Complex Genetics of Monogenic Familial Hypercholesterolemia

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Complex Genetics of Monogenic Familial Hypercholesterolemia

Complexe genetica van monogenetische familiaire hypercholesterolemie

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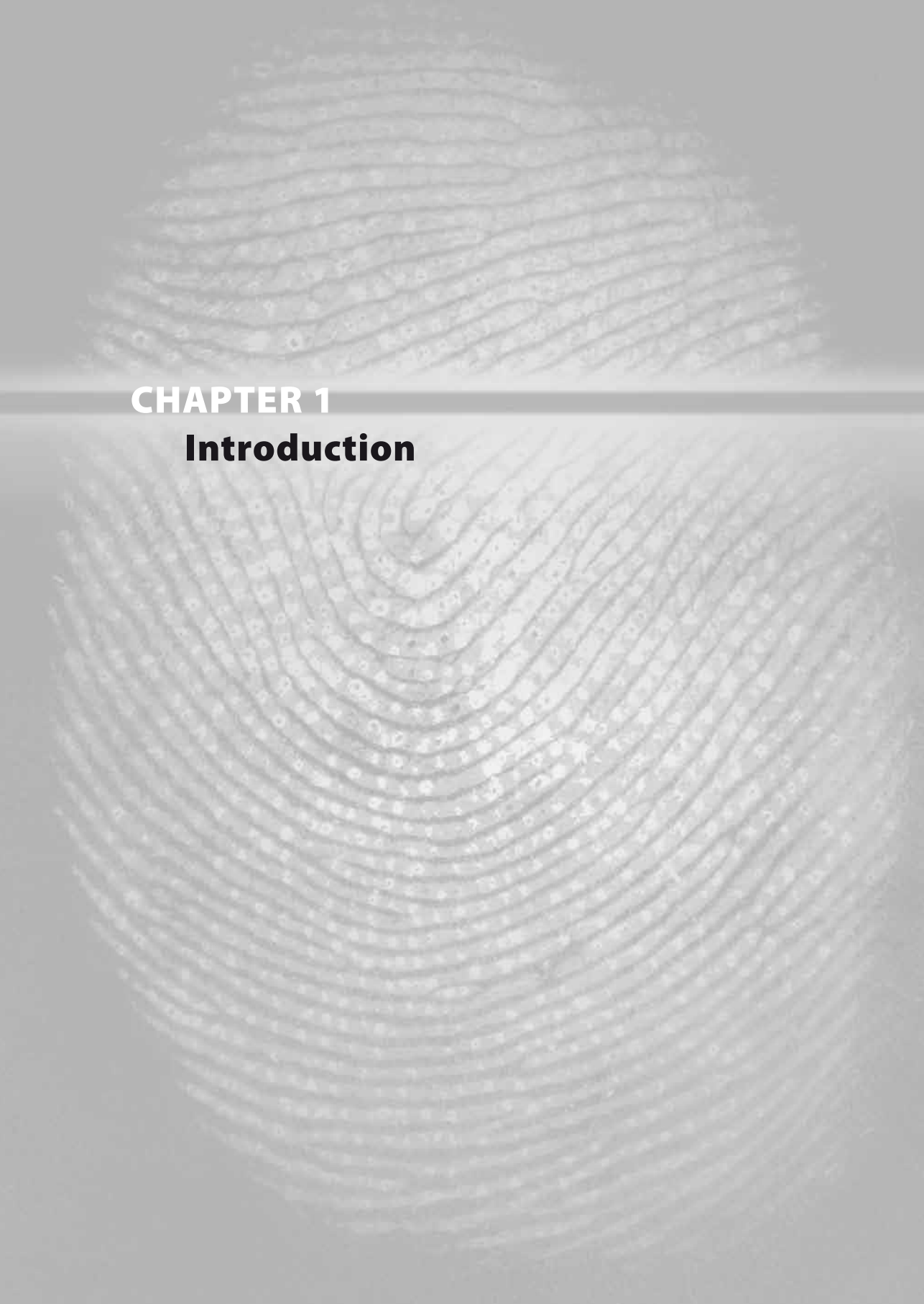

CHAPTER 1
Introduction

The etiology of cardiovascular disorders is complex and involves interaction of genetic, environmental, metabolic, and physiological factors. A small proportion of all cardiovascular cases results from rare single gene defects with strong effects on the phenotype. Biomedical research has been extremely successful in the genetic characterization of uncommon, monogenic cardiovascular disorders, thereby providing fundamental insights into the pathogenesis of cardiovascular disease (CVD). In 1985, Goldstein and Brown have been awarded the Nobel Prize for medicine for elucidating the molecular basis of familial hypercholesterolemia (FH).[1]

1.1 Familial hypercholesterolemia

FH is an autosomal dominant disorder of lipid metabolism caused by mutations in the gene coding for the low-density lipoprotein (LDL) receptor and leading to accelerated atherosclerosis and CVD at young age.[1] The LDL receptor is a transmembrane protein that regulates plasma cholesterol levels by uptake of LDL particles from the blood circulation (Figure 1). Mutations in the LDL receptor gene cause insufficient removal of circulating LDL particles, which stimulates the hepatic cholesterol production, resulting in raised plasma LDL cholesterol

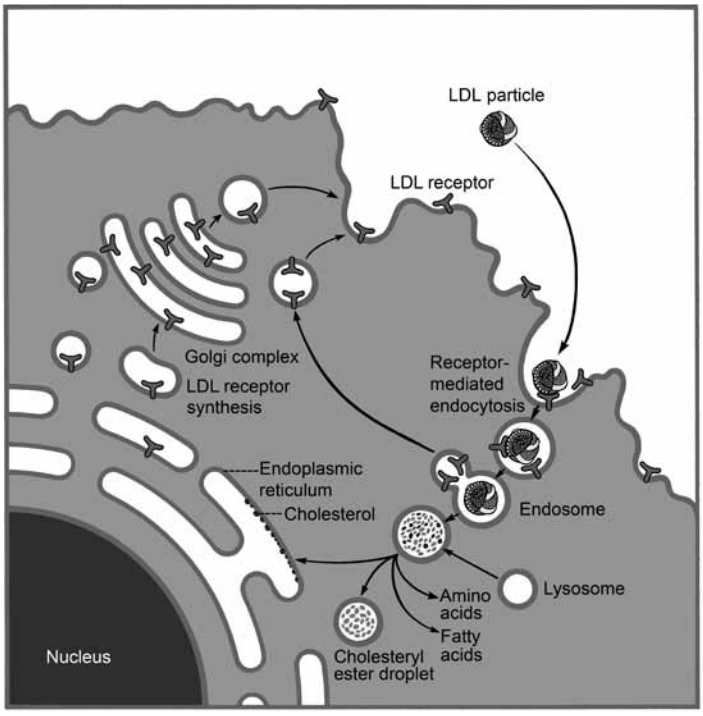

Figure 1. A schematic overview of the LDL receptor cycle in the hepatocyte.

concentrations. In the extremely rare (1/million) homozygous form of FH, the LDL receptor function is severely reduced or completely absent and plasma LDL cholesterol concentrations up to 26 mmol/L have been reported. Homozygous FH patients develop tendon xanthomas in childhood and massive atherosclerosis occurs frequently at a very young age. The heterozygous form of FH is considerably more common, with a prevalence of 1/500 persons in Western societies. Heterozygous FH patients have partially reduced LDL receptor function, leading to two- to fourfold elevated plasma LDL cholesterol levels. However, substantial variation is seen in the onset of atherosclerotic disease symptoms and cardiovascular death among patients with heterozygous FH.[2,3] After diagnosis, heterozygous patients are treated lifelong with inhibitors of β-hydroxy-β-methylglutaryl coenzyme A reductase (statins) to prevent premature CVD.

1.2 Risk of cardiovascular disease in FH

Approximately 5% of all patients with premature coronary heart disease are heterozygous for mutations in the LDL receptor gene. In studies before the widespread use of statins, approximately 52-85% of male and 32-58% of female heterozygous FH patients had documented CVD by the age of 60.[4,5] These frequencies are much lower in the present-day, statin-treated FH patients.[6,7] Typically, the mean age of CVD onset in untreated males was between 40 and 45 years and in females about ten years later. The burden of heterozygous FH, however, may have been preferentially studied in patients and families that presented themselves with premature symptoms of atherosclerosis. A mortality study in a large family-tree provided insight in the natural course of heterozygous FH, as the persons in the pedigree were not selected on the basis of clinical manifestations of FH.[2] The excess mortality from untreated heterozygous FH varied largely over time and between branches of the pedigree. Mortality was not increased in the 19th and early 20th century, rose to a peak in the 1930s to 1960s, and decreased thereafter (Figure 2). Interestingly, the mortality of the FH pedigree in the 1800s was even lower than in the general population; hypercholesterolemia may have conferred a survival advantage when infectious diseases were more prevalent. In multiple pedigrees with untreated heterozygous FH patients, it was estimated that 40% of the patients had a normal life expectancy whereas 60% suffered from premature death.[2,8] This emphasizes that this monogenic disorder has a complex burden, which is modified by other genes and environmental factors.[9]

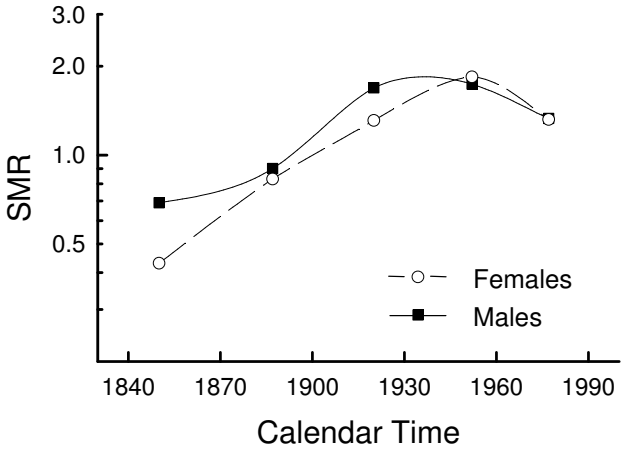

Figure 2. Mortality from heterozygous FH in a large pedigree according to sex and throughout the ages.(2) SMR indicates standardized mortality ratio. The mortality in the pedigree was compared with the mortality in the Dutch population standardized for age, gender, and calendar period; the SMR = 1.0 for the Dutch population.

1.3 Classical risk factors of cardiovascular disease in FH

Traditional cardiovascular risk factors account for most CVD cases in the general population,[10] but the influence of these factors in FH is less straightforward.[11] In FH, higher age and male gender are also clearly associated with increased CVD susceptibility.[12,13] In addition, raised levels of plasma high-density lipoprotein cholesterol were protective towards CVD in most FH studies.[14,15] However, conflicting data were reported about the contribution of other well-established cardiovascular risk factors such as smoking, diabetes, hypertension, and body mass index (BMI), partly due to small study samples.[11-14,16-22] The effect of an increased concentration of high-sensitivity C-reactive protein, fibrinogen, lipoprotein(a), and homocysteine on CVD risk in FH is still uncertain.[11,12,14,18,20,23-31] Raised LDL cholesterol level is the hallmark of FH, but variation of plasma LDL cholesterol concentration among FH patients does not predict CVD.[11,12,13,18,19,21,32]

The GIRaFH-study (**G**enetic **I**dentification of **R**isk F**a**ctors in **F**amilial **H**ypercholesterolemia) provided extensive data on the influence of classical risk factors to the development of CVD in FH.[33] A total of 2400 unrelated patients with FH, aged 18 years and older, were identified at lipid clinics throughout the Netherlands between 1989 and 2002. CVD was defined as coronary, cerebral, and peripheral artery disease. Male gender, smoking, hypertension, diabetes mellitus, low HDL cholesterol concentrations, and elevated lipoprotein(a) levels were independent predictors of an increased risk of CVD. These six risk factors explained 18.7% of the variation in CVD risk. Clearly, other still unknown and possibly genetic factors play a role in the development of CVD in FH patients. This thesis focuses on the influence of such genetic factors on susceptibility of CVD in individuals with heterozygous FH.

1.4 LDL receptor mutations

The LDL receptor gene is located on chromosome 19 at 19p13.1-p13.3. More than 800 sequence variations have been identified in this gene and the residual LDL receptor activity varies considerably among the different mutations.[34] LDL receptor mutations can be divided into six functional classes based on their phenotypic effects on the protein.[35]

- Class 1 mutations do not synthesize the LDL receptor protein.
- Class 2 mutations encode LDL receptor protein that is completely (class 2A) or partially (class 2B) blocked in its transport between the endoplasmic reticulum and the Golgi complex.
- Class 3 mutations produce LDL receptor protein that is effectively transported to the cell surface but does not properly bind with the LDL particles.
- Class 4 mutations lead to LDL receptor protein that successfully binds the LDL particles, but fail to internalize the receptor-LDL complex.
- Class 5 mutations cause LDL receptor protein that fails to release the internalized LDL particles in the endosome, a process that is essential for receptor recycling.
- Class 6 mutations encode rare LDL receptor proteins that do not bind properly to the cell membrane and, consequently, are released from the cell.

Classes 1 and 2A mutations are referred to as receptor-negative mutations or null alleles, whereas classes 2B to 6 mutations are named receptor-defective mutations. Studies in adult FH patients that analyzed the genotype-phenotype relationship at the LDL receptor locus have yielded conflicting results.[3,22,36-43] Selection on specific founder mutations, limited numbers of patients, different classifications of the LDL receptor mutation types, and interference of environmental factors can all account for these differences in outcome. In this thesis we assessed the influence of LDL receptor genotype on lipid concentrations, CVD risk, and response to statin therapy in children with FH.

1.5 Candidate genes of cardiovascular disease in FH

A growing number of genetic variants have been implicated in the development of CVD.[44] These genetic markers are not necessarily the cause of the disorder, but may be useful to improve risk assessment. Alternatively, genetic markers may provide important clues to disease pathophysiology and, therefore, may suggest new opportunities for therapeutic intervention.

Candidate gene studies focus on selected genes; they have a specific hypothesis and the association between the candidate gene and CVD susceptibility is tested. The variation in genes consists mostly of single nucleotide polymorphisms (SNPs). Essentially, candidate SNPs are chosen based on combinations of: (a) prior likelihood of being functional, (b) association

of the SNP with an atherosclerosis-related phenotype, and (c) technological considerations including the availability of high-throughput and lower costs pre-selected SNPs.[45] In addition, candidate gene studies are performed that use haplotypes (based on a set of SNPs that

Table 1. Candidate genes of cardiovascular disorders in FH

Gene	Polymorphism	Risk variant	N	Freq.	Phenotype	Effect	Ref.
ATP binding cassette A1	Arg219Lys	K variant	374	0.29	CHD	OR 0.63; p<0.05	(47)
Angiotensin-converting enzyme	insertion/deletion	DD genotype	213	0.33	MI	OR 2.57; p=0.02	(48)
					CHD	OR 2.21; p=0.02	(48)
		D allele	112	0.55	CHD	NS	(49)
		DD genotype	228	0.31	CHD	NS	(12)
Angiotensinogen	Met235Thr	T variant	112	0.25	CHD	NS	(49)
Angiotensin II type I receptor	A1166C	C allele	112	-	CHD	OR 3.10; p=0.04	(49)
Apolipoprotein E	E2/E3/E4 variants	E4 allele	706	0.09	CVD	NS	(50)
		E4 allele	236	-	CHD	NS	(14)
		E4 allele	93	0.09	CHD	NS	(51)
Estrogen receptor alpha	Haplotype-analysis:						
	T–1989G	GG genotype	295	0.17	CHD	OR 4.5; p=0.04	(52)
Cholesteryl ester transfer protein	Taq1 RFLP	B2/B2 genotype	300	0.20	CVD	NS	(53)
Lipoprotein lipase	Asn291Ser	NS+SS variants	1045	0.07	CVD	OR 3.89; p=0.003	(54)
	Asp9Asn	DN+NN variants	2091	0.05	CVD	OR 2.2; p=0.04	(55)
Methylenetetrahydrofolate reductase	C677T	TT genotype	199	0.11	age CHD	p<0.05	(56)
		TT genotype	249	24.5	CHD	NS	(57)
Paraoxonase1	Leu55Met	LL variant	187	0.46	IMT	NS	(58)
		LL variant	197	0.47	CVD	NS	(59)
	Gln192Arg	QQ variant	187	0.48	IMT	NS	(58)
		QQ variant	197	0.48	CVD	NS	(59)
		LL/QQ variant	187	-	IMT	p=0.002	(58)
	Haplotype-analysis:						
	G-824A	AA genotype	181	0.10	IMT	p=0.03	(60)
Paraoxonase2	Cys311Ser	CC+CS variants	197	0.37	CVD	p=0.01	(59)
Platelet glycoprotein IIIa	C1565T	T allele	80	0.23	CHD	NS	(61)

Abbreviations: CHD, coronary heart disease; MI, myocardial infarction; CVD, cardiovascular disease; IMT, intima-media thickness carotid artery; NS, non-significant.

occur together in the locus) to obtain more detailed information about the genetic variation of the locus in relation with CVD. SNP databases such as HAPMAP provide precognition about tagging SNPs for haplotyping the gene of interest for different ethnic populations.[46]

A variety of mostly small-scale single candidate gene association studies among FH populations have been published (Table 1).[12,14,47-61] Bertolini and co-workers assessed the single and the combined effects of 11 SNPs in 8 candidate genes involved in lipid metabolism on coronary artery disease (CAD) in 221 unrelated FH index cases and 349 FH relatives.[62] In these Italian FH patients, the fatty acid binding protein-2 54TT variant was an independent predictor of increased CAD risk after adjustment was made for clinically relevant risk factors. The 219RK and KK variants of the ATP binding cassette A1 were independently associated with decreased CAD.

Recently, the contribution to CVD risk of 65 polymorphisms in 36 candidate genes previously implicated in CVD via their influence on lipid metabolism, blood pressure regulation, coagulation and hemostasis, homocysteine metabolism, endothelial function, cell adhesion, inflammation, and plaque stability have been tested in the GIRaFH study.[63] In 1940 patients (80.1%) complete genotypes for all 65 polymorphisms could be obtained. The G20210A polymorphism in the prothrombin gene was strongly associated with an increased CVD risk (GA versus GG; $p<0.001$). Furthermore, the Met235Thr variant of angiotensinogen and the Thr347Ser variant of apolipoprotein A4 were associated with increased CVD risk, whereas the Ser311Cys variant of paraoxonase-2 and the C1100T variant of apolipoprotein C3 were associated with decreased CVD risk ($P<0.05$). In this thesis we investigated the role of three additional candidate genes on CVD susceptibility in this large cohort of FH patients.

1.6 Aims and outline of this thesis

Despite the monogenic background of heterozygous FH, the CVD "penetrance" varies enormously, depending only partially on the well-known classical risk factors. The principal aim of the studies described in this thesis is to investigate genetic factors that could potentially enhance the ability of clinicians to identify FH individuals with severely increased CVD risk, who will most likely benefit from targeted therapeutic intervention.

First, we examined the role of variations at the LDL receptor locus. Since LDL receptor genotype-phenotype analyses in adult FH patients have revealed conflicting results, we performed our analyses in children with FH. FH children might be better subjects to analyze the exact effects of LDL receptor genotypes, because they share a more homogeneous environment and do not yet have additional lipid disorders. We tested this hypothesis and assessed the relationship between the type of LDL receptor mutation and plasma lipid concentrations in FH children and the relation with the occurrence of CVD risk in the parents of these children. The LDL receptor genotype has also been implicated in the response to statin therapy.

After diagnosis, all FH patients are treated with statins but the response shows considerable interindividual variation. In this thesis, we studied whether LDL receptor genotype influenced the response to statin treatment in children with FH.

