4 is not a CHD risk factor in FH patients with an *LDLR* mutation
Differences in efficiency of reverse cholesterol transport might determine CHD risk in FH patients

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Nederlandse samenvatting

Familiaire hypercholesterolemie (erfelijk verhoogd cholesterol, afgekort FH) is een genetische aandoening die gekenmerkt wordt door een defect in de low-density lipoprotein (LDL) cholesterol receptor. Hierdoor wordt het schadelijke LDL-cholesterol (LDL-C) niet optimaal uit het bloed verwijderd. De lever kan de defecte receptor niet compenseren. In tegendeel, de lever wordt in deze situatie gestimuleerd om meer cholesterol geproduceerd. FH wordt daardoor gekenmerkt door extreem hoge LDL-C waarden met als gevolg een verhoogd risico op hart- en vaatziekten, waardoor al op zeer jonge leeftijd met name coronaire hartziekte (CHZ) optreedt. Diëet heeft weinig effect op de hoge cholesterolwaarden in FH-patiënten, omdat bij minder aanbod van cholesterol juist de eigen cholesterolproductie verder opgeschroefd wordt. Er zijn wel veel andere factoren die het risico op CHZ in FH-patiënten beïnvloeden. In dit proefschrift hebben we er een aantal van bestudeerd.

Wij hebben met name biologische risicofactoren bestudeerd, deze kan je meestal moeilijk beinvloeden. Dit in tegenstelling tot omgevingsfactoren zoals roken of ongezond eten. FH is tegenwoordig met statines goed te behandelen. Ook hier hebben we onderzoek naar gedaan: benadert het CHZ-risico van behandelde FH-patiënten normale waarden en wat zijn factoren die het effect van behandeling beïnvloeden?

DEEL I: FACTOREN DIE HET RISICO OP CHZ IN FH BEÏNVLOEDEN

In het eerste deel van dit proefschrift bestuderen we een aantal biologische risicofactoren op CHZ, voor een groot deel genetische risicofactoren. Van een genetische risicofactor is sprake, als een bepaalde variant van het DNA significant vaker aanwezig is in de groep met CHZ dan in de groep zonder. Vaak zorgt de DNA variant er dan voor dat een eiwit minder goed gemaakt of juist sneller afgebroken wordt. Dit kunnen eiwitten zijn die van belang zijn voor bijvoorbeeld het cholesterol opruimen uit de weefsels, of voor het vormen van een stolsel in de bloedvaten, of voor elk ander proces dat een rol speelt bij het

ontstaan van CHZ. De eerste genetische studies maakten gebruik van de zogenaamde kandidaatgenmethode: varianten om te bestuderen werden uitgezocht in genen waarvan al verwacht werd dat ze een rol speelden in de ziekte van interesse. In **hoofdstuk 2** laten we zien dat een bekende genetische risicofactor voor CHZ in de algemene populatie, namelijk de E4 variant van het Apolipoproteïne E, in FH-patiënten juist geen risicofactor is. Mogelijk beschermt de LDL-receptor mutatie de FH-patiënten tegen het hogere risico op CHZ in *APOE4* dragers.

Het nadeel van deze kandidaatgen methode was dat nieuwe belangrijke genen onontdekt bleven: kandidaatgenen werden gekozen op basis van bekende kennis. Hierin kwam verandering toen na de ontrafeling van de gehele genetische code chips ontwikkeld werden met honderdduizenden genetische varianten. Al deze DNA varianten konden tegelijk vergeleken worden tussen mensen met en zonder de ziekte van interesse. Dit was uiteraard wel een statistische valkuil: als je veel testen doet zal er altijd wat uitkomen op basis van toeval. De regels voor statistische significantie werden aangescherpt en er werden enorme studie-populaties samengevoegd om de kans om nieuwe varianten te vinden te vergroten. Bovendien werd duidelijk dat bevindingen bevestigd moeten worden met extra onderzoek. Op deze wijze kan je onderscheid maken welke DNA varianten echt leiden tot CHZ en welke varianten niet bevestigd kunnen worden in herhaald onderzoek en dus toevalsbevindingen waren in het eerste onderzoek.