The monogenetic background but large variation in CVD risk determines that FH is an exemplary model to analyze candidate genes involved in CVD. In the second part of this thesis, we investigated the effect of three candidate genes of CVD based on the correlation with an atherosclerosis-related phenotype (complement factor H gene) or their reported association with cardiovascular risk factors (adenosine triphosphate binding cassette G8 gene and glucocorticoid receptor gene). In the general discussion, the main findings of the studies presented in this thesis are summarized briefly. In addition, the limitations and potential pitfalls when conducting genetic association studies in FH and developing individual prognostic models are discussed and directions for future research are provided.

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CHAPTER 2

Effect of low-density lipoprotein receptor mutation on lipoproteins and cardiovascular disease risk: a parent-offspring study

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Abstract

Studies on the clinical consequences of different low-density lipoprotein (LDL) receptor genotypes in adult patients have yielded conflicting results. We hypothesized that children with familial hypercholesterolemia (FH) provide a better model to perform genotype-phenotype analyses than adults. We tested this hypothesis and assessed the effect of LDL receptor genotypes on lipoprotein levels and on parental risk of cardiovascular disease (CVD) in a pediatric FH cohort. We identified 75 different LDL receptor mutations in 645 children with heterozygous FH; in these children, null alleles were clearly associated with more elevated LDL cholesterol levels compared to receptor-defective mutations. Familial factors explained 50.4% of the variation in LDL cholesterol levels of this pediatric cohort compared to only 9.5% in adults. Parental CVD risk was not significantly different between carriers of null alleles and receptor-defective mutations (RR, 1.22; 95% CI, 0.76 to 1.95; p=0.4). The N543H/2393del9 mutation was associated with a less deteriorated lipid profile and the parents had less often CVD relative to parents with other mutations (RR, 0.39; 95% CI, 0.20 to 0.78; p=0.008). We could confirm that children with FH provide a better model to perform genotype-phenotype analyses. In particular, children with null alleles had significantly more elevated LDL cholesterol levels than carriers of other alleles but this was not associated with higher risk of CVD in the parents. Nonetheless, a specific LDL receptor mutation was associated with less deteriorated lipoprotein levels and a milder CVD risk.

Introduction

Familial hypercholesterolemia (FH) is a common disorder caused by mutations in the low-density lipoprotein (LDL) receptor gene. FH is characterized by elevated plasma LDL cholesterol (LDL-C) levels, tendinous xanthomas, and premature cardiovascular disease (CVD).[1] Despite the monogenic nature of the disorder, heterozygous FH shows large variability in phenotypic expression related to both environmental and genetic factors.[2] At present, more than 800 sequence variations in the LDL receptor gene have been identified and the residual LDL receptor activity varies considerably between mutations.[3-4]

Previous studies in adult heterozygous FH patients have assessed whether the residual LDL receptor function influenced lipoprotein metabolism and, consequently, cardiovascular risk. These studies have yielded conflicting results.[5-14] Additional familial factors were proposed to have greater influence on the clinical consequences of FH.[2,15] In effect, it is not clear whether variance of lipoprotein levels and CVD risk could be attributed to variation at the LDL receptor locus or to additional familial factors. For instance, adult FH patients referred to lipid clinics often suffered from additional lipid disorders.[16] Such additional lipid disorders are not yet expressed in childhood. Therefore, FH children might be more suited to analyze the exact effects of LDL receptor genotypes. Moreover, selection of FH children is not based on additional CVD risk factors.

The purposes of this study were (1) to test the hypothesis that children with FH provide a better model to perform genotype-phenotype analysis than adults and (2) to assess the assumed relationship between LDL receptor genotypes and lipoproteins in a large pediatric cohort and their relation with the occurrence of parental CVD.

Methods

Study populations

Our pediatric FH cohort consists of 851 children with FH who were referred consecutively to our pediatric Lipid Clinic between July 1989 and January 2003. The general characteristics of this pediatric cohort have been previously published.[17] At present, in our ongoing molecular screening program, a LDL receptor mutation was identified in 655 children. In 8 children, we found apolipoprotein B mutations and they were excluded from the analyses, as were 2 children with homozygous FH. We performed the lipoprotein analyses in 593 heterozygous FH children after exclusion of 52 children on lipid lowering medication. The selection procedure is shown in the flow chart of Figure 1. We analyzed the parental risk of CVD in 436 affected parents of index children (the first child of a sibship that visited our lipid clinic). The presence of CVD in the parents was documented and defined as: (1) angina pectoris confirmed with electrocardiographic exercise testing, (2) ≥ 70% stenosis on coronary angiography, (3)

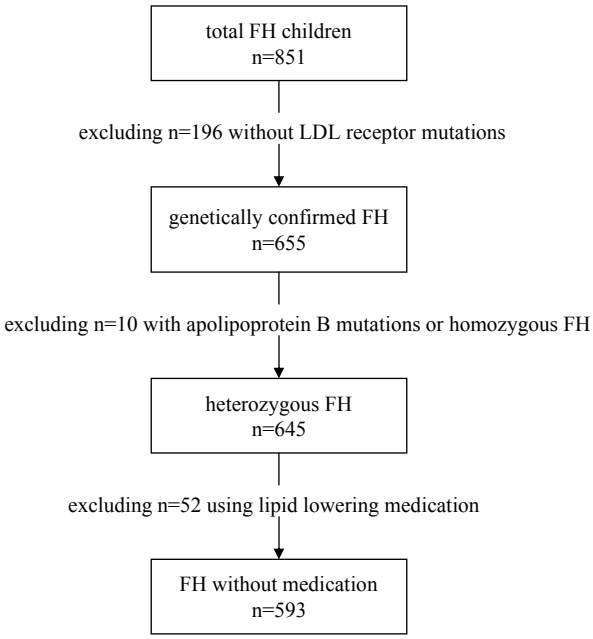

Figure 1. Flow chart of the selection procedure for the pediatric FH cohort.

myocardial infarction assessed by electrocardiography, or a deep and wide Q-wave as an electrocardiographic manifestation of an old infarction, or CPK-MB monitoring during the acute phase, (4) coronary bypass or percutaneous transluminal coronary angiography, or (5) stroke. CVD before the age of 60 years was considered premature CVD. The Institutional Review Board approved the study protocol, and informed consent was obtained from all children and parents.

The adult FH cohort from our national genetic testing program for FH has been described in detail and we presented genotype-phenotype data elsewhere.[8] In brief, we studied 399 untreated adult patients with an identified LDL receptor mutation after exclusion of all 66 index patients. Their mean age was 31.1 (± (SEM) 0.90) years, mean LDL-C was 5.65 (± 0.07) mmol/l, mean high-density lipoprotein cholesterol (HDL-C) was 1.06 (± 0.33), mean triglycerides (TG) was 1.49 (± 0.05), and 16.0 (± 4.0) % had CVD. In the present study, this adult FH cohort was used to calculate the sharing of familial factors among adult relatives (see statistical analysis).

Type of LDL receptor mutation

We compared lipid profiles between carriers of different types of mutations. Mutations can be divided into six functional classes based on their phenotypic effects on the protein. The mutation groups for our primary analyses were based on their functional class as reported in the literature: *(1)* the receptor-negative mutations or null alleles group contained all class

1 mutations, class 2A mutations, early stop-codons, and nonsense mutations, although the latter had often undetermined residual function; *(2)* the receptor-defective mutations group contained class 2B to 6 mutations; *(3)* the undetermined-receptor-activity mutations group contained all remaining mutations with undetermined mutational class. Secondary analyses were performed on seven mutations, frequently identified among the children.

Laboratory methods

Fasting plasma concentrations of total cholesterol (TC), TG, and HDL-C were measured using commercially available kids (Boehringer, Mannheim, Germany). LDL-C concentrations were calculated by the Friedewald formula.[18] All children had plasma TG concentrations below 4.5 mmol/L (398 mg/dL). Apolipoprotein A1 and apolipoprotein B100 were determined on a Behring nephelometer, BN 100 (Behring, Marburg, Germany). Lipoprotein (a) [Lp(a)] concentrations were measured using the Apo-Tek ELISA (Organon Teknika, Rockville MD, USA). Mutational analyses were performed with the use of polymerase chain reaction and restriction enzyme analysis as described previously.[19]

Statistical analysis

All data were analyzed using SPSS software (version 11.5, SPSS). In a linear mixed model, we calculated the intra-class correlations (i.e. the variance in LDL-C levels introduced by familial factors) in the pediatric and the adult FH cohort. In the pediatric FH cohort, we compared continuous data among specific LDL receptor mutations with one-way ANOVA. Statistical testing of TG and Lp(a) levels was performed after logarithmic transformation. We also performed analyses of the lipoproteins restricted to 398 index children without lipid lowering medication. Moreover, a sibpair analysis was performed to compare each index child with one sibling using the paired t-test. A total of 127 sibships consisted of two children, the remaining 30 sibships contained at least three children. In the latter, one affected sibling was randomly selected for the sibpair analysis. The effect of the nature of the mutation on lipoproteins was estimated in a linear mixed model including random familial effects and adjusted for age and gender. A total of 239 children were unrelated. The remaining 354 children were brothers and sisters and 72 of these sibships were also related in 35 pedigrees. Therefore, we made separate categorical variables for sibship and pedigree to adjust for family ties. In effect, we adjusted for first degree and higher degree relative.

In an affected-offspring model, we directly compared the parental risk of CVD in carriers of null alleles, receptor-defective mutations, and the N543H/2393del9 mutation with Cox regression and cumulative event-free survival was analyzed with the Kaplan-Meier method. The parental date of birth was used in the Cox regression to adjust for differences in life expectancy over calendar periods and adjustment for parental gender and family was made. Statistical significance was assessed at the 5% level of probability.

Results

Additional familial factors

In a linear mixed model, familial factors (intra-class correlation) explained 50.4% of the variance in LDL-C levels among the FH children. In the adult FH cohort, only 9.5% of the variance in LDL-C levels could be explained by familial factors. Table 1 shows the model in both the pediatric patients and the adult FH cohort: the LDL-C levels showed a much stronger correlation among related children than in adult siblings.

Table 1. LDL cholesterol levels explained by determinants and familial factors in a mixed linear model

Determinant	Effect on LDL-C	
	FH adults	**FH children**
	mmol/L (± 95% CI)	mmol/L ± (95% CI)
Mean age	0.25 (-0.18 to 0.67)	-0.39 (-0.73 to -0.06)
Male gender	-0.16 (-0.46 to 0.14)	-0.43 (-0.64 to -0.22)
Null allele	0.51 (-0.21 to 1.23)	1.13 (0.83 to 1.43)
Unexplained	5.69 (5.14 to 6.24)	5.91 (5.50 to 6.31)
Variance explained by familial factors*	9.5%	50.4%

FH=familial hypercholesterolemia, LDL-C=low-density lipoprotein cholesterol.

* intra-class correlation

LDL receptor gene mutations in FH children

The identified LDL receptor gene mutations are listed in Table 2. A total of 75 different mutations were detected in 645 children with heterozygous FH from 383 apparently unrelated families. We found 19 null alleles in 186 children, 26 receptor-defective mutations in 372 children, and 30 mutations with undetermined residual function in 87 children. Seven LDL receptor mutations were discovered in substantial numbers of children: the double mutation N543H/2393del9 was found in 141 children; two splice site defects at positions 1359-1 and 313+1/2 were detected in 106 and 64 children, respectively; the deletion of 2,5 kb at exon 7 and 8 is a large rearrangement and was found in 26 children; two missense mutations, V408M and E207K, were present in 38 and 31 children, respectively; and the W23X nonsense mutation was found in 28 children.

Lipoproteins

A total of 52 FH children were on lipid lowering medication and were excluded from the lipoprotein analyses. The characteristics and lipid profiles according to the type of LDL receptor mutation of the remaining 593 FH children are presented in Table 3. The mean age

Table 2. LDL receptor gene mutations in heterozygous FH children

Location	Mutation	Effect	Type	Ref.	Activity	Class	Receptor protein	n
Exon 1	M-21V	Translation initiation signal deleted	missense	[19]	<2%	1	negative	4
	M-21L	Translation initiation signal deleted	missense	[21]		1	negative	4
Exon 2	W23X	Trp→stop at 23	missense	[19]	2-5%	1	negative	28
	A29S	Ala→Ser at 29	missense	[19]		3-6	defective	2
Intron 2	191-2	3′-splice acceptor signal	splicing	[19]			unknown	10
Exon 3	R60C	Arg→Cys at 60	missense	[19]			unknown	1
Intron 3	313+1/2	5′-splice donor signal	splicing	[19]	2-5%	2	defective	64
	314-1	3′-splice acceptor signal	splicing	[19]			unknown	3
Exon 4	C134G	Cys→Gly at 134	missense	[4]	15-30%	2B	defective	1
	C146X	Cys→stop at 146	nonsense	[4]		1	negative	11
	C152W	Cys→Trp at 519	missense	[19]		2B	defective	2
	C163R	Cys→Arg at 163	missense	[19]			unknown	15
	646delTG	Stop at 195	frameshift	[19]			negative	1
	653delGGT	Gly at 197 deleted	frameshift	[19]	<2%	2B	defective	3
	D200G	Asp→Gly at 200	missense	[4]	<2%	2B	defective	1
	C201X	Cys→stop at 201	nonsense	[3]		1	negative	1
	D203V	Asp→Val at 203	missense	[19]			unknown	2
	D206E	Asp→Glu at 206	missense	[19]	5-15%	2B	defective	1
	E207K	Glu→Lys at 207	missense	[4]	<2%	2B	defective	31
Exon 5	E219X	Glu→stop at 219	nonsense	Novel		1	negative	2
	C234R	Cys→Arg at 234	missense	[19]			unknown	1
	D245E	Asp→Glu at 245	missense	[4]	15-30%	2B	defective	1
	C249X	Cys→stop at 249	nonsense	Novel		1	negative	1
Exon 6	K273E	Lys→Glu at 273	missense	[19]			unknown	1
	S285L	Ser→Leu at 285	missense	[19]	2-5%	2B	defective	15
	C292Y	Cys→Tyr at 292	missense	[19]			unknown	2
Exon 7	G314V	Gly→Val at 314	missense	[19]			unknown	3
	C317G	Cys→Gly at 317	missense	[19]		2B	defective	2
	G322S	Gly→Ser at 322	missense	[4]	15-30%	2B/5	defective	1
	R329X	Arg→stop at 329	nonsense	[19]		1	negative	6
	C331W	Cys→Trp at 331	missense	[19]	9%		unknown	1
Intron 7	1061-8	3′-splice acceptor signal	splicing	[19]			unknown	2
	D333G	Asp→Gly at 333	missense	[4]	15-30%	2B/5	defective	4
Exon 8	I334V	Ile→Val at 334	missense	[19]			unknown	1
	E336K	Glu→Lys at 336	missense	[4]		2B/5	defective	1
	C356Y	Gly→Tyr at 356	missense	[19]			unknown	1
	C371X	Cys→stop at 371	nonsense	[19]		1	negative	7
Exon 9	A378D	Ala→Asp at 378	missense	[19]		3-6	defective	3
	R395W	Arg→Trp at 395	missense	[19]			unknown	2

Location	Mutation	Effect	Type	Ref.	Activity	Class	Receptor protein	n
	N407K	Asn→Lys at 407	missense	[19]			unknown	1
	V408M	Val→Met at 408	missense	[4]	<2%	5	defective	38
	A410T	Ala→Thr at 410	missense	[19]	5-15%	3-6	defective	4
	D412Y	Asp→Tyr at 412	missense	[19]			unknown	3
	V415A	Val→Ala at 415	missense	[19]		3-6	defective	3
	W422C	Trp→Cys at 422	missense	[19]	5-15%	3-6	defective	1
	I430T	Ile→Thr at 430	missense	[19]			unknown	1
Intron 9	1358+1	5′-splice donor signal	splicing	[19]		1	negative	3
	1359-1	3′-splice acceptor signal	splicing	[19]		1	negative	106
Exon 10	W462R	Trp→Arg at 462	missense	[20]		2B/5	defective	1
	1480del121bp	Stop at 486	frameshift	Novel			negative	4
	1486delGG	Stop at 513	frameshift	[19]			negative	2
Exon 11	G525V	Gly→Val at 525	missense	Novel			unknown	2
	G528D	Gly→Asp at 528	missense	[4]	<2%	2A	negative	2
Exon 12	W556R	Trp→Arg at 556	missense	[19]			unknown	1
	1759delA	Stop at 643	frameshift	[19]			negative	1
	G571E	Gly→Glu at 571	missense	[4]	5-15%	5	defective	6
	I577L	Ile→Leu at 577	missense	[19]			unknown	1
	L590F	Leu→Phe at 590	missense	[19]		2A	negative	1
Exon 13	R612C	Arg→Cys at 612	missense	[19]		3-6	defective	1
Exon 14	2032del12	Del Gln-Tyr-Leu-Cys at 657-660	frameshift	[19]			unknown	4
	P664L	Pro→Leu at 664	missense	[4]	15-30%	2B	defective	17
	P664T	Pro→Thr at 664	missense	[19]			unknown	1
	C690S	Cys→Ser at 690	missense	[19]			unknown	3
Exon 15	2204ins13	Stop at 715	frameshift	Novel			negative	1
Exon 16	2343del5	Stop at 765	frameshift	[19]			negative	2
Intron 16	2389+1	5′-splice donor signal	splicing	[19]			unknown	4
	2390-2	3′-splice acceptor signal	splicing	[19]			unknown	2
Exon 17	2411insG	Stop at 795	frameshift	[19]			negative	2
	V806I	Val→Ile at 806	missense	[4]	15-30%	4A	defective	3
Double Mutation	K290R/ C292W	Lys→Arg at 290/ Cys→ Trp at 292	missense	[19]			unknown	3
	N543H/ 2393del9bp	Asn→ His at 543/ Del Leu-Val-Phe at 778-780	missense	[19]	25%	2B	defective	141
Large rearrangements		Deletion of 2.5kb		[4]	2-5%	3&5	defective	26
		Insertion of 10kb		[19]			unknown	9
		Insertion of 4,4kb		[19]			unknown	2
		Insertion of 4kb		Novel			unknown	1

Table 3. Characteristics of FH children with null alleles, receptor-defective mutations, and mutations with undetermined receptor activity

Parameter	Undetermined receptor activity	Null alleles*	Receptor-defective mutations	p-value†
	(n=80)	(n=172)	(n=341)	
Age, y (range)	10.4 (3.0-18.5)	10.6 (1.4-19.9)	10.6 (1.4-19.3)	1.0
Gender, m / f	38 / 42	79 / 93	170 / 171	0.4
TC (mmol/L)	7.82 ± 0.17	8.04 ± 0.11	6.86 ± 0.08	<0.001
LDL-C (mmol/L)	6.27 ± 0.16	6.40 ± 0.11	5.23 ± 0.07	<0.001
HDL-C (mmol/L)	1.21 ± 0.03	1.26 ± 0.02	1.29 ± 0.02	0.7‡
TC/HDL-C	6.65 ± 0.18	6.65 ± 0.15	5.57 ± 0.09	<0.001
TG (mmol/L)	0.73 ± 0.04	0.82 ± 0.03	0.74 ± 0.02	0.03§
Apo A-I (g/L)	1.23 ± 0.03	1.26 ± 0.02	1.26 ± 0.01	1.0
Apo B100 (g/L)	1.77 ± 0.05	1.75 ± 0.03	1.47 ± 0.02	<0.001
Lipoprotein (a) (mg/L)	184 ± 26	196 ± 19	184 ± 12	0.6§

TC=total cholesterol, LDL=low-density lipoprotein, HDL=high-density lipoprotein, Apo A-I=apolipoprotein A-I, Apo B100=apolipoprotein B100, TG=triglyceride, values are given as means ± standard error of the mean (SEM). We observed similar distributions of smoking, BMI, and stigmata (data not shown) in the three mutational groups.

*The 5 nonsense mutations that were classified as null alleles had similar distributions of baseline characteristics compared with receptor-negative mutations (data not shown).

†Comparison between null alleles and receptor-defective mutations.

‡Statistical analysis adjusted for individual serum triglyceride levels did not change the result (data not shown).