In **hoofdstuk 3** beschrijven we een genetische studie die gebruik maakt van zo'n chip met ruim 550,000 genetische varianten tegelijkertijd. Wij hebben hier een truc gebruikt om de kans dat onze positieve bevindingen juist-positief zouden zijn, dat wil zeggen niet alleen positief ten gevolge van het grote aantal testen maar een echte bevinding, te vergroten: we hebben oude FH-patiënten zonder CHZ vergeleken met de jongste met CHZ. Door dit grote contrast dachten we dat het makkelijker zou zijn om verschillen op te sporen. De genetische risicofactoren die we zo vastgesteld hebben, hebben we in een aantal andere populaties getest. Helaas bleken ze in deze populaties geen relatie te hebben met CHZ, en hebben we dus geen nieuwe risicovarianten opgespoord. We denken dat er veel risicovarianten zijn met zulke kleine effecten, dat het heel moeilijk is om deze op te sporen. De risicovarianten die je met zo'n chip kunt opsporen, hebben frequenties boven de 5%. De veel zeldzamere LDL receptor mutaties gaven onverwachts een heel sterk signaal in onze studie als we deze vergeleken met mensen zonder FH. Hiermee tonen we aan dat zeldzame genetische varianten in zulke studies ook signalen kunnen produceren via de vaker voorkomende varianten op de chip: dit wordt 'synthetische associaties' genoemd(**hoofdstuk 4**). We begrijpen nog niet volledig hoe dat ontstaat, want de signalen lagen in de DNA streng heel ver van de mutatie in het LDL receptor gen: tot 2,4 megabasen, dat is een signaal 2,400,0000 bouwstenen van de feitelijke mutatie.

In **hoofdstuk 5** kijken we naar variatie in de functie van het 'goede' cholesterol, het high-density lipoprotein (HDL). HDL zorgt ervoor dat cholesterol vanuit de cellen in de periferie, zoals in de vaatwand waar het aderverkalking kan gaan vormen, naar de lever gebracht wordt om het cholesterol met de gal uit te scheiden via de darm. Dit wordt het 'reverse cholesterol transport' genoemd. We hebben plasma van mannen met FH met en

zonder CHZ vergeleken met dat van hun broers zonder FH en zonder CHZ en ontdekt dat mensen met FH zonder CHZ cholesterol beter door cellen uit kunnen laten scheiden dan hun broers. Mogelijk is dit een compensatie-mechanisme dat er voor zorgt dat deze FH-patiënten geen CHZ krijgen, ondanks de hoge LDL-C waarden.

In **hoofdstuk 6** tenslotte, hebben we bestudeerd of CHZ-risico verschilt afhankelijk of FH van vader of van moeder geërfd wordt. De aanleiding van deze studie was de hypothese van Barker, die aan de hand van kinderen in Engeland vaststelde dat invloeden tijdens de zwangerschap mogelijk een effect hebben op de kans op ziekten later in het leven, zoals diabetes mellitus en hart- en vaatziekten. FH-patiënten die FH van hun moeder erven, worden al vroeg in de zwangerschap blootgesteld aan de effecten van hoge cholesterolwaarden. Door gebruik te maken van een grote stamboom die terugvoerde tot de 19e eeuw, konden we vaststellen dat de mortaliteit inderdaad hoger was in FH-patiënten die FH van hun moeder hadden geërfd, dan in FH-patiënten die FH van hun vader hadden geërfd. Dit is een belangrijk gegeven dat meer studie behoeft, want zwangeren met FH worden alleen in uitzonderlijke gevallen behandeld wegens de mogelijke nadelige effecten van statines voor de foetus. Maar de effecten van hoge cholesterolwaarden bij de moeder op de foetus zijn momenteel onderbelicht.

DEEL II: BEHANDELING VAN FH.

In het tweede deel richten we ons op de behandeling van FH. Begin jaren ’90 kwamen statines op de markt. Statines remmen een belangrijk enzym van de cholesterolproductie. Omdat deze cholesterolproductie juist in FH sterk verhoogd is, zijn statines zeer effectief in FH. In de eerste klinische trials liet behandeling met statines al zulke indrukwekkende verlagingen van het cholesterol zien, dat het niet ethisch werd beschouwd af te wachten of dit effect zich zou vertalen in een effect op CHZ-risico. Hierdoor was het lang onduidelijk of het risico nu daadwerkelijk genormaliseerd was, wat vooral gevolgen had voor de verzekerbaarheid van behandelde FH-patiënten. In **hoofdstuk 7** bestuderen we een grote populatie FH-patiënten om zo’n trial te simuleren: we konden dit doen omdat niet iedereen gelijk statines kreeg op de eerste dag dat ze beschikbaar waren, maar vaak zat er enige vertraging in. Deze vertraging hebben we gebruikt om het effect van statines te bestuderen. De CHZ-risicoreductie bleek ongeveer 80% te zijn. Het bleek, zoals we verwachtten, dat juist de mensen die het hoogste CHZ-risico hadden, doordat ze gemiddeld ouder waren en gemiddeld de hoogste cholesterolwaarden en vaker hoge bloeddruk hadden, het eerst behandeld werden. Daarom denken we niet dat ons effect een overschatting is, maar eerder een onderschatting. Als we de behandelde 50+ers in onze FH-populatie vergeleken met een cohort van 50+ers uit de algemene Rotterdamse populatie bleek dat het risico op een hartaanval niet meer significant verschillend was.

Het cohort dat we hiervoor gebruikt hebben, waren allemaal zogenaamde index-patiënten: patiënten die zelf naar de dokter waren gegaan in verband met een hoog cholesterol of hart- en vaatziekten. In Nederland worden ook de familieleden van index-

patiënten actief opgespoord via cascadescreening. Algemeen wordt aangenomen dat FH-patiënten die zo opgespoord worden een lager CHZ-risico hebben dan de mensen die zelf naar de dokter zijn gegaan. We wilden graag weten of dit inderdaad zo was en wat het effect van statines in deze groep was: in principe worden ook deze mensen immers na het stellen van de diagnose levenslang met statines behandeld. We bestudeerden meer dan 11,000 mensen die op deze manier opgespoord zijn en vergeleken ze met hun familieleden zonder de mutatie en de index-patiënten: we stelden vast dat het CHZ-risico niet significant verschillend is tussen de index-patiënten en de patiënten opgespoord via cascadescreening, en dat er 3 patiënten behandeld moeten worden om één geval van CHZ te voorkomen.

Niet iedereen reageert even goed op therapie. De wetenschap die zich bezighoudt met genetische verschillen die de reactie op medicatie beïnvloeden wordt pharmacogenetica genoemd. In **hoofdstuk 9** presenteren we een pharmacogenetische studie waarin we laten zien dat een bepaalde variant ('TT') van een bepaald gen (ATP-binding Cassete A1 of *ABCA1*) een hoger risico op CHZ met zich meebrengt, maar dat door een beter effect op statines, dit risicoverschil in behandelde patiënten niet meer te zien is. Dit lijkt met name te komen door een sterkere verhoging van het 'goede' HDL cholesterol bij gebruik van statines in deze 'TT'-groep.

In de algemene discussie in **hoofdstuk 10**, bespreken we al deze hoofdstukken nogmaals en bekijken de resultaten kritisch. We concluderen dat we een aantal voor FH belangrijke CHZ-risicofactoren gevonden hebben, maar voorspellen wie wel en geen CHZ krijgt is nog steeds niet mogelijk. Zo lang predictie niet mogelijk is, moeten alle FH-patiënten behandeld worden, vooralsnog bij voorkeur met statines, die bewezen effectief en veilig zijn. Er is echter toch behoefte aan nieuwe therapieën met name voor mensen die FH van zowel hun vader als hun moeder geërfd hebben, al CHZ hebben doorgemaakt, bijwerkingen hebben van statines of zwanger zijn. De resultaten van genetische studies moeten gebruikt worden om nieuwe aangrijpingspunten voor therapie vast te stellen.

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Mijn hardloopmaatjes Jeroen en Geert-Jan, een stukje met jullie rennen doet altijd goed. De marathon in 2008 blijft een hoogtepunt. Ik hoop dat we nog lang samen blijven rennen en sporten en jullie mijn gezeur al rennende aan blijven horen. Ooit nog een keertje New York? Volgend jaar??

Hier wil ik ook graag onze grootste supporter Els (& Ton) bedanken. Lieve Els, jouw onvoorwaardelijke steun en belangstelling doen me altijd goed.

Dan wil ik een paar belangrijke vriendinnen hier noemen. Linda, het was een grote eer je paranimf te zijn. Jouw relativeringsvermogen helpt me vaak; ik wou dat ik er maar een fractie van had. Hieke, al zo lang mijn maatje, fijn dat we nu ook vakliteratuur kunnen delen al loop je wel een paar honderd jaar achter. Susan, het is altijd een hele reis naar Groningen, maar het voelt wel altijd als een soort minivakantie, zeker met Guus en Google als attracties!

(Ex-)Collega’s in het IJsselland, zowel arts-assistenten als staf (als de rest), bedankt voor het fijne werkklimaat, ik heb met veel plezier in het IJsselland gewerkt en veel geleerd. Sanne, dank voor het checken van mijn proefdrukken tijdes je "verlof". Marcella, ik hoop dat we nog lang samen blijven mopperen bij een biertje, sandwich of in de sneeuw...

Tot slot mijn familie, altijd belangstellend en geduldig, dank voor al jullie steun en belangstelling. Pappa en mamma, altijd trots en nooit twijfelend aan mijn kunnen, dank voor alle support. Opa en oma, fijn dat jullie er nog bij kunnen zijn! Van knakworst tussen de middag naar pelgrimbier op mijn promotie! Lieve Mirjam, van jou kan ik altijd op aan, misschien binnenkort eindelijk weer aan de pilates? Roel, fijn dat het weer goed met je

gaat, en ik ben ook trots op jou. Family in Singapore, it is great to be with you, hopefully we can visit you again soon.