§Statistical testing after logarithmic transformation.

was 10.6 years in the three groups. The 172 children with null alleles had significantly more elevated mean LDL-C levels (difference (± SEM), 1.16 ± 0.13 mmol/L; p<0.001) and higher mean TG levels (difference, 0.08 ± 0.04 mmol/L; p=0.03) compared to the 341 children with receptor-defective mutations. The carriers of mutations with undetermined receptor activity had intermediate LDL-C and TG levels. Mean HDL-C levels were similar in the three groups.

An analysis restricted to index children showed similar relations between lipoproteins and LDL receptor genotype (data not shown). A sibpair analysis showed no statistical difference in terms of characteristics or lipoprotein levels between index children and affected siblings: the mean difference in LDL-C was 0.02 ± 0.11 mmol/L; p=0.9. In Figure 2, a comparison of the mean LDL-C levels is shown between the mutation types and between the seven specific mutations adjusted for age, gender, and family ties. The mean LDL-C level in carriers of null alleles was 6.44 ± 0.22 mmol/L, significantly more elevated than the 5.31 ± 0.37 mmol/L in the carriers of receptor-defective mutations (p<0.001).

Table 4 shows lipid profiles of the seven most frequent mutations. Carriers of the N543H/2393del9 mutation had significantly less increased mean LDL-C levels (difference, 1.76 ± 0.12 mmol/L; p<0.001) and mean TG levels (difference, 0.14 ± 0.04 mmol/L; p<0.001) and showed a tendency towards higher HDL-C levels (difference, 0.08 ± 0.03 mmol/L; p=0.09) compared to carriers of other mutations. In line with our unadjusted results, the carriers of the N543H/2393del9 mutation had significantly less increased mean LDL-C levels compared with

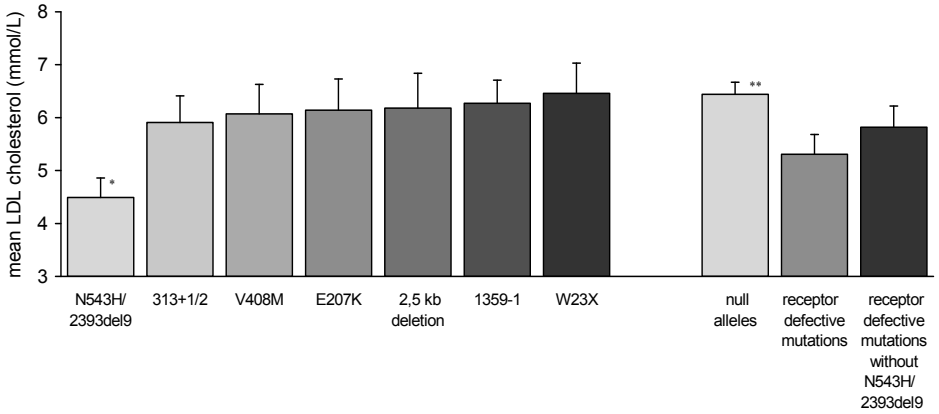

Figure 2. Mean LDL-C according to type of LDL receptor mutations adjusted for age and gender.

* Significant ($p<0.001$) difference between carriers of the N543H/2393del9 mutation and carriers of other mutations

** Significant ($p<0.001$) difference between carriers of null alleles and receptor-defective mutations with and without the N543H/2393del9 mutation

Table 4. Characteristics of 7 most frequent mutations

Parameter	N543H/ 2393del9	313+1/2	V408M	E207K	2,5 kb deletion	1359-1	W23X	p-value*
	defective (n=139)	defective (n=53)	defective (n=35)	defective (n=27)	defective (n=22)	null allele (n=100)	null allele (n=28)	
Age, y (range)	10.4 (1.4-19.3)	11.0 (4.5-17.9)	11.7 (2.0-16.6)	10.0 (4.1-18.0)	10.2 (2.5-18.0)	10.5 (1.4-19.9)	11.3 (3.2-18.0)	0.4
Gender, m /f	81 / 58	21 / 32	18 / 17	13 / 14	6 / 16	50 / 50	10 / 18	0.009
TC (mmol/L)	6.04 ± 0.09	7.55 ± 0.19	7.46 ± 0.20	7.73 ± 0.23	8.14 ± 0.33	7.82 ± 0.15	8.24 ± 0.25	<0.001
LDL-C (mmol/L)	4.41 ± 0.08	5.90 ± 0.18	5.94 ± 0.19	6.17 ± 0.22	6.54 ± 0.30	6.21 ± 0.15	6.51 ± 0.26	<0.001
HDL-C (mmol/L)	1.33 ± 0.02	1.30 ± 0.04	1.17 ± 0.05	1.24 ± 0.05	1.17 ± 0.04	1.26 ± 0.03	1.27 ± 0.04	0.09†
TC/HDL-C	4.73 ± 0.10	6.02 ± 0.20	6.70 ± 0.28	6.55 ± 0.37	7.10 ± 0.33	6.47 ± 0.19	6.76 ± 0.34	<0.001
TG (mmol/L)	0.66 ± 0.03	0.76 ± 0.05	0.79 ± 0.06	0.76 ± 0.07	0.98 ± 0.12	0.78 ± 0.04	0.94 ± 0.09	<0.001‡
Apo A-I (g/L)	1.28 ± 0.02	1.25 ± 0.03	1.20 ± 0.04	1.22 ± 0.02	1.23 ± 0.04	1.24 ± 0.02	1.31 ± 0.03	0.08†
Apo B100 (g/L)	1.23 ± 0.02	1.68 ± 0.05	1.71 ± 0.05	1.70 ± 0.07	1.84 ± 0.08	1.70 ± 0.05	1.85 ± 0.09	<0.001
Lipoprotein (a) (mg/L)	185 ± 18	147 ± 25	208 ± 42	165 ± 48	227 ± 40	160 ± 21	210 ± 40	0.4‡

TC=total cholesterol, LDL=low-density lipoprotein, HDL=high-density lipoprotein, Apo A-I=apolipoprotein A-I, Apo B100=apolipoprotein B100, TG=triglyceride, values are given as means ± standard error of the mean (SEM).

*Comparison between N543H/2393del9 mutation and other mutations.

†Statistical analysis after additional adjusted for individual serum triglyceride (data not shown).

‡Statistical testing after logarithmic transformation.

carriers of other alleles (4.49 ± 0.37 mmol/L versus 6.17 ± 0.22 mmol/L; $p<0.001$). Moreover, carriers of the two null alleles, 1359-1 and W23X, had significantly more elevated mean LDL-C levels relative to other mutations (6.27 ± 0.44 mmol/L versus 5.42 ± 0.24 mmol/L; $p<0.001$ and 6.46 ± 0.57 mmol/L versus 5.55 ± 0.25 mmol/L; $p=0.006$, respectively). We compared

Table 5. Null alleles versus receptor-defective mutations without the N543H/2393del9 mutation

Parameter	Null alleles (n=172)	Receptor-defective mutations (n=202)	p-value
Age, y (range)	10.6 (1.4-19.9)	10.8 (2.0-18.0)	0.7
Gender, m / f	79 / 93	89 / 113	0.7
TC (mmol/L)	8.04 ± 0.11	7.43 ± 0.10	<0.001
LDL-C (mmol/L)	6.40 ± 0.11	5.80 ± 0.09	<0.001
HDL-C (mmol/L)	1.26 ± 0.02	1.27 ± 0.02	1.0*
TC/HDL-C	6.65 ± 0.15	6.14 ± 0.12	0.007
TG (mmol/L)	0.82 ± 0.03	0.80 ± 0.03	0.6†
Apo A-I (g/L)	1.26 ± 0.01	1.25 ± 0.01	0.5
Apo B100 (g/L)	1.75 ± 0.03	1.64 ± 0.03	0.01
Lipoprotein (a) (mg/L)	196 ± 19	182 ± 15	0.5†

TC=total cholesterol, LDL=low-density lipoprotein, HDL=high-density lipoprotein, Apo A-I=apolipoprotein A-I, Apo B100=apolipoprotein B100, TG=triglyceride, values are given as means ± standard error of the mean (SEM).

*Adjustment for individual serum triglyceride levels did not change the result (data not shown).

†Statistical testing after logarithmic transformation.

the lipoproteins between null alleles and receptor-defective mutations after exclusion of the carriers of the N543H/2393del9 mutation (Table 5). Nonetheless, the difference in mean LDL-C levels between null alleles and receptor-defective mutations remained significant (difference, 0.60 ± 0.14 mmol/L; p<0.001). In fact, after adjustment for age, gender, and specific family ties, mean LDL-C levels were 6.44 ± 0.24 mmol/L and 5.82 ± 0.40 mmol/L for null alleles and receptor-defective mutations, respectively (p<0.001).

Parental history of CVD

We restricted the analyses of the relation between the nature of the mutation and parental history of premature CVD to 436 index children with FH. The number of events in FH parents was 38 in 5341 person years for null alleles, 44 in 6519 person years for receptor-defective mutations (N534H/2393del9 mutation excluded), 24 in 2651 person years for mutations with undetermined residual function, and 12 in 3852 person years for the N534H/2393del9 mutation. After exclusion of the carriers of the N534H/2393del9 mutation, the CVD risk was 1.22 of parents with null alleles relative to receptor-defective mutations (95% CI, 0.76 to 1.95; p=0.4). Strikingly, parents with the N543H/2393del9 mutation had significantly less often CVD than the parents with other mutations (RR, 0.39; 95% CI, 0.20 to 0.78; p=0.008). In figure 3, the Kaplan-Meier curves show the CVD free survival of parents with null alleles, receptor-defective mutations, and the N534H/del2393del9 mutation, respectively. CVD free survival was significant better in the N543H/2393del9 mutation compared with null alleles and receptor-negative mutations (log rank test, p=0.02).

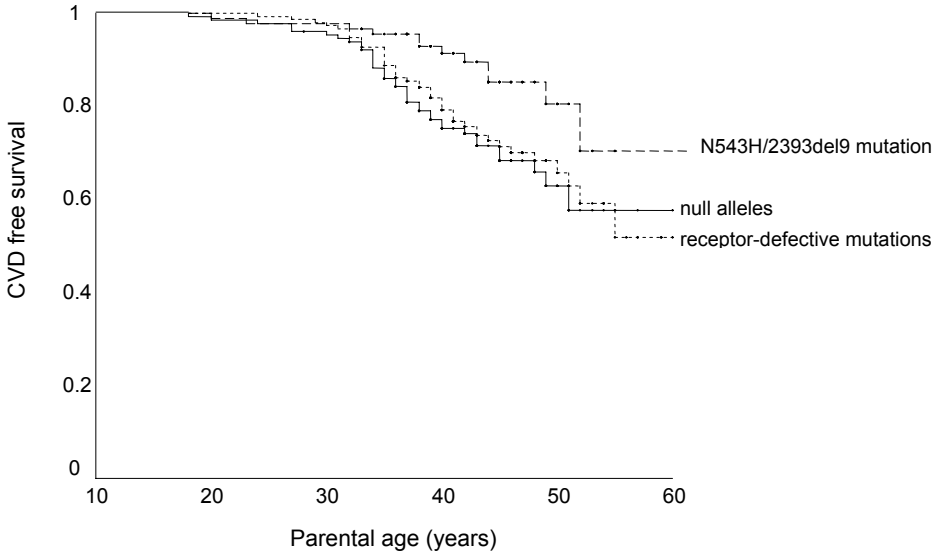

Figure 3. Kaplan-Meier curves for CVD free survival among parents with different types of LDL receptor mutations. CVD free survival was significant better in the N543H/2393del9 mutation compared with null alleles and receptor-negative mutations (log rank test; p=0.02).

Discussion

In the present study, we could confirm that FH children provide a better model than adults to analyze the relation between the type of LDL receptor mutation and lipoproteins: the LDL-C levels among related FH children were strongly correlated compared to a relative weak correlation among adult relatives with the disorder. Likely, children refrain from unfavorable lifestyles and share their environment to a greater extent than related adults.[20] The consequences of LDL receptor genotypes could, therefore, be estimated against a more homogeneous background in our pediatric FH cohort. A similar analysis has been reported in genetically confirmed FH children, but these children were selected on specific founder mutations.[21]

In line with most reports, we found that carriers of null alleles had significantly more elevated LDL-C levels than carriers of receptor-defective mutations.[5-7,9,10,13] In an adult FH cohort, we recently observed no difference of LDL-C levels between carriers of null alleles and other alleles after exclusion of families with the N543H/2393del9 mutation.[8] Exclusion of carriers with this mutation, however, did not change the results in our pediatric FH cohort. The analyses of lipoprotein levels were performed before the potential onset of additional lipid disorders in the children. Such lipid disorders may have influenced our and other analyses in adults. Moreover, the present results are in agreement with the only other study in FH children that showed significantly lower LDL-C levels in carriers of a receptor-defective mutation compared to carriers of two null alleles.[21]

Contrary to some previous studies, we found no significant differences in CVD risk between null and other alleles.[5,10-12] The present results are, however, in line with our findings among

FH adults: in a family study, we excluded all probands to avoid selection on CVD and found no difference in CVD risk between carriers of null alleles and other alleles.[8] The results of the latter studies and two recent mortality studies suggest that additional factors are of greater relevance towards the burden of FH than heterogeneity at the LDL receptor locus.[2,8,15] Instead of further genotype-phenotype analyses, research in FH patients should better focus on the identification of these additional risk factors.

The *type* of mutation did not significantly contribute to variation of CVD risk, but carriers of a *specific* mutation had clearly less increased CVD risk. This N543H/2393del9 mutation is a combination of a missense mutation in exon 11 and a deletion of 9 base pairs in exon 17 linked on the same allele. This allelic combination of mutations reduces the LDL-C uptake by 75%, suggesting a defect in transport of LDL receptor.[22] Recently, we have found in the larger group of children with clinical FH that more severely increased LDL-C levels could identify the FH children from families at highest risk for CVD.[17] In particular, FH children with LDL-C levels equal to or above 6.23 mmol/L were 1.7 times more likely to have a parent with FH with premature onset of CVD than those with LDL-C levels below 6.23 mmol/L. In this latter study, however, the difference in parental CVD risk could be due to the selection of children with the N543H/2393del9 mutation. Our present genotype-phenotype analysis was performed in a subselection from this earlier report and only 4.3% of the children with this specific mutation had a LDL-C level equal to or above 6.23 mmol/L compared to 44.5% in children with other LDL receptor mutations. Therefore, our earlier finding on the LDL-C levels may be based on the presence of this specific mutation.[17] This finding of relative mild FH in carriers of the N543H/2393del9 mutation is in agreement with observations in Spanish FH patients carrying the same mutation.[23]

Limitations of genotype-phenotype analyses in FH include the relatively large group of mutations with unknown functional class and residual receptor function. We divided the mutations into a null alleles group, a receptor-defective group, and a remaining group with undetermined mutational class. These groups were based on the functional class or specific properties of a mutation as reported in the literature. This classification has been used in several other studies and has resulted in well-defined groups. However, a disadvantage is that we excluded a relatively large group of mutations with undetermined receptor function from the analyses.

In summary, we conclude that FH children provide a better model to analyze the effects of LDL receptor genotypes on the lipid profile than adults. The specific N543H/2393del9 mutation associates with a milder lipid phenotype and less increased parental CVD risk compared to other mutations. However, the variation in lipid profile poorly explains the differences in CVD risk in carriers of other mutations. Therefore, future research in FH patients should focus on the identification of additional risk factors in the pathogenesis of CVD.

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CHAPTER 3

LDL receptor genotype and response to pravastatin in children with familial hypercholesterolemia: substudy of an intima-media thickness trial

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Abstract

Background: The lipid lowering effects of statin therapy show considerable interindividual variation in patients with familial hypercholesterolemia (FH). Whether or not the type of low-density lipoprotein (LDL) receptor mutation predicts the response to statin treatment is not yet established. We analyzed the relationship between LDL receptor genotype and response to pravastatin treatment in children with FH using carotid intima-media thickness (IMT) to measure efficacy.

Methods and Results: In a randomized, placebo-controlled, double-blind, two-year trial with pravastatin, 193 children had genetically confirmed FH and were included in the present sub-study. At baseline, children with null alleles had higher LDL cholesterol levels (difference, 0.94 ± (SEM) 0.19 mmol/L; $p<0.001$) and a greater carotid IMT (difference, 0.019 ± 0.01 mm; $p=0.02$) compared to children with receptor-defective mutations. The decrease in carotid IMT during the trial was not significantly different in children with null alleles and receptor-defective mutations (0.018 ± 0.012 mm and 0.012 ± 0.010 mm; two-way covariance analysis $p=0.7$). After two-year treatment, the children with null alleles continued to have greater carotid IMT than children with receptor-defective mutations (difference, 0.016 ± 0.01 mm; $p=0.02$). LDL cholesterol-lowering showed a tendency to less reduction in carriers of null alleles compared to carriers of receptor-defective mutations (1.30 ± 0.25 mmol/L and 1.85 ± 0.20 mmol/L; two-way covariance analysis $p=0.08$).

Conclusions: In FH children, we found that the null allele genotype was associated with a greater carotid IMT, higher LDL cholesterol levels, and a non-significant tendency to attenuated LDL cholesterol-lowering, when compared to receptor-defective mutations. Null alleles identify FH patients at the highest cardiovascular disease risk who may benefit from more aggressive treatment, started in childhood.

Introduction

Familial hypercholesterolemia (FH) is a common metabolic disorder caused by mutations in the low-density lipoprotein (LDL) receptor gene.[1] The disorder is characterized by severely elevated LDL cholesterol levels from birth onwards. Consequently, FH patients have an increased risk of cardiovascular disease. After diagnosis, heterozygous patients are treated lifelong with inhibitors of β-hydroxy-β-methylglutaryl coenzyme A reductase (statins) to prevent premature cardiovascular disease.[2]

The lipid lowering response to statin therapy, however, shows considerable interindividual variation.[3] In clinical practice, noncompliance with prescribed medication is an important cause of variation in statin response.[4] In addition, several investigators have assessed whether specific LDL receptor mutations affect the lipid lowering response to statin therapy.[3,5-11] Understanding this relationship could result in a more individual approach of the treatment of FH patients. However, these studies have yielded conflicting results. Selection on specific founder mutations, limited numbers of patients, different classifications of the LDL receptor mutation types, and a variety of treatment strategies *without randomization* made it difficult to compare the results of these studies. In the present subgroup of a randomized, placebo-controlled clinical trial with pravastatin in FH children, we analyzed a large number of different LDL receptor mutations. Recently, we have found that studying children with FH provide more accurate information on genotype-phenotype interactions than studying adults probably because of a lower chronic exposure to known environmental factors that alter lipid levels.[12] The randomization was used to reduce the influence of confounding factors. In addition, our observations were controlled for placebo effects, which enabled us to determine the natural course of the specific LDL receptor mutations during the two-year follow-up.

At present, genotype-phenotype studies have focussed on the lipid lowering response to statin therapy instead of analyzing the effects of statins on the atherosclerotic process. In adults, carotid intima-media thickness (IMT) has been accepted as a validated marker for atherosclerosis and future cardiovascular outcome.[13-16] There are clear indications that carotid IMT is a marker of the increased atherosclerotic burden in childhood as well.[17] In a randomized statin trial, we measured both carotid IMT and lipid concentrations and the purpose of the present subgroup analysis was to determine whether LDL receptor genotype influenced the response to pravastatin treatment in children with heterozygous FH.

Methods

Study design

The FH children in the present subgroup analysis were participants in a single center clinical trial carried out in The Netherlands. The study has been described in detail elsewhere.[17,18]

In brief, it was a prospective, randomized, placebo-controlled, double-blind trial to assess the effect of two years of treatment with pravastatin on the carotid IMT in 214 children with heterozygous FH, aged between 8 and 18 years. After consenting, we randomized children to receive pravastatin once daily or matching placebo. In the active treatment group, children younger than 14 years of age received 20 mg pravastatin and in those 14 years and older pravastatin 40 mg was given. We monitored study drug compliance by tablet counting. In the present genotype-phenotype substudy, we included all 193 children whose LDL receptor mutation had been identified (Figure 1). The Institutional Review Board approved the study protocol, and informed consent was obtained from all children and parents.

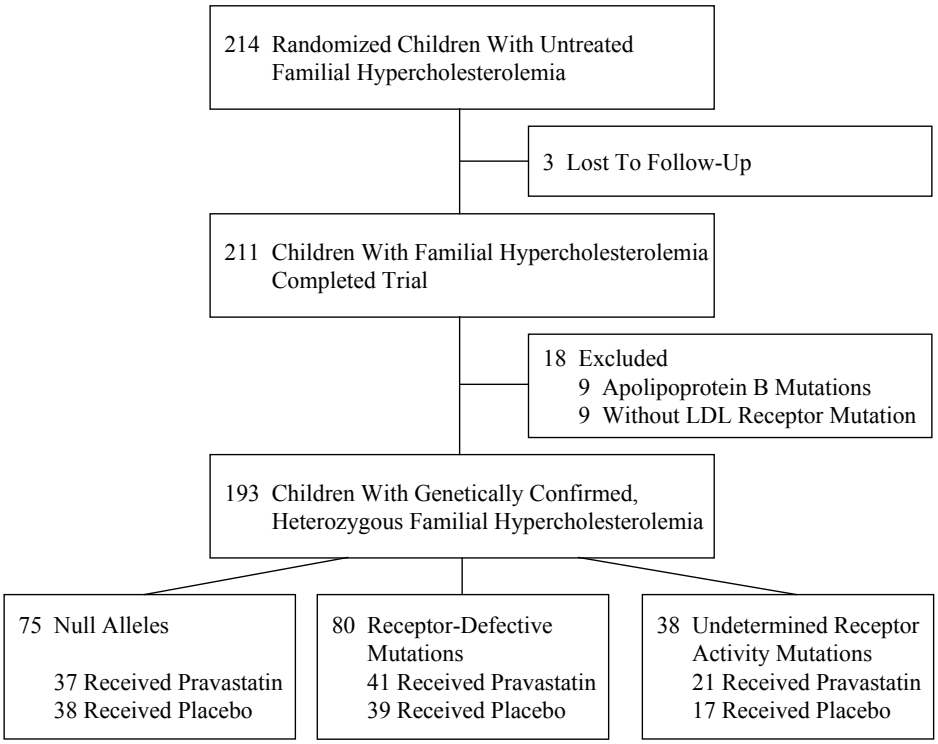

Figure 1. Flow of study participants in the genotype-phenotype substudy.

Type of LDL receptor mutation

We classified the LDL receptor mutations into mutation groups based on their functional class as reported in the literature: *(a)* the receptor-negative mutations or null alleles contained all class 1 mutations, class 2A mutations, large rearrangements (except the 2.5kb deletion which is a class 3 and 5 mutation), mutations resulting in a deletion of the translation initiation signal, and early stop-codons; *(b)* the receptor-defective mutations contained class 2B to 6 mutations; *(c)* the undetermined-receptor-activity mutations contained all remaining mutations with undetermined mutational class. A total of 49 different mutations were

detected in 193 children with heterozygous FH. We found 17 null alleles in 75 children, 14 receptor-defective mutations in 80 children, and 18 mutations with undetermined residual function in 38 children (Figure 1).

Intima-media thickness

The primary efficacy outcome of this substudy was defined as the difference in change from baseline in mean carotid IMT between the placebo and pravastatin group at two years of follow-up compared between null alleles and receptor-defective mutations. A single experienced sonographer performed all B-mode ultrasound examinations. The far walls of the left and right common carotid artery (CCA), carotid bulb (BULB), and internal carotid artery (ICA) were imaged. The digital images were analyzed off-line by one image analyst. For a given segment, IMT was defined as the average of the left and right IMT measurements. Mean carotid IMT was defined as the mean of the CCA, BULB, and ICA far wall segments. The quantitative IMT measurements have been described in detail elsewhere.[17,18]

Laboratory methods

Plasma concentrations of total cholesterol (TC), triglycerides (TG), and high-density lipoprotein (HDL) cholesterol were measured using standard (automated) methods after a 12-hour overnight fast. LDL cholesterol concentrations were calculated by the Friedewald formula.[19] All children had plasma TG concentrations consistently below 4.0 mmol/L. Mutational analyses were performed with standard methods as described previously.[20]

Statistical analysis

All statistical analyses were carried out with SPSS software (version 11.5, SPSS). We compared children with null alleles and receptor-defective mutations for relevant clinical characteristics, lipid concentrations, and carotid IMT. Differences among LDL receptor genotype groups were analyzed with Students' t-test for continuous data. Since TG concentrations had a skewed distribution, the analyses were based on log-transformed data. To examine the relationship between LDL receptor genotype and baseline carotid IMT, independent of lipid concentrations, we used a multivariate linear regression analysis adjusted for initial LDL cholesterol levels.

We have performed a placebo-controlled subgroup analysis to determine the effect of genotype on efficacy of pravastatin treatment. First, we calculated for each participant the change from baseline in carotid IMT and lipid concentrations during the trial. Secondly, we used a linear regression model to analyze the difference between the placebo and pravastatin group in carotid IMT and lipids change during the trial for each genotype group separately (IMT/lipids change during the trial = $\beta_0 + \beta_{\text{treatment group}}$ * treatment group). The $\beta_{\text{treatment group}}$ is the difference between the pravastatin group and the placebo group in IMT/lipids change during the trial. As a result of randomization for the trial and of randomization by nature of the

genotypes, we did not expect differences of age and sex distributions between the genotype groups. Nonetheless, we did perform multivariate linear regression analysis to adjust for age and sex and found similar results (data not shown). Thirdly, the interaction between genotype (null alleles or receptor-defective mutations) and treatment (placebo or pravastatin) was statistically tested with two-way covariance analysis. Since LDL receptor genotype was associated with baseline lipid levels and carotid IMT, we did not adjust for baseline values in multivariate linear regression analysis and two-way covariance analysis. Nonetheless, when we adjusted for baseline values, similar trends were found between LDL receptor genotype and response to treatment (data not shown). Throughout, a two-tailed P value of less than 0.05 was interpreted as indicating a statistical significant difference.

Results

Patient characteristics

The baseline characteristics according to the type of LDL receptor mutation are presented in Table 1. Study drug compliance was similar among the LDL receptor genotype groups. Children with null alleles and receptor-defective mutations were evenly distributed over placebo-treated and pravastatin-treated groups. As expected, children with null alleles had significantly more elevated mean TC levels (difference (± SEM), 0.94 ± 0.20 mmol/L; p<0.001) as well as mean LDL cholesterol levels (difference, 0.97 ± 0.20 mmol/L; p<0.001) compared to children with receptor-defective mutations. Moreover, carriers of null alleles had a greater mean carotid IMT (difference, 0.020 ± 0.01 mm; p=0.01) and mean IMT of the ICA segment (difference, 0.022 ± 0.01 mm; p=0.03) compared to carriers of receptor-defective mutations. Mean IMT of the CCA and BULB segments also tended to be higher in children with null alleles but this was not significant (difference, 0.021 ± 0.01 mm; p=0.06 and difference, 0.017 ± 0.01 mm; p=0.08, respectively). Furthermore, after adjustment for baseline LDL cholesterol levels, the difference in mean carotid IMT between carriers of null alleles and receptor-defective mutations was 0.018 ± 0.01 mm (p=0.04).

Changes in carotid IMT

Figure 2 shows mean differences of the changes in carotid IMT during the trial between the placebo and pravastatin group according to the LDL receptor genotype. The decrease of mean carotid IMT as well as of mean IMT in the CCA, the BULB, and the ICA segments between children who received placebo and pravastatin treatment was not significantly different in carriers of null alleles compared to carriers of receptor-defective mutations (two-way covariance analysis; p=0.7, p=0.4, p=0.3, and p=0.7, respectively). The changes in carotid IMT in Figure 2 were not adjusted for the changes in LDL cholesterol concentrations. Adjustment for changes in LDL cholesterol levels during the trial did not influence the decrease of mean

Table 1. Baseline characteristics of children with null alleles, receptor-defective mutations, and mutations with undetermined receptor activity

Variable	Undetermined receptor activity	Null alleles	Receptor-defective mutations	p-value*
	(n=38)	(n=75)	(n=80)	
Age, years (range)	12.4 (8.0-18.5)	13.4 (8.4-18.0)	12.6 (8.1-18.5)	0.1
Gender, male/female	16 / 22	33 / 42	44 / 36	0.2
BMI, kg/m2	20.3 ± 0.6	19.5 ± 0.4	18.9 ± 0.4	0.3
Study drug compliance, %	88.5 ± 2.3	89.4 ± 1.8	90.2 ± 1.3	0.7
Lipids in mmol/L				
TC	7.98 ± 0.22	8.30 ± 0.16	7.36 ± 0.12	<0.001
LDL cholesterol	6.42 ± 0.21	6.66 ± 0.15	5.70 ± 0.12	<0.001
HDL cholesterol	1.21 ± 0.04	1.23 ± 0.03	1.26 ± 0.03	0.5†
TG	0.78 ± 0.07	0.92 ± 0.06	0.90 ± 0.06	0.9‡
IMT in mm				
CCA	0.520 ± 0.01	0.521 ± 0.01	0.501 ± 0.01	0.06
BULB	0.529 ± 0.08	0.532 ± 0.01	0.515 ± 0.01	0.08
ICA	0.429 ± 0.01	0.455 ± 0.01	0.433 ± 0.01	0.03
mean carotid	0.492 ± 0.01	0.503 ± 0.01	0.483 ± 0.01	0.01
IMT in mm§				
CCA	0.486 ± 0.07	0.510 ± 0.05	0.503 ± 0.05	0.09
BULB	0.549 ± 0.07	0.513 ± 0.05	0.511 ± 0.04	0.2
ICA	0.430 ± 0.07	0.431 ± 0.05	0.421 ± 0.04	0.08
mean carotid	0.489 ± 0.05	0.485 ± 0.04	0.479 ± 0.03	0.04

BMI=body mass index, TC=total cholesterol, LDL=low-density lipoprotein, HDL=high-density lipoprotein, TG=triglyceride, IMT=intima-media thickness, CCA=common carotid artery, BULB=carotid bulb, ICA=internal carotid artery, mean carotid=mean of the CCA, BULB, and ICA. All values are given as mean ± standard error of the mean (SEM).

*Comparison between null alleles and receptor-defective mutations.

†Statistical analysis adjusted for individual serum triglyceride levels did not change the result (data not shown).

‡Statistical testing after logarithmic transformation.

§Carotid IMT adjusted for initial LDL cholesterol levels.

carotid IMT among carriers of null alleles and carriers of receptor-defective mutations: both 0.014 ± 0.01 mm (two-way covariance analysis; p=0.6). However, after two-year treatment, children with null alleles had a consistently greater mean carotid IMT (difference, 0.016 ± 0.01 mm; p=0.02) and mean IMT of the CCA segment (difference, 0.019 ± 0.01 mm; p=0.04) compared to children with receptor-defective mutations. Mean IMT of the BULB and the ICA segments after the treatment period tended to be higher in children with null alleles but this did not reach significance (difference, 0.017 ± 0.01 mm; p=0.07 for both segments).

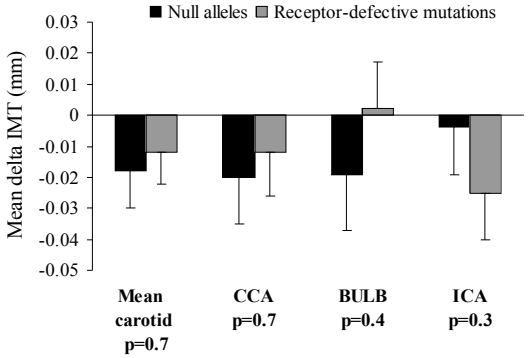

Figure 2. Mean differences of the changes in carotid IMT during the trial between the placebo and pravastatin group in children with null alleles and receptor-defective mutations.

IMT=intima-media thickness, CCA=common carotid artery, BULB=carotid bulb, ICA=internal carotid artery, mean carotid=mean of the CCA, BULB, and ICA. Error bars indicate standard error of the mean difference. P values for the interaction between genotype (null alleles or receptor-defective mutations) and treatment (placebo or pravastatin) were calculated using two-way covariance analysis

Changes in lipid concentrations

Mean differences of the changes in lipid concentrations during the trial between the placebo and pravastatin group according to the LDL receptor genotype are presented in Figure 3. Children with null alleles tended to less reduction of TC and LDL cholesterol levels during the trial compared to children with receptor-defective mutations, but this did not reach statistical significance (two-way covariance analysis; both p=0.08). After two-year treatment, mean TC in carriers of null alleles was 7.62 ± 0.20 mmol/L and mean LDL cholesterol was 5.94 ± 0.19 mmol/L, and remained significantly more elevated than the 6.60 ± 0.17 mmol/L and 4.90 ± 0.17 mmol/L in carriers of receptor-defective mutations (both p<0.001).

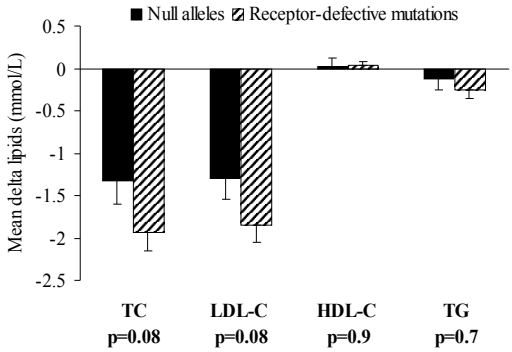

Figure 3. Mean differences of the changes in lipid concentrations during the trial between the placebo and pravastatin group in children with null alleles and receptor-defective mutations.

TC=total cholesterol, LDL=low-density lipoprotein, HDL=high-density lipoprotein, TG=triglyceride. Error bars indicate standard error of the mean difference. P values for the interaction between genotype (null alleles or receptor-defective mutations) and treatment (placebo or pravastatin) were calculated using two-way covariance analysis

Pravastatin increased HDL cholesterol levels and reduced TG levels to a similar extent in both LDL receptor genotype groups (two-way covariance analysis; p=0.9 and p=0.7, respectively).

Discussion

In this subgroup study of a randomized, placebo-controlled, two-year trial with pravastatin in heterozygous FH children, we showed that LDL receptor genotype was significantly associated with the carotid IMT, independent of LDL cholesterol levels. Although the reduction of LDL cholesterol levels by pravastatin treatment tended to be less in carriers of null alleles, we observed no significant difference in change of carotid IMT during the trial between the two LDL receptor genotype groups. However, at baseline and after two-year treatment, carotid IMT and lipid profile were more unfavorable in children with null alleles compared to children with receptor-defective mutations.

Our present analysis is the first genotype-phenotype study in FH that demonstrated the influence of statin therapy on carotid IMT. In adults, numerous studies have shown that an increase in IMT of the carotid artery is associated with an increased risk of myocardial infarction and stroke, and that a decrease in carotid IMT due to drug treatment is associated with a decrease in the incidence of vascular events.[13-16] Therefore, noninvasive B-mode carotid IMT has now been accepted as a validated marker for the process of atherosclerosis in adults. There are clear indications that carotid IMT is a marker for atherosclerosis in childhood as well. Children with FH have a 5-fold more rapid increase of carotid arterial wall IMT during childhood than their unaffected siblings.[17] This increase led to a significant deviation in terms of IMT values from the age of 12 years onwards. Therefore, it might be suggested to measure carotid IMT as a marker of the increased atherosclerotic burden in children.

In this placebo-controlled trial, the response to pravastatin treatment on carotid IMT was not significantly different between the two genotype groups. Nonetheless, carriers of null alleles had a greater carotid IMT than carriers of receptor-defective mutations at baseline and this unfavorable difference was largely maintained during treatment. After correction for LDL cholesterol levels, the differences in carotid IMT between the LDL receptor genotype groups became smaller and less significant. The lower significance may partly be based on loss of statistical power as the result of an additional co-variable (LDL cholesterol) in the multivariate analysis. The small decrease of the difference in carotid IMT between the genotype groups after correction for LDL cholesterol levels, however, suggest that the greater carotid IMT in carriers of null alleles was partly but not solely the result of higher LDL cholesterol levels. Knowledge of LDL receptor genotype may therefore improve clinical decision-making: untreated *and* treated children carrying null alleles exhibit a more increased risk of cardiovascular disease that may be partially independent of their more increased LDL cholesterol levels. Carriers of null alleles may have to a certain extent irreversible atherosclerosis, but hopefully

they just need more aggressive statin treatment. Clearly, the carotid IMT of children with null alleles was reduced during pravastatin treatment. Unfortunately, we could not assess the relationship between LDL receptor genotype and responses to increasing doses of statins in our substudy. In future research, the effect of more aggressive and earlier statin treatment in children with null alleles should be investigated. Studies on efficacy and safety in FH children of stronger statins and high dosages are starting and some are ongoing. In addition to statin treatment, a healthy lifestyle should be advised, because our results suggest that cholesterol independent mechanisms affect the carotid IMT and more aggressive lipid lowering may have a disappointing effect especially in carriers of null alleles.

In the present study, the reductions of TC and LDL cholesterol levels during pravastatin treatment were not significantly different in the two genotype groups. Previous genotype-phenotype studies in adult FH patients have yielded conflicting results.[3,5-11] In a recent study, we showed that children with FH are better suited for genotype-phenotype analysis than adults.[12] In a linear mixed model, we calculated the contribution of familial factors to the variance of LDL cholesterol levels in a pediatric and an adult FH cohort (intra-class correlation). Familial factors explained 50.4% of the variance in LDL cholesterol levels among the FH children and only 9.5% in adult FH patients. Hence, the LDL cholesterol levels showed a much stronger correlation among related children than in adult siblings. Likely, children have a lower chronic exposure to known environmental factors that alter lipid levels. Moreover, we used placebo-controlled data and, therefore, information was available about the natural course of the specific LDL receptor mutation on carotid IMT and lipid profile during the two-year follow-up. Adjusting for the natural course reduces bias in the analyses of the relationship between LDL receptor genotype and treatment response: placebo effect and secular trends did not influence our observations.

Our subgroup analysis had not enough power to observe small differences in the carotid IMT responses between the two LDL receptor genotype groups with significance. The question arises whether or not we have made a type II error: a difference is not observed with statistical significance because of lack of power due to small numbers. However, we did not find a difference between the point-estimates. Recently, Schultz and Grimes emphasized that methodological rigor to eliminate bias, properly report to avoid misinterpretation, and always publish results to avert publication bias is more important than insufficient sample size.[21] Moreover, the methodological advantages enable that such analyses could ultimately be combined in meta-analysis. We have estimated that a meta-analysis to test the results of this hypothesis-generating study should be considered when 2600 or more children have been included in statin IMT trials. Future studies on genotype effects should also maintain the placebo-controlled data of the trial to enable such a meta-analysis.

In summary, we conclude that LDL receptor genotype was significantly associated with the carotid IMT before and during treatment with pravastatin in heterozygous FH children, independent of LDL cholesterol levels. Selection of null alleles identifies children with the

highest cardiovascular disease risk who may benefit by more aggressive as well as earlier lipid lowering treatment.

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CHAPTER 4

CFH Y402H decreases cardiovascular disease risk in patients with familial hypercholesterolemia

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Abstract

Background: The Y402H polymorphism of complement factor H (CFH) has been strongly and consistently related with increased risk of age-related macular degeneration (AMD). Previous work has suggests that AMD and atherosclerosis share similar pathogenic pathways. However, association studies of the CFH variant with cardiovascular disease (CVD) yielded conflicting results, probably because they were underpowered.