Lieve Maurits, misschien ben jij wel het allerblijst dat mijn proefschrift af is. Ik ben blij dat je het volgehouden hebt. Hopelijk hebben we nu wat meer tijd om samen leuke dingen te doen. Misschien tijd om een eigen racefiets te kopen?

Tot slot Marijke, wat was het geweldig geweest, al die jaren nog samen in Rotterdam. In gedachten blijf je er altijd bij.

List of Publications

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Cardiovasc Revasc Med 2006; 7:165-172.

About the author

Jorie Versmissen werd geboren op 17 juli 1981 in Vlaardingen. Na het Stedelijk Gymnasium in Schiedam, ging ze in 1999 geneeskunde studeren aan de Erasmus Universiteit in Rotterdam. Omdat ze al direct naast de klinische kant ook door de moleculaire en wetenschappelijke kant van de geneeskunde gefascineerd raakte, besloot ze in 2000 het master programma 'Molecular Medicine' te gaan volgen, dat ze in 2004 afsloot met een master thesis. Hierna liep ze haar co-schappen waarna ze in februari 2007 begon met haar promotie-onderzoek op de afdeling inwendige geneeskunde bij Prof. Eric Sijbrands, genaamd 'The whole-genome and classical risk factors in cardiovascular disease'. In september-december 2009 verrichte ze een deel van haar onderzoek aan de University of California San Francisco. Op 1 mei 2010 is zij in het IJsselland ziekenhuis in Capelle aan den IJssel begonnen aan haar opleiding tot internist (Opleiders: Prof J.L.C.M. van Saase en Dr. H.E. van der Wiel). In haar vrije tijd kookt en eet Jorie graag, wat ze gelukkig afwisselt met rennen en fietsen.

Jorie Versmissen was born in Vlaardingen on July 17th, 1981. She visited the 'Stedelijk Gymnasium' in Schiedam, followed by studying Medicine at the Erasmus University in Rotterdam. Because she got fascinated by the molecular and scientific aspects as much as by the clinical aspects of medicine, she participated in the master programme 'Molecular Medicine'. After finishing her master thesis in 2004, she did her internships. In February 2007 she started her PhD project at the department of Prof. Eric Sijbrands, called 'The whole-genome and classical risk factors in cardiovascular disease'. In September-December 2009 she went to San Francisco to be a research fellow at the University of California San Francisco. May 1st, 2010 she started her residencies in Internal Medicine at the IJsselland Hospital in Capelle aan den IJssel. Jorie's main hobbies are the perfect combination of eating and cooking, and running and biking.

ECTS PORTFOLIO

Coronary Heart Disease in Familial Hypercholesterolemia

Jorie Versmissen

Department: Internal Medicine
Research School: Cardiovascular Research School Erasmus University Rotterdam (COEUR)
Promotor: Prof.dr. E.J.G. Sijbrands
PhD-period: 2007-2012

PhD training	Year	ECTS
General academic skills		
Biomedical English Writing and Communication	2008	1.5
Research Integrity	2008	0.3
Research Skills		
Courses MGC and MolMed during MSc program	2003	4.5
Genetic Epidemiology (Principles, GE03 and GE of complex disease)- NIHES	2007	2.8
UCSC and Ensembl genome browsing-MolMed	2007	1.2
Coeur courses: Molecular Biology, Epidemiology of CVD	2007	3
Classical Methods for Data-Analyses-NIHES	2007	6
Coeur Research Seminars	2007-2010	2.8
NWO talentendag	2009	0.3
Symposia and conferences		
CBG 'Genome variation and complex phenotypes', Amsterdam	2007	0.6
Wetenschapsdagen Inwendige Geneeskunde, Antwerpen*	2008-2010	1.8
American Heart Association Scientific Sessions, New Orleans**	2008	1.5
DAS (Dutch Atherosclerosis Society) symposium, Ermelo***	2008, 2009	1.2
Benelux Nuclear receptors meeting, Utrecht	2008	0.3
ATVB Scientific Sessions, San Francisco***	2010	0.9
European Lipoprotein Club, Tutzing **	2010	1.2

*Poster presentation 2008 and 2009, Oral presentation 2010, Participant organizing committee 2009
** Oral presentation;
*** Poster presentation

Teaching		
Junior Med School: lecturing, supervision 3-week research program	2008,2009	2
Supervision Medical students participating in extracurricular research	2009-2010	1