Methodology and Findings: We determined whether or not the Y402H polymorphism influenced CVD risk in a multicenter cohort study involving 2016 unrelated patients with familial hypercholesterolemia (FH), who have an extremely increased susceptibility to CVD. We identified 261 individuals who were homozygous for the polymorphism (CC genotype; 12.9%), 929 individuals who were heterozygous (TC genotype; 46.1%), and 826 individuals carried the wild type (TT genotype; 41.0%). During 95,115 person years, 644 patients had a cardiovascular event. Carriers of the CC genotype had a decreased risk of CVD (relative risk 0.67, 95% confidence interval 0.51 – 0.87; p = 0.003) relative to the other genotype groups. This association was unaltered after adjustment for clinically relevant cardiovascular risk factors or age effects. Separate analyses for coronary, cerebral, and peripheral artery disease revealed similar decreased risk estimates for the CC genotype.

Conclusion: Among patients with FH, the Y402H CFH variant was associated with less susceptibility to CVD. This suggests that the inconsistencies about this relationship are the result of context dependent expression. The studies with significant findings are in balance and it cannot be excluded that it were all chance findings. Therefore, adequately powered replication studies should be performed to dissect the association with CVD.

Introduction

Age-related macular degeneration (AMD) is a progressive late-onset disease affecting central vision and is the leading cause of irreversible blindness in the elderly Western population.[1] Recently, three independent genomic studies identified the Y402H (rs 1061170) polymorphism of complement factor H (CFH) that may account for almost half of all AMD cases.[2-4] Several replication studies established the association of this CFH variant with increased risk of AMD in different ethnic populations and different clinical subtypes of the disease.[5-10]

Epidemiologic, genetic, and pathological evidence suggest that similar pathways may be involved in the etiologies of AMD and atherosclerosis.[11] Atherosclerosis is a chronic inflammatory disorder and its initiation and progression are thought to be influenced by activation of the complement system.[12,13] The complement system is the innate component of the immune response and consists of a cascade of plasma and membrane-bound proteins that *(i)* protects the host from invading microorganisms, *(ii)* removes debris and damaged tissues, and *(iii)* enhances cell mediated inflammatory reactions.[14] A number of studies have convincingly shown that, although complement activation is nearly absent in normal arteries, it is extensively activated in atherosclerotic lesions.[15-17]

During complement activation, host cells are protected from being damaged as innocent bystanders by regulatory proteins, including CFH.[18] Immunohistochemical studies have detected CFH in early human coronary artery lesions.[19] Interestingly, CFH was exclusively located in the superficial layer of the arterial intima, suggesting that it may protect the arterial wall from damage by excess complement activation. Several variants in the CFH gene have been identified, but the Y402H polymorphism is of particular interest because it is located within the binding site for heparin and C-reactive protein.[18] It is proposed that the replacement of tyrosine for histidine at position 402 in exon 9 may affect the binding properties of CFH on host surfaces, potentially influencing complement activation, immune responses, and inflammation.[20]

To date, three studies have been conducted on potential associations between the Y402H polymorphism and cardiovascular disease (CVD).[21-23] In contrast with the strong and consistent findings in AMD, however, they have yielded conflicting results. This inconsistency may be due to heterogeneity in the cardiovascular phenotype and variations in population and study design. An alternative reason for these variable findings, though, is false negative results because subsequent studies were underpowered. Consequently, we postulate that the association between this CFH variant and CVD should be assessed in a population at increased risk of CVD with the matching high power to detect potential small effects of the genetic modifier.

Patients with heterozygous familial hypercholesterolemia (FH) have severely elevated low-density lipoprotein (LDL) cholesterol levels and as a result they belong to those at highest risk of CVD.[24] The atherosclerotic burden of FH shows, however, large variation and many

untreated patients reach a normal life span. [25] The disorder is considered to be an exemplary model to analyze secondary (or modifier) genes as well as environmental factors involved in CVD.[26]

We hypothesized that the Y402H polymorphism in CFH influences susceptibility to CVD. Therefore, we assessed the relationship between this CFH variant and risk of CVD in a large cohort of patients with heterozygous FH. Furthermore, we determined the influence of the polymorphism on the risk of coronary, cerebral, and peripheral arterial events separately.

Methods

Study design and patients

The study population and data collection of the FH cohort have been described in detail elsewhere.[27] In brief, lipid clinics throughout The Netherlands submitted DNA samples of clinically suspected patients with FH to a central laboratory for LDL receptor mutation analysis. From this database, we randomly selected a cohort of 4000 out of 9300 patients, who were collected between 1989 and 2002. We excluded subjects with secondary causes of hypercholesterolemia and those with hypercholesterolemia caused by other genetic defects, such as familial defective apolipoprotein B. A total of 2400 unrelated patients, aged 18 years and older, fulfilled the diagnostic criteria for FH as they were previously published.[27] DNA was available of 2016 patients with heterozygous FH for the present analyses. Of these patients, over 99% were Caucasian and 1055 (52.3%) individuals had a documented LDL receptor mutation. The Institutional Review Board of each participating hospital approved the study protocol and informed consent was obtained from all patients.

We assessed cardiovascular risk factors using the following definitions. Smoking was defined as ever smoking. Hypertension was defined as systolic blood pressure >140 mmHg or diastolic blood pressure >90 mmHg at three consecutive office visits. Patients on antihypertensive medication were only added to the hypertension group, when a documented diagnosis was available. Diabetes mellitus was defined as patients using anti-diabetic medication or fasting plasma glucose >6.9 mmol/L. Lipid levels were determined in fasting patients not using lipid-lowering medication for at least 6 weeks. Total cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides were measured by standard methods. LDL cholesterol was calculated with the Friedewald equation.

Cardiovascular disease

The primary clinical endpoint of the present genetic association study was CVD, defined as fatal or non-fatal CVD using internationally accepted criteria. Cardiovascular disease was defined as: (I) myocardial infarction, proven by at least two of the following: (a) classical symptoms >15 minutes, (b) specific electrocardiographic abnormalities, (c) elevated cardiac

enzymes (>2 times upper limit of normal); (II) percutaneous coronary intervention or other invasive procedures; (III) coronary artery bypass grafting; (IV) angina pectoris, defined 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, (d) a more than 70 percent stenosis on a coronary angiogram; (V) ischaemic stroke, demonstrated by CT- or MRI scan; (VI) documented transient ischaemic attack; (VII) peripheral arterial bypass grafting; (VIII) peripheral percutaneous transluminal angioplasty or other percutaneous invasive intervention; (IX) intermittent claudication, defined as classical symptoms with at least one unequivocal result of one of the following: (a) ankle/arm index <0.9 or (b) a stenosis >50 percent on an angiogram or duplex scan. Secondary endpoints of the study were coronary heart disease, cerebral artery disease, and peripheral artery disease. Coronary heart disease was defined as: *(i)* myocardial infarction, *(ii)* percutaneous coronary intervention or other invasive procedures, *(iii)* coronary artery bypass grafting, and *(iv)* angina pectoris. Cerebral artery disease was defined as: *(i)* ischaemic stroke and *(ii)* documented transient ischaemic attack. Peripheral artery disease was defined as: *(i)* peripheral arterial bypass grafting, *(ii)* peripheral percutaneous transluminal angioplasty or other percutaneous invasive intervention, and *(iii)* intermittent claudication.

Molecular analysis

Genomic DNA was extracted from peripheral blood leukocytes according to standard protocols.[28] Considering the concordance of previous association studies for CFH Y402H in AMD populations, we investigated specifically this polymorphism. The Y402H polymorphism in CFH was detected with molecular analysis of the CFH gene with the standard protocol of the 7900HT Taqman (Applied Biosystems, Foster City, USA). Two oligonucleotide primers were used to identify the replacement of thymine (T) by cytosine (C) on position 1277.[2]

Statistical analyses

All data were analyzed using SPSS for Windows software package version 11.5.0 (SPSS Inc., Chicago, IL, USA). Deviations of the genotype distribution from that expected for a population in Hardy-Weinberg equilibrium were tested using chi-square statistics with one degree of freedom. Genotype and allele frequencies among patients with and without history of CVD were compared with the chi-square test. Differences between CFH genotypes were tested with chi-square statistics for dichotomous variables and independent sample t-test for continuous variables. If dependent variables were not normally distributed, logarithmic transformations were applied. To adjust statistical tests for age and gender, we used multiple linear regression analysis.

Cox proportional hazard regression analysis was used to determine the association between the CFH variant and the occurrence of CVD. 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. Follow-up started at birth and ended at the first occurrence of established fatal or non-fatal CVD. Patients without CVD were censored at the date of the last lipid clinic visit or at the date of death attributable to other causes. CFH genotype was analysed according to an additive, dominant or recessive genetic model, respectively. The recessive genetic model (homozygous carriers relative to heterozygous and wild type carriers) had the highest log-likelihood and was used in the present analyses (data not shown). The cumulative CVD free survival, without adjustment for co-variables, was illustrated with the Kaplan-Meier method. Initially, we adjusted in the multiple Cox regression analysis for variables that are independent of the polymorphism: year of birth, gender, and smoking. Additionally, adjustment was made for all other variables that were significant risk factors of CVD in Cox regression analysis. The presence of the homozygous CFH variant in young patients, who did not yet express their high cardiovascular risk, could potentially have led to lower risk estimates. Therefore, we tested the effect of age by separate analyses of age tertiles and by adjusting for age tertiles in the Cox regression analysis. Statistical significance was assessed at the 5% level.

Results

Genotype and allele frequencies

We observed homozygosity for the Y402H polymorphism in 261 (CC genotype; 12.9%) patients, heterozygosity in 929 (TC genotype; 46.1%) patients, and 826 (TT genotype; 41.0%) patients carried the wild type. Genotype distributions in the total cohort and in the patients with and without history of CVD were in Hardy-Weinberg equilibrium ($p>0.18$).

To compare our results with previous case-control studies, we reported all genotype and allele frequencies in Table 1. The distribution of CFH genotypes was significantly different between patients with and without history of CVD ($p=0.003$) as a result of higher frequency of the CC genotype in patients without history of CVD ($p=0.001$).

Table 1. Genotype and allele frequencies for the T→C variation in CFH gene coding for Y402H variant

Genotype or Allele	Patients with CVD N (frequency)	Patients without CVD N (frequency)
TT	270 (0.419)	556 (0.405)
TC	314 (0.488)	615 (0.448)
CC	60 (0.093)	201 (0.147)
T allele	854 (0.663)	1727 (0.629)
C allele	434 (0.337)	1017 (0.371)

CFH denotes complement factor H; CVD denotes history of cardiovascular disease.

Table 2. Characteristics of 2016 patients with familial hypercholesterolemia according to the genotypes of the CFH Y402H polymorphism

Characteristic	TT genotype (n=826)	TC genotype (n=929)	CC genotype (n=261)	p-value*
Age at first lipid clinic visit (years)	45.2 (± 0.4)	44.7 (± 0.4)	43.9 (± 0.8)	0.2
Age at last lipid clinic visit (years)	50.3 (± 0.4)	49.8 (± 0.4)	48.9 (±0.9)	0.2
Males (%)	47.3 (± 1.7)	48.5 (± 1.6)	44.8 (± 3.1)	0.3
Smoking, ever (%)	73.5 (± 1.6)	74.1 (± 1.5)	67.5 (± 3.0)	0.05†
Hypertension (%)	9.9 (± 1.0)	8.4 (± 0.9)	7.7 (± 1.7)	0.7†
Diabetes (%)	5.6 (± 0.8)	6.2 (± 0.8)	5.4 (± 1.4)	0.9†
Body mass index (kg/m2)	25.1 (± 0.1)	25.1 (± 0.1)	24.9 (± 0.2)	0.5†
Total cholesterol (mmol/L)	9.44 (± 0.07)	9.60 (± 0.07)	9.47 (± 0.13)	0.8†
Low-density lipoprotein cholesterol (mmol/L)	7.30 (± 0.08)	7.40 (± 0.07)	7.42 (± 0.13)	0.6†
High-density lipoprotein cholesterol (mmol/L)	1.22 (± 0.01)	1.23 (± 0.01)	1.19 (± 0.02)	0.07†
Triglycerides (mmol/L)	1.80 (± 0.04)	1.81 (± 0.04)	1.66 (± 0.07)	0.02†‡

CFH denotes complement factor H.

Values are given as means ± standard error of the mean (SEM).

*Comparison between the CC genotype and other genotypes.

†Adjusted for age and gender.

‡Statistical testing after logarithmic transformation.

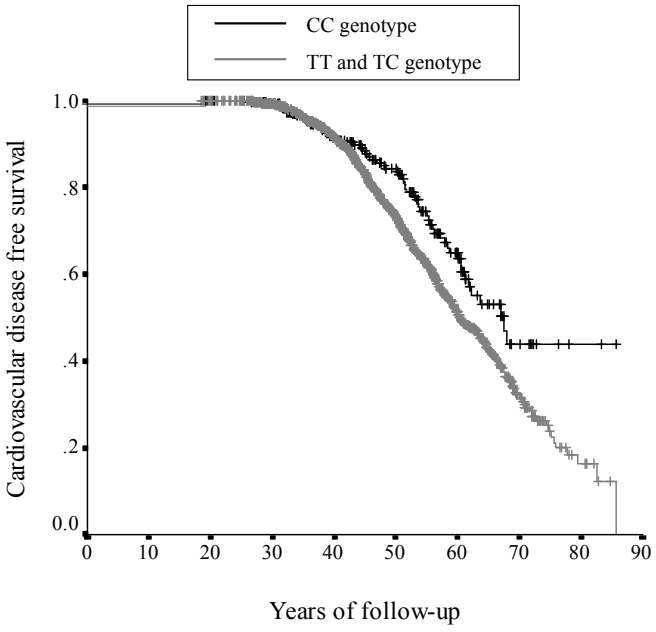

Figure 1. Kaplan-Meier curves for cardiovascular disease free survival among familial hypercholesterolemia patients with the CC genotype and TT/TC genotypes of complement factor H gene. Carriers of the CC genotype had a decreased risk of CVD compared with carriers of other genotypes (RR 0.67, 95% CI 0.51 – 0.87; p=0.003).

Table 3. Relative risk of cardiovascular disease estimated with Cox regression analyses in 2016 patients with familial hypercholesterolemia

Variables	Relative Risk (95% CI)		p-value
Analyses of single variables			
Year of birth	1.07	(1.06 to 1.08)	<0.001
Males	2.96	(2.51 to 3.48)	<0.001
Smoking	1.90	(1.54 to 2.34)	<0.001
Diabetes mellitus	1.31	(1.03 to 1.66)	0.03
Hypertension	1.13	(0.91 to 1.40)	0.3
Body mass index	1.03	(1.00 to 1.06)	0.03
Low-density lipoprotein cholesterol	0.97	(0.92 to 1.01)	0.1
High-density lipoprotein cholesterol	0.37	(0.27 to 0.49)	<0.001
Triglycerides	1.23	(1.08 to 1.39)	0.001
CC genotype of CFH gene	0.67	(0.51 to 0.87)	0.003
Analyses of CC genotype of CFH gene adjusted for			
Year of birth, gender, and smoking	0.68	(0.51 to 0.89)	0.005
Year of birth, gender, smoking, diabetes, body mass index, high-density lipoprotein cholesterol, triglycerides	0.54	(0.36 to 0.79)	0.002
Analyses of CC genotype of CFH gene and age tertiles*			
19 – 43 years	1.12	(0.57 to 2.18)	0.7
44 – 55 years	0.50	(0.29 to 0.86)	0.01
56 – 86 years	0.74	(0.51 to 1.06)	0.1

CFH denotes complement factor H.

*Multiple Cox regression analyses of CC genotype of CFH gene adjusted for year of birth, gender, and smoking according to the age tertiles in the cohort.

Characteristics of patients

Comparisons of general characteristics among the genotypes are presented in Table 2. Patients with the CC genotype had significant lower plasma triglyceride levels (difference (±SEM), 0.14 ± 0.07 mmol/L; p=0.01). We found no significant differences in demographics, the presence of smokers, hypertension, and diabetes mellitus, body mass index (BMI), and other plasma lipid concentrations between the CC genotype and other genotypes.

CFH genotype and CVD

During 95,115 person years, 644 patients had their first cardiovascular event. Mean age of onset of CVD was 48.5 ± 10.7 years. In Figure 1, the Kaplan-Meier curves show the CVD free survival of patients with the CC genotype and TT/TC genotype. Without adjustment for co-variables, carriers of the CC genotype had a decreased risk of CVD compared with carriers of other genotypes (RR 0.67, 95% CI 0.51 – 0.87; p=0.003). As shown in Table 3, year of birth, male sex, smoking, the presence of diabetes, higher BMI, lower levels of HDL cholesterol, and higher triglyceride levels were significantly associated with an increased cardiovascular risk

in univariate analyses. In Cox regression analysis with adjustment for year of birth, gender, and smoking, the CC genotype was significantly associated with 0.68 decreased risk of CVD (p=0.005; Table 3). Additional adjustment for other risk factors did not change the effect of CC genotype on the decreased susceptibility to CVD in our population (p=0.002). Furthermore, we found similar results among men and women (data not shown).

Age effects

The average (± SD) age at end of follow-up of patients without CVD was 46.6 ± 12.8 years and this was significantly lower than the mean age (48.5 ± 10.7 years) at onset of CVD in the other group. The presence of the homozygous CFH variant in young patients, who did not yet express their high cardiovascular risk, could potentially have led to lower risk estimates. To test for effects of the CC genotype on CVD risk with age, we stratified the cohort by age tertiles and performed separate Cox regression analyses in these three age strata. The results of these analyses are shown in Table 3. In the lower age tertile, the CC genotype was not associated with CVD. The point-estimates of the relative risks in the two upper age tertiles were similar to the findings in the total cohort. Actually, we may have underestimated the cardioprotective effect of the CC genotype in our total cohort as a result of the presence of young patients. Therefore, we performed an analyses restricted to the upper two age tertiles adjusting for year of birth, gender, and smoking and found a relative risk of 0.63 (95% CI, 0.47 to 0.86; p=0.003). Finally, we performed a Cox regression analysis with adjustment for year of birth, gender, smoking, and age tertiles: patients with the CC genotype had 0.68 (95% CI, 0.52 to 0.89; p=0.006) times less frequent CVD relative to patients with the other genotypes.

CFH genotype and secondary outcomes

As expected, coronary heart disease was the most prevalent cardiovascular event in the population: in 549 (85.2%) of the FH patients with a history of CVD the first event was coronary heart disease, in 39 (6.1%) patients the first event was cerebral artery disease, and peripheral artery disease was the first event in 56 (8.7%) patients. The relationship between the CC genotype of the CFH gene and these three major cardiovascular events has been shown in Figure 2. The CC genotype was significantly associated with decreased risk of coronary heart disease (RR 0.69, 95% CI 0.52 – 0.93; p=0.01). Although we found no significant association between the CC genotype and other major cardiovascular events, probably as a result of lower prevalence in our cohort, the relative risk of carriers with the CC genotype was 0.39 for cerebral artery disease (95% CI, 0.14 – 1.06; p=0.07) and 0.69 for peripheral artery disease (95% CI, 0.33 – 1.42; p=0.3).

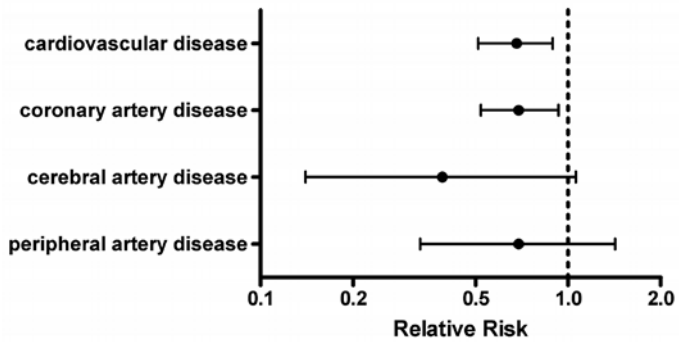

Figure 2. Effects of CC genotype of complement factor H gene on cardiovascular disease, coronary heart disease, cerebral artery disease, and peripheral artery disease in patients with familial hypercholesterolemia.

Discussion

In this large, multicenter cohort study of high-risk patients with heterozygous FH, we demonstrated that the CC genotype of the CFH gene, present in ~13% of the population, was significantly associated with a 2-fold decreased risk of CVD. Adjustment for clinically relevant cardiovascular risk factors and age effects did not influence the effect. We also analyzed the risk of coronary, cerebral, and peripheral events separately and found similar decreased relative risks in carriers of the CC genotype.

Atherosclerosis is characterized by a strong inflammatory component.[12] Data is accumulating that activation of the complement system is an important link between inflammation and atherogenesis.[13] Complement activation can arise through the classical, alternative, or lectin pathway.[14] All three pathways initiate activation of a C3 convertase enzyme which leads to the production of C3a and C3b and then to the terminal C5b-9 membrane attack complex, causing cell lysis. Complement activation is nearly absent in normal arteries, but is considerably active in atherosclerotic lesions as demonstrated by C5b-9 depositions in atherosclerotic plaques;[15] the extent of deposition was correlated with the severity of the lesion.[16] Furthermore, the expression of messenger RNA for complement proteins was up regulated in vascular cells of atherosclerotic lesions,[17] and elevated levels of activated complement products were found in plasma and atherosclerotic plaques of patients with myocardial infarction.[29]

During complement activation, host cells are protected from being damaged by CFH. CFH modulates the complement cascade initiated by the alternative pathway: it inhibits the activation of C3 to C3a and C3b and inactivates existing C3b, thereby preventing uncontrolled complement activation.[18] CFH was also present in human coronary artery lesions but was exclusively located in the superficial layer of the arterial intima, suggesting that CFH may protect the arterial wall from damage by excess complement activation.[19]

CFH is encoded by a member of the Regulator of Complement Activation (RCA) gene cluster, a group of closely linked genes located on chromosome 1q32 coding for several of the

complement regulatory proteins. Mutations in the CFH gene are extremely rare and are associated with renal diseases like glomerulonephritis and atypical hemolytic uremic syndrome. A large number of polymorphisms have been identified in CFH, but their potential influence on the level of expression or function of CFH are unknown.[19] Interestingly, the Y402H polymorphism is located within the binding site for heparin and C-reactive protein. Recently, it has been demonstrated that the wild type and the 402H variant differentially recognize heparin in vitro.[30] This functional alteration may affect binding of CFH to the proteoglycan layer on the arterial intima, potentially influencing complement activation and inflammation in the atherosclerotic plaque.

Three previous studies also assessed the relationship between this CFH variant and cardiovascular events, but they have yielded conflicting results.[21-23] In the Physicians' Health Study cohort, no association of the CFH Y402H polymorphism with risk of incident myocardial infarction and ischaemic stroke was observed.[21] In addition, the authors examined the relationship between the CFH variant and plasma C-reactive protein levels in a subpopulation. Although they found no significant association, the C-reactive protein concentrations were lowest in individuals with the CC genotype (median mg/L: TT, 1.39; TC, 1.10; CC, 1.00). In the second case-control study, Goverdhan et al. reported no significant association between the CFH variant and coronary artery disease.[22] In contrast, in the Rotterdam Study, a prospective population-based cohort study in the elderly, carriers of the CC genotype had a hazard ratio of 1.77 (95% CI 1.23 to 2.55) for myocardial infarction.[23] All these previous studies, however, were performed in populations that were on average 20 years older than individuals in our study. Our patients had much more severely increased LDL cholesterol levels: such values were hardly or not present at all in the other studies. Moreover, most studies were underpowered to detect the modest effects that are expected for genetic susceptibility for a complex disorder such as CVD.

The strength of the present study lies in the availability of a well-documented, large cohort of patients with severe hypercholesterolemia resulting in a 8.5 times increased CVD risk.[24] However, the risk shows considerable variation among patients with FH: 40% of untreated patients reach a normal life span whereas excess mortality caused by CVD occurs in the remaining 60%.[25] Clearly secondary genetic factors and life style determine the burden from this monogenic disorder.[31] The monogenic background and the large variation of CVD risk offer a unique opportunity to analyze secondary genes involved in CVD.[26]

A limitation of our study is that the influence of the Y402H polymorphism on potential intermediate traits such as C-reactive protein levels and other inflammation markers could not be determined. In addition, we were unable to replicate our findings in a second independent sample of patients with FH. To our knowledge, however, the present FH cohort is the only one with well-documented clinical outcome measures. The cardioprotective effect of the CFH variant may be restricted to hypercholesterolemic patients. Furthermore, association studies would ideally be performed using haplotype-based analysis in stead of single

nucleotide polymorphism-based analysis. The CFH Y402H polymorphism, however, was the only genetic variant consistently associated with AMD in previous reports and therefore we restricted our analysis to this polymorphism. An advantage of this single nucleotide polymorphism-based approach is that we had no problems regarding multiple testing, as is often the case in genetic association studies.

Interestingly, individuals with the Y402H polymorphism in CFH have an increased susceptibility to AMD,[2-10] but homozygous carriers of the CFH variant had a decreased risk of CVD in our FH subjects. Differences in angiogenesis may be a possible explanation for this opposite effects of the CFH variant in cardiovascular disease and AMD. Insufficient collateral blood vessel formation increases the burden of ischemic vascular diseases,[32] whereas neovascularisation clearly increases the severity of AMD.[1] It is, therefore, tempting to hypothesize that CFH is involved in the process of neovascularisation.

The exact reason for the conflicting results on the polymorphism in CVD research is unknown. The expression of the CFH variant may be context dependent, but it cannot be excluded that the significant results so far were based on chance findings. Obviously, future research is warranted to acquire more detailed characterization of the CFH gene and to establish the functional consequences of the CFH Y402H variant and its impact on complement activation, neovascularisation, and complex diseases such as AMD and atherosclerosis.

In conclusion, this large cohort study shows a significant 2-fold decreased risk of CVD, as well as coronary heart disease, in severe hypercholesterolemic patients homozygous for the CC genotype of CFH gene. The association was not explained by known cardiovascular risk factors such as age, gender, smoking, diabetes, and cholesterol levels, suggesting that the CC genotype is a modifier gene of CVD.

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CHAPTER 5

ABCG8 gene polymorphisms, plasma cholesterol concentrations, and risk of cardiovascular disease in familial hypercholesterolemia

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Submitted

Abstract

Background: Elevated plasma plant sterol concentrations may be a risk factor of cardiovascular disease (CVD). Polymorphisms in the ABCG8 gene have been identified that contribute to the variation in plasma concentrations of plant sterols. However, data on the direct relationship of ABCG8 gene polymorphisms with CVD are lacking.

Methodology and Findings: Therefore, we examined associations between the D19H and T400K polymorphisms in the ABCG8 gene and cardiovascular events in a large, multicenter, cohort study of 2012 patients with heterozygous familial hypercholesterolemia (FH). A total of 253 individuals carried one or two alleles of the D19H variant and 580 individuals the T400K variant. During 94,809 person years, 648 (32.2%) subjects developed CVD. We defined CVD as coronary (n=553), cerebral (n=37), or peripheral artery disease (n=58). In a Cox proportional hazard regression model adjusted for year of birth, gender, and smoking, the D19H polymorphism was not associated with total CVD risk ($p>0.1$). However, after additional adjustment for plasma HDL cholesterol and triglycerides levels there was evidence of an association with higher risk of coronary heart disease (RR 1.40 confidence interval 1.03–1.92; $p=0.03$). We observed no relationship between the T400K polymorphism and cardiovascular endpoints ($p>0.1$).

Conclusion: In conclusion, our data suggest that these genetic variations in the ABCG8 gene are not a major contributor for differences in CVD risk among patients with FH.

Introduction

Increased plasma cholesterol levels are among the most important risk factors of cardiovascular disease (CVD). Cholesterol is the most common sterol of animal origin and its levels are regulated by cholesterol absorption and synthesis. Sterols of plant origin are structurally similar to cholesterol but they cannot be synthesized endogenously and are completely derived from the diet. The major plant sterols, sitosterol and campesterol, are normally present in very low amounts in the blood and are at least 100-fold less abundant than cholesterol.[1]

The accumulation of plant sterols is actively prevented by two members of the adenosine triphosphate binding cassette (ABC) transporter family, ABCG5 and ABCG8.[2,3] Both proteins are expressed in the intestine and in the liver, where they dimerize to form active membrane transporters. They function to limit the intestinal absorption of dietary sterols by effluxing sterols from enterocytes to the small intestinal lumen and promote the excretion of sterols from the liver into the bile. Mutations in either one of the two genes that encode for these transporters cause sitosterolemia. This is a rare autosomal recessive disorder characterized by increased intestinal absorption and markedly impaired biliary secretion of plant sterols.[4] Patients with sitosterolemia have plasma and tissue levels of plant sterols that are >50-fold increased and they develop CVD at a young age.[5,6]

High plasma concentrations of plant sterols have been suggested to increase CVD risk also in nonsitosterolemic subjects. Elevated plasma sitosterol and campesterol levels and/or their ratios to cholesterol have been associated with a personal or family history of CVD in both hypercholesterolemic and normocholesterolemic individuals.[7-10] Moreover, the higher the ratio of sitosterol and campesterol to cholesterol in serum, the higher was their ratio also in the carotid artery wall of individuals undergoing carotid endarterectomy.[11]

Plasma plant sterol concentrations vary over a 5- to 10-fold range among individuals, but are very stable within individuals and they are highly heritable.[12] It was found that common DNA sequence polymorphisms in the ABCG8 gene contributed to the variation in plasma concentrations of plant sterols such as sitosterol and campesterol.[12-14] In addition, ABCG8 gene variants were also associated with plasma total cholesterol and LDL cholesterol levels.[13-16] Although plant sterol concentrations have been related to CVD and common polymorphisms in ABCG8 have been identified that associated with plant sterol levels, the direct influence of ABCG8 polymorphisms on atherosclerotic diseases is yet unknown.

The effect of a single polymorphism on genetic susceptibility for a complex disorder such as CVD is a priori expected to be modest.[17] The association between ABCG8 variants with CVD should, therefore, preferably be examined in a population at increased risk of CVD with the matching high power to detect potential small effects of the genetic variant. Patients with heterozygous familial hypercholesterolemia (FH) have severely elevated plasma low-density lipoprotein (LDL) cholesterol levels, leading to accelerated atherosclerosis and an increased risk of premature CVD.[18,19] Despite the monogenic cause, the cardiovascular morbidity and

mortality of FH shows considerable variation related to both environmental and genetic factors.[20-22] The disorder is considered to be an exemplary model to analyze secondary (or modifier) genes involved in CVD.[23]

We hypothesized that the presence of common polymorphic variants in the ABCG8 gene may effect variation in CVD risk. We therefore examined the relationship between polymorphisms in the ABCG8 gene and plasma cholesterol concentrations as well as susceptibility to CVD in patients heterozygous for FH.

Methods

Study design and study population

The exact selection procedure and data collection of the FH cohort have been previously published.[24] In brief, lipid clinics throughout The Netherlands submit DNA samples from clinically suspected FH patients to a central laboratory for LDL receptor mutation analysis. Between 1989 and 2002, a total of 9300 DNA samples were collected and we randomly selected a cohort of 4000 subjects from this database. FH was diagnosed according to internationally established criteria.[25] A total of 2400 unrelated patients, aged 18 years and older, fulfilled the diagnostic criteria for heterozygous FH. Of these patients, over 99% were Caucasian. The Institutional Review Board of each participating hospital approved the study protocol and informed consent was obtained from all patients.

Data collection

Phenotypic data were obtained by reviewing the patient's medical records by a trained team of data collectors.[26] Smoking was classified as ever having smoked. Body mass index (BMI) was calculated from height and length (kg/m^2). Hypertension was defined as a systolic blood pressure >140 mmHg or a diastolic blood pressure >90 mmHg at three consecutive office visits or patients with a documented diagnosis and using antihypertensive medication. Diabetes mellitus was defined as patients with fasting plasma glucose >6.9 mmol/L or patients using anti-diabetic medication.

All laboratory parameters, as stated in the medical record, were determined in fasting patients not using lipid-lowering medication for at least 6 weeks. Total cholesterol, high-density lipoprotein (HDL) cholesterol and triglyceride levels were measured by standard enzymatic methods. LDL cholesterol concentrations were calculated with the Friedewald formula.

Cardiovascular disease

Primary outcome of the study was fatal or non-fatal CVD. CVD was defined as coronary, cerebral, or peripheral artery disease using internationally accepted criteria as published before.[24] Secondary endpoints of the study were coronary heart disease, cerebral artery disease, and

peripheral artery disease. Coronary heart disease was defined as: *(i)* myocardial infarction, *(ii)* percutaneous coronary intervention or other invasive procedures, *(iii)* coronary artery bypass grafting, and *(iv)* angina pectoris. Cerebral artery disease was defined as: *(i)* ischaemic stroke and *(ii)* documented transient ischaemic attack. Peripheral artery disease was defined as: *(i)* peripheral arterial bypass grafting, *(ii)* peripheral percutaneous transluminal angioplasty or other percutaneous invasive intervention, and *(iii)* intermittent claudication.

Molecular analysis

Genomic DNA was extracted from peripheral blood leukocytes according to a standard protocol.[27] We decided to analyse only those ABCG5 and/or ABCG8 polymorphisms that showed most consistently associations with plasma plant sterol and cholesterol concentrations in earlier studies among Caucasian populations.[12-16] This resulted in the selection of two polymorphisms in ABCG8: D19H (substitution of histidine for aspartic acid at amino acid 19 in exon 1) and T400K (substitution of thyrosine for lysine at amino acid 400 in exon 8). Genotype determination was performed using fluorescence-based assay-by-design allelic discrimination method using Taqman Universal PCR master mix (Applied Biosystems, Foster City, USA), Taqman SNP Genotyping Assays, and a Taqman ABI Prism 7900 Sequence Detection System (Applied Biosystems). Taqman SNP Genotyping Assay ID for the D19H was C_26135643_10 and for the T400K C_375061_10 (Applied Biosystems). Reaction components and amplification parameters were based on the manufacturer's instructions using an annealing temperature of 60°. Results were scored blinded for CVD status. From the 2400 heterozygous FH patients who comprised the study cohort, complete ABCG8 genotypes were available of 2012 individuals.

Statistical analysis

All data were analyzed using SPSS for Windows software package version 11.5.0 (SPSS Inc., Chicago, IL, USA). Genotype and allele frequencies were compared with values predicted by Hardy-Weinberg equilibrium using the chi-square test. Because of the limited number of individuals homozygous for the ABCG8 polymorphisms, heterozygous and homozygous carriers of the minor allele were combined in the analyses. Chi-square test was applied to evaluate differences in genotype distributions between individuals without and with a history of CVD. Differences in plasma cholesterol concentrations and patient characteristics among ABCG8 genotypes were analyzed with chi-square statistics. Statistical testing of triglyceride concentrations was performed after logarithmic transformation because of its skewed distribution. Multiple linear regression analysis was used to adjust statistical tests for the effects of age, gender, and smoking.

The association between the D19H and T400K polymorphisms in ABCG8 and the occurrence of fatal or non-fatal CVD was assessed using a Cox proportional hazard regression analysis. Follow-up started at birth and ended at the first occurrence of established fatal or non-fatal

CVD. Patients without CVD were censored at the date of the last lipid clinic visit or at the date of death attributable to other causes than CVD. Initially, we adjusted for variables that are independent of the polymorphisms in ABCG8: year of birth, age, and smoking. Polymorphisms might express their effects via for example hypertension, diabetes mellitus, obesity, or dyslipidemia. To evaluate the influence of the polymorphisms in ABCG8 on intermediate CVD traits, we additionally adjusted for variables that were significant risk factors of CVD in the Cox regression analysis. A two-tailed p-value of less than 0.05 was considered a statistical significant result.

Results

Patient characteristics and genotype frequencies

Characteristics of all 2012 subjects with heterozygous FH are shown in Table 1. Table 2 shows ABCG8 genotype and allele frequencies. For the D19H polymorphism, we observed homozygosity (HH) in 9 (0.4%) patients, heterozygosity (DH) in 244 (11.8%) patients, and 1812 (87.7%) patients were homozygous for the wild type allele (DD). For the T400K polymorphism, 48 (2.3%) patients were homozygous (KK), 532 (25.9%) patients were heterozygous (TK), and in 1473 (71.7%) patients the homozygous wild type allele (TT) was detected. Genotype distributions in the total cohort and in patients without and with a history of CVD were in Hardy-Weinberg equilibrium for both ABCG8 genotypes ($p > 0.2$).

Using chi-square analyses, ABCG8 genotype distributions were similar between individuals without and with a history of CVD (D19H, $p = 0.9$; T400K, $p = 0.1$)

Table 1. Characteristics of 2012 individuals with heterozygous FH

Characteristic	
Age at first lipid clinic visit (years)	44.8 (± 0.3)
Age at last lipid clinic visit (years)	49.8 (± 0.3)
Males (%)	48.7 (± 1.1)
Smoking, ever (%)	73.2 (± 1.0)
Hypertension (%)	9.0 (± 0.6)
Diabetes (%)	5.8 (± 0.5)
Body mass index (kg/m2)	25.1 (± 0.1)
Total cholesterol (mmol/L)	9.50 (± 0.05)
Low-density lipoprotein cholesterol (mmol/L)	7.33 (± 0.05)
High-density lipoprotein cholesterol (mmol/L)	1.22 (± 0.01)
Triglycerides (mmol/L)	1.81 (± 0.03)

Values are given as means ± standard error of the mean (SEM).

Table 2. Genotype and allele distributions for D19H and T400K polymorphisms in ABCG8

	D19H		T400K	
	CVD –	CVD +	CVD –	CVD +
	N (%)	N (%)	N (%)	N (%)
Genotypes				
Wild type	1198 (87.8)	570 (88.0)	965 (70.7)	479 (73.9)
Heterozygous	161 (11.8)	76 (11.7)	372 (27.3)	151 (23.3)
Homozygous	5 (0.4)	2 (0.3)	27 (2.0)	18 (2.8)
Total	1364 (100.0)	648 (100.0)	1364 (100.0)	648 (100.0)
Alleles				
Wild type allele	2557 (93.7)	1216 (93.8)	2302 (84.4)	1109 (85.6)
Minor allele	171 (6.3)	80 (6.2)	426 (15.6)	187 (14.4)
Total	2728 (100.0)	1296 (100.0)	2728 (100.0)	1296 (100.0)

CVD indicates history of cardiovascular disease.

ABCG8 genotypes and plasma cholesterol concentrations

Plasma cholesterol concentrations according to D19H genotypes are illustrated in Figure 1 and among T400K genotypes in Figure 2. We found no significant differences in plasma lipid concentrations among D19H and T400K genotypes. Additional adjustment for year of birth, gender, and smoking did not change the results. Patients homozygous or heterozygous for the T400K polymorphism had significantly less often hypertension compared to carriers of the wild type alleles (7.0% vs. 9.8%; $p = 0.03$). In addition, demographics, gender, BMI, the

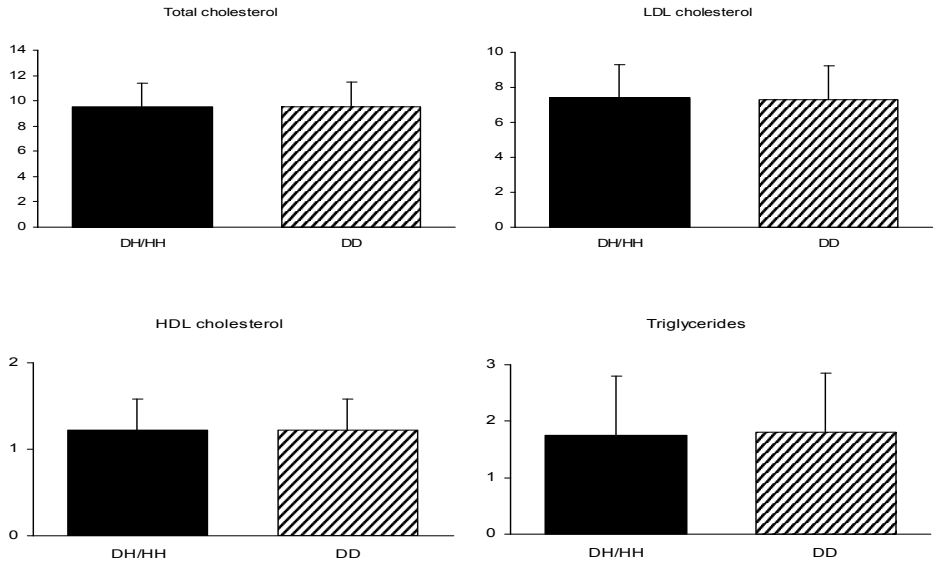

Figure 1. Mean plasma cholesterol concentrations ± standard deviation in the different genotypes of the ABCG8 D19H polymorphism. Cholesterol levels are expressed in mmol/L.

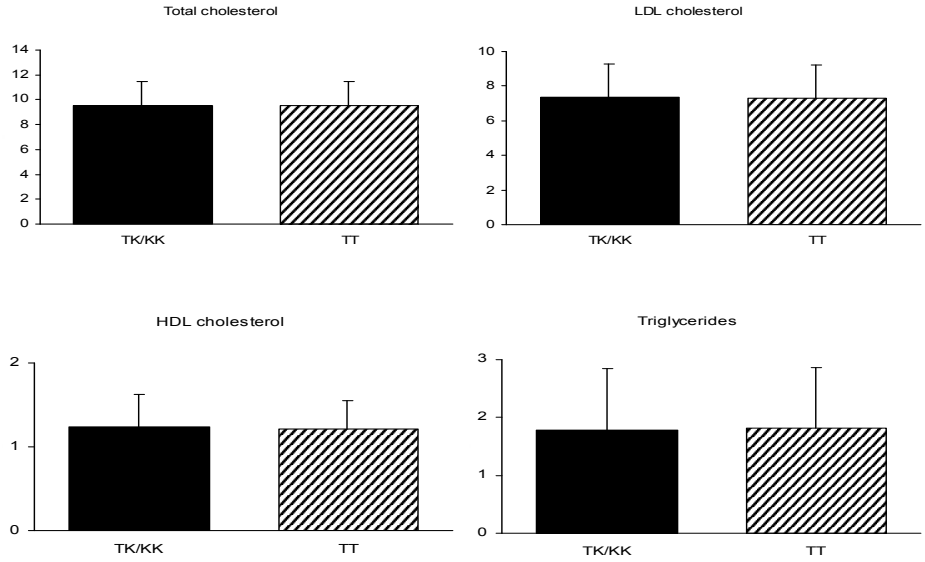

Figure 2. Mean plasma cholesterol concentrations ± standard deviation in the different genotypes of the ABCG8 T400K polymorphism. Cholesterol levels are expressed in mmol/L.

presence of smokers and diabetes mellitus were comparable among the ABCG8 genotype groups (data not shown).

ABCG8 genotypes and risk of CVD

During 94,809 person years, 648 (32.2%) FH individuals had their first cardiovascular event. Mean age of onset of CVD was 48.4 ± 10.9 years. Separate analyses of a number of variables, which may act as confounder or intermediate trait in the relationship between the ABCG8 variants and CVD susceptibility, are presented in Table 3. Year of birth, male gender, smoking, diabetes, lower plasma HDL cholesterol concentrations, and higher plasma triglyceride levels were significantly associated with increased CVD risk. As shown in Table 4, we found no evidence for associations of the D19H or T400K polymorphisms with total CVD risk.

Table 3. Risk factors of cardiovascular disease estimated with Cox regression analyses

Variables	Relative Risk (95% CI)		p-value
Year of birth	1.07	(1.06 to 1.09)	<0.001
Males	3.03	(2.57 to 3.58)	<0.001
Smoking	1.94	(1.57 to 2.39)	<0.001
Diabetes mellitus	1.31	(1.03 to 1.67)	0.03
Hypertension	1.14	(0.92 to 1.41)	0.2
Body mass index	1.02	(0.99 to 1.05)	0.1
Low-density lipoprotein cholesterol	0.96	(0.92 to 1.01)	0.1
High-density lipoprotein cholesterol	0.36	(0.26 to 0.48)	<0.001
Triglycerides	1.22	(1.08 to 1.38)	0.002

Table 4. Relative risk of cardiovascular events estimated with Cox regression analyses in 2012 individuals with FH

	Crude model			Adjusted model		
	Relative risk (95% CI)		p-value	Relative risk (95% CI)		p-value
ABCG8 D19H polymorphism						
Cardiovascular disease	1.13	(0.89 – 1.46)	0.3	1.24	(0.92 – 1.68)	0.2
Coronary heart disease	1.27	(0.98 – 1.64)	0.07	1.40	(1.03 – 1.92)	0.03
ABCG8 T400K polymorphism						
Cardiovascular disease	0.88	(0.73 – 1.05)	0.2	0.87	(0.69 – 1.08)	0.2
Coronary heart disease	0.87	(0.72 – 1.06)	0.2	0.83	(0.66 – 1.05)	0.1

Crude: adjusted for year of birth, gender, and smoking; adjusted: further adjusted for diabetes, plasma HDL cholesterol and triglycerides concentrations.

ABCG8 genotypes and risk of secondary cardiovascular endpoints

Coronary artery disease was the most frequent cardiovascular event: in 553 (85.3%) subjects it was the first event. Cerebral artery disease occurred as the first event in only 37 (5.7%) persons. In 58 (9.0%) patients the first event was peripheral artery disease. The relationship between ABCG8 genotypes and coronary heart disease is shown in Table 4. The D19H polymorphism was associated with increased risk of coronary heart disease, but only after adjustment for year of birth, gender, smoking, diabetes, HDL cholesterol, and triglycerides levels in multiple Cox regression analyses (adjusted model). We found no significant relationship of the D19H variant with susceptibility of cerebral artery disease and peripheral artery disease (data not shown). Nor did we find associations between T400K polymorphism and any secondary cardiovascular endpoint.

The average (± SD) age at end of follow-up of patients without CVD was 46.5 ± 12.7 years and this was significantly lower than the mean age (48.4 ± 10.9 years) at onset of CVD in the other group. Therefore, we tested the effect of age by adjusting for age tertiles in the Cox regression analysis, but this did not essentially change the outcome measures (data not shown).

Discussion

In this large cohort study of high-risk patients with FH, we found that the D19H polymorphism was not associated with plasma cholesterol levels nor with total CVD risk, but there was evidence of an association with higher risk of coronary heart disease. We observed no significant association between the T400K polymorphism and plasma cholesterol levels or cardiovascular endpoints.

Our present analysis is the first study that examined the association between polymorphisms in the ABCG8 gene and risk of CVD. The important role of elevated plant sterol levels in humans is illustrated by sitosterolemia in which plasma levels of plant sterols are increased

>50-fold due to non-function of either the ABCG5 or ABCG8 protein, leading to severe atherosclerotic arterial disease at a young age.[5,6] Although mutations that cause sitosterolemia are extremely rare, more common sequence variants in ABCG8 have been identified that have more subtle effects on plasma plant sterol levels as well as plasma cholesterol concentrations.[12] In addition, several studies have reported that also in nonsitosterolemic subjects elevated plasma concentrations of plant sterols are associated with cardiovascular events, suggesting that plant sterols are atherogenic.[7-10]

Although the D19H polymorphism was not associated with plasma cholesterol levels in our FH cohort, heterozygous and homozygous carriers of this polymorphism had a slightly higher risk of coronary heart disease than carriers of the wild type. Previous studies have consistently demonstrated associations of the 19H allele with lower plasma sitosterol and campesterol concentrations.[12,13] Hence, it was speculated that the substitution of histidine for aspartic acid at amino acid 19 increases the transporter function of ABCG8. The enhanced efflux of sterols from enterocytes to small intestinal lumen and augmented excretion of sterols from the liver into the bile would eventually lead to lower plasma sterol concentrations. However, the effect of this amino acid substitution on ABCG8 function was never directly determined in an *in vitro* assay. Results about the relationship of the D19H polymorphism with plasma cholesterol levels were more contradicting: one study has found an association of the 19H allele with lower plasma total and LDL cholesterol levels,[13] whereas other genetic association studies have failed to confirm this relationship.[12,16]

Based on the association with lower plasma plant sterol concentrations, we had a priori expected that carriers of the D19H variant would have had a decreased risk of cardiovascular events. Despite the clinical phenotype of sitosterolemia and several (relatively small) studies that found an association of elevated plasma plant sterol levels with CVD, other research groups have linked a high plant sterol content of the diet to atheroprotection. In the Asian diet, for example, the main protein is soy (which is rich in plant sterols), and this has been postulated as one of the possible explanations for the lower incidence of CVD in some Asian populations.[28] In addition, adding plant sterols to margarine and other foods is currently used to optimize cardiovascular risk profile by decreasing plasma cholesterol concentrations.[29]

A possible explanation for the lack of a significant association in the primary endpoint total CVD with the D19H polymorphism is that coronary heart disease is probably a more homogeneous phenotype than the combined endpoint CVD. On the other hand, the minor allele frequency of the D19H polymorphism in our FH cohort was 6.2% and the association with coronary heart disease was only significant after correction for a number of cardiovascular risk factors. Post-hoc analyses in such small sub-populations should always be interpreted with caution and our observations should, therefore, be replicated in larger population-based studies.

We found no association between the T400K polymorphism and any cardiovascular event nor with plasma cholesterol concentrations. Previous studies have shown that this

polymorphism was associated with lower sitosterol to cholesterol ratios, but these findings were not as consistent as for the D19H variant.[12-14] In line with our observations, none of these studies found a significant relationship between the T400K variant and plasma cholesterol levels.

Although the D19H variant was associated with a slightly higher risk of coronary heart disease, we have to conclude that these two ABCG8 variants are not major contributors of CVD or coronary heart disease susceptibility in our study. The severe hypercholesterolemia in our patients might offer a clue as to why we observe these results. The potential beneficial effect of these ABCG8 polymorphisms may be partly due to lower total and LDL cholesterol levels.[13-16] We did not observe, however, such differences; the dominant dyslipidemia of FH may outweigh potential beneficial effects on cholesterol metabolism. In the general population, variants in the ABCG8 gene may be better risk predictors for cardiovascular events.

The strengths of our study are that we included a high-risk study population and that we carefully selected our polymorphisms. FH patients have an 8.5 times increased risk of CVD.[19] Although all FH patients have severe hypercholesterolemia, excess mortality caused by CVD occurs in 60% of the untreated patients whereas 40% reach a normal life span.[20] Clearly secondary genetic factors and environmental factors determine CVD risk in FH.[20-22] The monogenic background and the large variation of CVD risk offer a unique opportunity to analyze secondary genes involved in CVD.[23] Moreover, we restricted the number of genetic variants (and thereby the number of null hypotheses) by selecting only the two ABCG8 polymorphisms that showed most consistently associations with plasma plant sterol and cholesterol concentrations in earlier studies among Caucasian populations.[12-16]

Several potential limitations of the present study have to be mentioned. All phenotypic data were retrospectively obtained by reviewing the patient's medical records and certain information of interest, such as dietary habits and plasma plant sterol levels, were not available. Consequently, we could not assess the influence of plasma plant sterol concentrations as a potential intermediate trait in the relationship between ABCG8 polymorphisms and CVD risk. Moreover, we could not replicate our findings in a second independent study population, because to our knowledge this is the only large FH cohort with such well-defined clinical endpoints. Finally, our study population consists of Caucasian individuals with FH, so these data cannot be generalized to other ethnic groups and the general population.

In conclusion, in this large, multicenter, cohort study amongst high-risk individuals with FH, we found a modest effect of the ABCG8 D19H gene variant on risk of coronary heart disease, but we did not observe a relationship between the ABCG8 T400K polymorphism and cardiovascular endpoints. Our data suggest that these two genetic variations in the ABCG8 gene are not major contributors for differences in CVD susceptibility among FH individuals.

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CHAPTER 6

A functional polymorphism in the glucocorticoid receptor gene and its relation to cardiovascular disease risk in familial hypercholesterolemia

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Abstract

Context: Individuals with the functional ER22/23EK variant in the glucocorticoid receptor gene are relatively resistant to the downstream consequences of glucocorticoids. Evidence suggests that carriers have a more favorable cardiovascular risk profile, but the relationship between this ER22/23EK variant and cardiovascular disease has not been hitherto assessed.
Objective: We, therefore, determined whether carriership of the ER22/23EK improves cardiovascular disease risk in patients with severe hypercholesterolemia.
Design, Setting, and Participants: In a multicenter cohort study, 2024 patients with heterozygous familial hypercholesterolemia, aged 18 years and older, were genotyped for the ER22/23EK polymorphism. Patients were identified at lipid clinics throughout the Netherlands between 1989 and 2002.
Main Outcome Measures: The primary outcome measure was cardiovascular disease.
Results: Seventy-six (7.8%) out of 977 men and 72 (6.9%) of 1047 women were carriers of the ER22/23EK variant. A total of 395 men and 247 women had a cardiovascular event. In contrast to expected results, we observed no significant association of the ER22/23EK variant with cardiovascular disease risk (men, RR 0.75, 95% CI 0.50 – 1.14, p = 0.2; women, RR 1.37, 95% CI 0.82 – 2.28, p = 0.2). However, we found a significant interaction between gender and the polymorphism on cardiovascular disease (p = 0.02).
Conclusions: In this large cohort of individuals with very high-risk of cardiovascular disease, the association between the functional ER22/23EK polymorphism and cardiovascular risk was not significant overall, though it varied significantly by gender.

Introduction

Familial hypercholesterolemia (FH) is an autosomal dominant disorder caused by mutations in the low-density lipoprotein (LDL) receptor gene.[1] The prevalence of heterozygotes in Caucasian populations averages about 1:500, positioning FH among the most common monogenetic disorders in man.[2] Patients with FH have severely increased LDL cholesterol levels and a pronounced susceptibility to premature cardiovascular disease (CVD).[1,2] Despite the monogenetic nature of this disorder, however, large variation in CVD risk is observed.[3,4] Age, gender, smoking, body mass index (BMI), and the presence of hypertension and diabetes mellitus contribute to this variation of CVD risk.[5,6] In addition, polymorphisms in candidate genes have also been shown to modulate the expression of the clinical phenotype,[7-15] supporting the concept that the hereditary component of CVD is determined by multiple genes, each explaining only a limited proportion of the risk.[16] The present challenge is to identify those markers that improve risk prediction in FH in order to tailor preventive strategies to the individual risk level.

Variants in the glucocorticoid receptor gene have been associated with a plethora of cardiovascular risk factors.[17] One of these variants consists of two linked, single nucleotide changes in codon 22 and 23 of exon 2. The mutation in codon 22 at nucleotide position 198 does not result in an amino acid change (both coding for a glutamic acid (E)), whereas the other sequence change in codon 23 at nucleotide position 200 causes a change from arginine (R) to lysine (K). Therefore, this variant has been named ER22/23EK. Upon dexamethasone suppression testing, carriers of the ER22/23EK variant express higher serum cortisol concentrations as well as a smaller decrease in cortisol levels, suggesting a relative resistance to glucocorticoids.[18] Recently, the pathophysiological basis of this resistance was elucidated.[19,20] Alternative translation initiation occurs that results in two isoforms of the glucocorticoid receptor: a long (GR-A) and a short (GR-B) isoform.[21] The GR-B protein has stronger gene transcription activating effects.[20] The ER22/23EK polymorphism affects translation, resulting in a shift towards the less active GR-A variant.[19,20] In fact, association studies have shown that carriers have: *(i)* lower plasma total and LDL cholesterol levels; *(ii)* increased insulin sensitivity; *(iii)* beneficial body composition; and *(iv)* lower plasma C-reactive protein levels and a better survival.[18,22,23]

These findings suggest that carriers of this functional ER22/23EK variant in the glucocorticoid receptor gene may have a reduced risk of CVD. Nevertheless, this relationship has not been assessed. In the present study, we therefore investigated the effect of this polymorphism on CVD in patients heterozygous for FH.

Materials and Methods

Study design and study population

Between 1989 and 2002, Lipid Clinics throughout the Netherlands submitted blood samples of 9,300 patients who were clinically suspected for FH to a central laboratory for LDL receptor mutation analysis.[6] Out of this database, we randomly selected 4,000 patients and diagnosed FH according to previously published criteria.[6] We excluded subjects with secondary causes of hypercholesterolemia and those with hypercholesterolemia caused by other genetic defects, such as familial defective apolipoprotein B. A total of 2,400 unrelated patients aged 18 years and older fulfilled the diagnostic criteria for heterozygous FH. Over 99% of these patients were Caucasian. The Institutional Review Board of each participating hospital approved the study protocol and informed consent was obtained from all patients.

Data collection

Patients' medical records were used to acquire information about age, gender, smoking, BMI, and the presence of hypertension (patients with a documented diagnosis using antihypertensive medication or a systolic blood pressure >140 mmHg or a diastolic blood pressure >90 mmHg at three consecutive office visits) and diabetes mellitus (patients using anti-diabetic medication or fasting plasma glucose >6.9 mmol/L). Additional information was sought from general practitioners and hospitals that patients had visited formerly and with questionnaires to ensure data completeness.

Lipid levels were determined in fasting patients not using lipid-lowering medication for at least 6 weeks. Total cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides were measured by standard methods. LDL cholesterol was calculated with the Friedewald formula. Forty patients had triglyceride levels > 4.5 mmol/L and in these individuals LDL-C concentrations were directly measured by standard methods.

CVD and CHD definitions

CVD was defined as coronary, cerebral, or peripheral artery disease using internationally accepted criteria as published before.[6] Coronary heart disease (CHD) was defined by the presence of: *(I)* myocardial infarction, *(II)* percutaneous coronary intervention or other invasive procedures, *(III)* coronary artery bypass grafting, *(IV)* angina pectoris.

Molecular analysis

DNA was available of 2024 heterozygous FH patients for the present analyses. Genomic DNA was extracted from peripheral blood leukocytes according to a standard protocol.[24] The ER22/23EK polymorphism in the glucocorticoid receptor gene was detected by allelic discrimination using Taqman Universal PCR master mix (Applied Biosystems, Foster City, USA), primers (forward: 5'AGAAGAAAACCCCAGCAGTGT-3' and reverse: 5'-

CAGTAGCTCCTCCTCTTAGGGTTTTA-3′), probes (Applied Biosystems), and a Taqman ABI Prism 7900 Sequence Detection System (Applied Biosystems). Used probes were 5′-FAM-CACATCTCCCTTTTCCTGA-3′ and 5′-VIC-CACATCTCCCCTCTCCTGA-3′ (Applied Biosystems). Reaction components and amplification parameters were based on the manufacturer's instructions using an annealing temperature of 60°.

Statistical analysis

All data were analyzed using SPSS for Windows software package version 11.5.0 (SPSS Inc., Chicago, IL, USA). Since the hormone dynamics and the risk of CVD differ considerably between men and women, we stratified our analyses by gender. As expected, the number of ER22/23EK homozygotes was limited; the analyses of heterozygous and homozygous carriers were therefore combined. Contingency tables were used with chi-square tests to compare observed genotype frequencies with those expected under Hardy-Weinberg equilibrium. Differences between ER22/23EK carriers and noncarriers and men and women were tested with chi-square statistics for dichotomous variables or independent sample t-test for continuous variables. Statistical testing of triglyceride levels was performed after logarithmic transformation. We used multiple logistic regression analysis to adjust statistical tests for age.

The association between the ER22/23EK polymorphism and the occurrence of CVD and CHD was evaluated using a Cox proportional hazard regression analysis. 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. Follow-up started at birth and ended at the first occurrence of established fatal or non-fatal CVD. Patients without CVD were censored at the date of the last lipid clinic visit or at the date of death attributable to other causes than CVD. In the primary model, we adjusted for year of birth and smoking. Additional variables, which had significant effects on CVD risk in univariate Cox regression analyses, were investigated in the secondary model. Young patients with the polymorphism may not have had the chance to express their high CVD risk. Therefore, we tested the effect of age by adjusting for age tertiles in the Cox regression analysis. The interaction between the ER22/23EK variant and gender in the total population was statistically tested in the Cox regression analysis with adjustment for variables that were significantly different between genders. Throughout, a two-tailed p value of less than 0.05 was interpreted as statistical significant.

Results

Patient characteristics

In 977 men heterozygous for FH, 76 (7.8%) individuals were heterozygous for the ER22/23EK polymorphism but no homozygous carriers were detected. In 1047 women heterozygous for FH, 70 (6.7%) were heterozygous for the ER22/23EK variant whereas 2 (0.2%) were

Table 1. Genotype and allele frequencies for the ER22/23EK polymorphism in the glucocorticoid receptor gene

	Men		Women	
	CVD	No CVD	CVD	No CVD
	N (%)	N (%)	N (%)	N (%)
Genotypes				
Wild type	368 (93.2)	533 (91.6)	231 (93.5)	744 (93.0)
Heterozygous ER22/23EK	27 (6.8)	49 (8.4)	14 (5.7)	56 (7.0)
Homozygous ER22/23EK	0 (0.0)	0 (0.0)	2 (0.8)	0 (0.0)
Total	395 (100.0)	582 (100.0)	247 (100.0)	800 (100.0)
Alleles				
Wild type allele	763 (96.6)	1115 (95.8)	476 (96.4)	1544 (96.5)
ER22/23EK allele	27 (3.4)	49 (4.2)	18 (3.6)	56 (3.5)
Total	790 (100.0)	1164 (100.0)	494 (100.0)	1600 (100.0)

Abbreviations: CVD, history of cardiovascular disease.

Table 2. Characteristics of male and female familial hypercholesterolemia patients with and without the ER22/23EK polymorphism in the glucocorticoid receptor gene

	Men			Women		
	Noncarriers	ER22/23EK Carriers	p-value*	Noncarriers	ER22/23EK Carriers	p-value*
	(n=901)	(n=76)		(n=975)	(n=72)	
Age at first lipid clinic visit (years)	43.6 (± 0.4)	43.3 (± 1.2)	0.8	46.2 (± 0.4)	41.5 (± 1.6)	0.006
Age at last lipid clinic visit (years)	48.7 (± 0.4)	47.7 ± (1.2)	0.4	51.0 (± 0.5)	47.7 ± (1.7)	0.07
Smoking, ever (%)	79.3 (± 1.4)	76.1 (± 5.1)	0.6	67.7 (± 1.6)	57.6 (± 6.1)	0.08
Hypertension (%)	8.6 (± 0.9)	6.8 (± 2.9)	0.6	10.1 (± 1.0)	8.5 (± 3.3)	0.9
Diabetes mellitus (%)	5.5 (± 0.8)	3.9 (± 2.2)	0.7	6.3 (± 0.8)	6.9 (± 3.0)	0.6
Body mass index (kg/m2)	25.5 (± 0.1)	25.7 (± 0.4)	0.5	24.8 (± 0.1)	24.3 (± 0.5)	0.4
Total cholesterol (mmol/L)	9.36 (± 0.07)	9.41 (± 0.24)	0.8	9.67 (± 0.06)	9.76 (± 0.28)	0.4
LDL cholesterol (mmol/L)	7.24 (± 0.07)	7.22 (± 0.26)	0.9	7.46 (± 0.07)	7.70 (± 0.27)	0.2
HDL cholesterol (mmol/L)	1.09 (± 0.01)	1.14 (± 0.04)	0.3	1.32 (± 0.01)	1.30 (± 0.05)	0.6
Triglycerides (mmol/L)	1.96 (± 0.03)	2.15 (± 0.11)	0.4†	1.64 (± 0.03)	1.64 (± 0.13)	0.9†

Abbreviations: LDL indicates low-density lipoprotein; HDL, high-density lipoprotein.

Values are given as means ± standard error of the mean (SEM).

SI conversion factors: to convert total cholesterol, LDL cholesterol, and HDL cholesterol to mg/dL, multiply by 38.67; for triglycerides, multiply by 89.15.

*Additional adjustment for age.

†Statistical testing after logarithmic transformation.

homozygous. In women with CVD, the ER22/23EK variant deviated from Hardy-Weinberg equilibrium ($\chi^2 = 9.182$; $p = 0.002$) due to the small number of homozygotes; omitting one of them resulted in equilibrium. The genotype distributions in the total cohort and in all other subgroups (Table 1) did not differ from Hardy-Weinberg equilibrium ($\chi^2 < 1.610$; $P > 0.2$).

The clinical and biochemical characteristics of carriers and noncarriers of the ER22/23EK polymorphism are presented in Table 2. In men, no differences existed between patients with and without ER22/23EK. Female carriers of ER22/23EK were 4.6 ± (SD) 1.7 years younger at first Lipid Clinic visit compared with women without the polymorphism (p = 0.006). Other variables were not significantly different between female carriers and noncarriers.

Males visited the Lipid Clinic 2.3 ± (SEM) 0.6 years earlier, were more often smokers (difference, 12.0 ± 2.1%), had a higher BMI (difference, 0.7 ± 0.2 kg/m^2), lower plasma LDL cholesterol (difference, 0.24 ± 0.09 mmol/L) and HDL cholesterol (difference, 0.23 ± 0.02 mmol/L) levels, and higher plasma triglyceride concentrations (difference, 0.34 ± 0.05 mmol/L) compared with women; all as expected.

Risk factors for CVD

During 44.044 person years, 395 (40.4%) men had onset of CVD and a total of 247 (23.6%) women had their first cardiovascular event during 51.331 person years. The mean age of onset of CVD (± SD) was 45.7 ± 9.3 years in men and 52.8 ± 11.6 years in women.

Separate analyses of a number of variables, which may act as confounder or intermediate trait in the relationship between the ER22/23EK polymorphism and CVD risk, are presented in Table 3. In men and women, year of birth, smoking, and lower plasma HDL cholesterol concentrations were significantly associated with increased CVD risk. The presence of diabetes mellitus also contributed to an increased risk of CVD in women but not in men. Hypertension, BMI, plasma LDL cholesterol levels, and triglyceride concentrations were not significantly associated with CVD among men or women.

Table 3. Risk factors of cardiovascular disease in 977 men and 1047 women with familial hypercholesterolemia

Variables	Men			Women		
	Relative Risk (95% CI)		p-value	Relative Risk (95% CI)		p-value
Year of birth	1.05	(1.04 to 1.07)	<0.001	1.08	(1.06 to 1.10)	<0.001
Smoking	1.56	(1.14 to 2.15)	0.006	1.53	(1.15 to 2.05)	0.004
Diabetes mellitus	1.06	(0.76 to 1.48)	0.7	1.68	(1.19 to 2.37)	0.004
Hypertension	1.32	(1.00 to 1.75)	0.05	1.10	(0.79 to 1.52)	0.6
Body mass index	1.00	(0.97 to 1.04)	0.8	1.04	(1.00 to 1.08)	0.07
LDL cholesterol	0.99	(0.92 to 1.06)	0.7	1.00	(0.93 to 1.08)	0.9
HDL cholesterol	0.53	(0.35 to 0.80)	0.002	0.47	(0.29 to 0.75)	0.001
Triglycerides	1.15	(0.99 to 1.35)	0.08*	1.02	(0.83 to 1.26)	0.8*

Abbreviations: LDL, low-density lipoprotein; HDL, high-density lipoprotein.
*Statistical testing after logarithmic transformation.

CVD and the ER22/23EK polymorphism

In the total population, we found no significant association between the ER22/23EK variant and CVD with adjustment for year of birth, gender, and smoking (RR 0.92, 95% CI 0.67 – 1.27; p = 0.6). Unadjusted survival rates of men and women with and without the

Table 4. Cardiovascular disease free survival of patients with familial hypercholesterolemia according to the ER22/23EK polymorphism in the glucocorticoid receptor gene

	Men		Women	
	Noncarriers	ER22/23EK Carriers	Noncarriers	ER22/23EK Carriers
CVD free survival*				
40 years	62.9 (1.9)	63.0 (6.5)	87.1 (1.3)	85.7 (5.2)
50 years	35.2 (2.4)	43.9 (8.5)	70.1 (2.0)	63.3 (8.7)
60 years	20.5 (2.9)	31.4 (12.2)	49.2 (2.8)	41.0 (11.8)
Median survival time	54.7	56.8	69.6	66.0

Abbreviations: CVD, history of cardiovascular disease.

*Cumulative CVD free survival probability ± standard error of the mean (SEM).

Figure 1. Cardiovascular disease hazard curve according to the ER22/23EK polymorphism in 977 men and 1047 women with heterozygous familial hypercholesterolemia.

ER22/23EK polymorphism are shown in Table 4. Figure 1 illustrates the association between the ER22/23EK polymorphism and the cumulative incidence of CVD during lifetime follow-up in patients with FH after adjustment for year of birth and smoking and stratified by gender. The ER22/23EK polymorphism was not significantly associated with CVD in either gender: male carriers had a 0.75 (95% CI 0.50 – 1.14; p = 0.2) times decreased risk of CVD, whereas female carriers had a 1.37 (95% CI 0.82 – 2.28; p = 0.2) times increased CVD risk. As shown in Table 5, additional adjustment of plasma HDL cholesterol concentrations in men and plasma HDL cholesterol concentrations and diabetes mellitus in women did not change the results. To test for effects of the ER22/23EK variant on CVD risk with age, we repeated the multiple Cox regression analyses of the primary model with additional adjustment for age tertiles. After this additional adjustment, the CVD risk estimates remained virtually identical in men (RR 0.75, 95% CI 0.49 – 1.13; p = 0.2) and in women (RR 1.41, 95% CI 0.84 – 2.35; p = 0.2).

Table 5. Relative risks for cardiovascular disease and coronary heart disease according to ER22/23EK genotype

	Relative Risk (95% Confidence Interval)			
Genotype	**Univariate**	**Model 1**	**Model 2**	**Model 3**
Men CVD				
Wild type	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
ER22/23EK	0.86 (0.58 – 1.27)	0.75 (0.50 – 1.14)	0.73 (0.46 – 1.17)	0.75 (0.49 – 1.13)
Women CVD				
Wild type	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
ER22/23EK	1.27 (0.76 – 2.10)	1.37 (0.82 – 2.28)	1.35 (0.74 – 2.44)	1.41 (0.84 – 2.35)
Men CHD				
Wild type	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
ER22/23EK	0.83 (0.55 – 1.25)	0.72 (0.46 – 1.12)	0.72 (0.44 – 1.19)	0.71 (0.46 – 1.11)
Women CHD				
Wild type	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
ER22/23EK	1.45 (0.86 – 2.45)	1.60 (0.94 – 2.73)	1.62 (0.86 – 3.02)	1.64 (0.96 – 2.80)

Abbreviations: CVD, history of cardiovascular disease; CHD, coronary heart disease.

Model 1: adjusted for year of birth and smoking.

Model 2: adjusted for year of birth, smoking, and HDL cholesterol levels in men and adjusted for year of birth, smoking, HDL cholesterol levels, and diabetes in women.

Model 3: adjusted for year of birth, smoking, and age tertiles.

Although we found no significant relationship between the ER22/23EK variant and CVD overall, the effect of the polymorphism on CVD risk seemed opposite in men and women. Therefore, we tested the interaction between the ER22/23EK variant and gender in the total population using Cox regression analysis with adjustment for variables that were significantly different between genders (age, smoking, BMI, plasma LDL cholesterol, HDL cholesterol, and triglyceride levels). Indeed, the effect of the ER22/23EK variant on CVD risk was significantly different for men and women ($p = 0.02$).

CHD and the ER22/23EK polymorphism

CHD was the most frequent cardiovascular event: in 350 (88.6%) men and 200 (81.0%) women CHD was the first event. Cerebral artery disease occurred as the first event in only 20 (5.1%) men and 20 (8.1%) women; in 25 (6.3%) men and 27 (10.9%) women the first event was peripheral artery disease.

In the total population, again we observed no significant relationship between the polymorphism and CHD after adjustment for year of birth, gender, or smoking (RR 0.93, 95% CI 0.67 – 1.31; $p = 0.7$). With adjustment for year of birth and smoking, the ER22/23EK polymorphism was not significantly associated with CHD in men (RR 0.72, 95% CI 0.46 – 1.12; $p = 0.1$) or women (RR 1.60, 95% CI 0.94 – 2.73; $p = 0.09$). Additional adjustment for additional variables resulted in similar risk estimates (Table 5). In line with the results for CVD, the effect of the ER22/23EK variant on CHD risk was significantly different between men and women ($p = 0.006$).

Discussion

We hypothesized that the functional ER22/23EK variant in the glucocorticoid receptor gene might decrease CVD risk. In this large cohort at severely increased risk, however, the association between the ER22/23EK variant and the occurrence of CVD or CHD was not significant overall. Nonetheless, in a post hoc analysis we found opposite effects of the polymorphism on CVD and CHD in men and women.

Exogenous as well as endogenous glucocorticoid excess in humans contributes to the development of hypertension, dylipidemia, impaired glucose tolerance, central adiposity, and may imbalance thrombosis and fibrinolysis.[25,26] Glucocorticoids exert their function primarily via binding to the cytoplasmic glucocorticoid receptor, which then transfers into the nucleus where it enhances or represses transcription of specific target genes.[27] The ER22/23EK polymorphism in the glucocorticoid receptor gene results in a relative glucocorticoid resistance and has been associated with a beneficial cardiovascular risk profile defined by lower plasma total and LDL cholesterol levels, increased insulin sensitivity, and beneficial body composition.[18,22] In addition, carriers had lower plasma C-reactive protein levels as well as better overall survival.[23] In our FH cohort, 7.3% of the individuals carried at least one copy of the variant, in concordance with genotype frequencies described in previous studies (5.2 – 8.9%). In contrast to expected results, we found no significant association between the ER22/23EK polymorphism and CVD or CHD risk. The severe hypercholesterolemia in our patients might offer a clue as to why we observe these results. The potential beneficial effect of this polymorphism may be partly due to lower LDL cholesterol levels.[18] We did not observe, however, such differences; the dominant dyslipidemia of FH may outweigh potential beneficial effects on cholesterol metabolism. In the general population, variants in the glucocorticoid receptor gene may be better risk predictors for CVD. Alternatively, we made a type II error in the separate analyses of men and women: differences were not observed with statistical significance because of lack of power due to small numbers. The result of our interaction analysis between the polymorphism and gender in the total population support the latter alternative. Based on literature, we had a priori hypothesized that the ER22/23EK variant decreased CVD risk and the study was powered to detect a significant association between the polymorphism and cardiovascular risk in the total population.

Although we found no significant relationship between the ER22/23EK polymorphism and CVD or CHD overall, the influence of the genetic variant appears to be different between sexes. This variant has been preferentially analyzed in male individuals and most association studies did not analyze men and women separately. In a cohort of young-adults followed from the age of 13 to 36 years, the body composition in carriers of the ER22/23EK variant was indeed different between sexes: only male carriers were taller, leaner, and had more muscle strength.[22] The reason for these differential effects of the ER22/23EK variant between men and women is yet unknown. In rodents, the hypothalamic-pituitary-adrenal axis responds to

variations in circulating sex steroid concentrations.[28] This regulation differs between sexes: estrogen primarily exerts stimulatory effects on stress-induced ACTH and glucocorticoid release, whereas testosterone inhibits stress-related hypothalamic-pituitary-adrenal axis activity.[28-30] As speculated previously, in a relative glucocorticoid resistance condition, as is the case for ER22/23EK carriers, the high circulating estrogen levels in women may annul the potential beneficial effects of the ER22/23EK variant.[22] Otherwise, high variability in the expression of genes located at the X-chromosome between men and women has been reported and may also account for these gender differences.[31] An example of such a gene is Mediator subunit MED14. Garabedian and co-workers found that MED14 interacts with the glucocorticoid receptor and increases its transcriptional activation in a gene-specific manner.[32,33] Interestingly, MED14 is X-linked and fails to undergo X-chromosome inactivation. Therefore, the researchers suggested that MED14 levels could be higher in females than in males, which may represent a mechanism underlying gender-specific differences in the expression of glucocorticoid receptor target genes.[33] These observations support that separate analyses of men and women are indicated for studies on the ER22/23EK variant.

The monogenetic background but large variation in CVD risk determines the strength of the present study. Patients with heterozygous FH have 8.5 times increased cardiovascular risk.[4] The atherosclerotic burden of the disorder, however, exhibits wide variation and many untreated FH patients experience little or no excess mortality.[3] As in the general population, CVD in FH patients is the result of a dynamic interplay among multiple genes in addition to gene-environment interactions.[3-5] The disorder is therefore considered to be an exemplary model to analyze secondary (or modifier) genes involved in CVD.

However, there are also some limitations to our association study. It depended on medical records, questionnaires, and information retrospectively obtained from physicians as the primary source of data. Certain information of interest, such as postmenopausal status, was not available. Furthermore, the influence of the ER22/23EK polymorphism on potential intermediate traits (e.g. dexamethasone suppression test, insulin and C-reactive protein levels, body composition, and hormone levels) could not be determined. Our study included patients that were referred to lipid clinics and this could lead to selection bias in two different ways. First, patients with the most detrimental genetic profiles might have died before referral, although we did not observe such premature deaths in a previously reported mortality analysis.[3] Nonetheless, we cannot exclude that polymorphisms causing early death could have been missed, leading to underestimation of the risk. Secondly, patients that presented themselves with premature symptoms of atherosclerosis are more easily referred to lipid clinics; this could lead to selection on CVD in our cohort study. However, we used data of a nationwide screening program and to prevent selection biases we only selected patients from the 48 larger outpatient lipid clinics that are characterized by clinically more diverse patient populations. Finally, our findings in heterozygous FH cannot be extrapolated to other populations. The

observed opposite effects of the ER22/23EK variant on CVD and CHD in our FH cohort should be validated or excluded in wider population-based studies.

In conclusion, in this large, retrospective cohort study amongst patients with heterozygous FH, the association between the functional ER22/23EK polymorphism and CVD risk or CHD risk was not significant overall, though it varied significantly by gender.

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CHAPTER 7

Susceptibility to cardiovascular disease in patients with familial hypercholesterolemia is modified by haplotypes of the glucocorticoid receptor gene

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Abstract

Introduction: Glucocorticoids contribute to the development of hypertension, dyslipidemia, impaired glucose tolerance, and central adiposity. Several polymorphisms within the glucocorticoid receptor (GR) gene have been reported to alter glucocorticoid sensitivity and have been associated with risk factors for cardiovascular disease (CVD). The relationship between these GR variants and CVD has not been investigated.

Aim of the study: We determined whether the combined four functional polymorphisms in the GR gene influence CVD risk in patients with severe hypercholesterolemia.

Materials and Methods: In a multicenter cohort study, 1,830 patients with heterozygous familial hypercholesterolemia, aged 18 years and older, were genotyped for the ER22/23EK, N363S, *Bcl*I, and GR-9β variants. Patients were identified at lipid clinics throughout the Netherlands. The primary outcome measure was CVD defined as coronary, cerebral, and peripheral artery disease.

Results: A total of 359 (40.8%) men had onset of CVD and 224 (23.6%) women had their first cardiovascular event. We analyzed the combined effect of all GR variants by reconstructing haplotypes and using a Cox proportional hazards regression model with adjustment for year of birth and smoking. The most frequent haplotype (45%) consisted of the wild type alleles of all four polymorphisms. Compared to this haplotype, the GR-9β haplotype, with only variation at the GR-9β site, was associated with a 41% higher CVD risk (p=0.04) and the *Bcl*I haplotype, with only variation at the *Bcl*I site, with a 34% higher CVD risk (p=0.03) in men only. In women, none of the haplotypes was significantly related with CVD. We found no significant association of any individual GR variant with CVD risk among men and women. No statistical significant differences in cardiovascular risk factors were found between the GR genotype groups.

Conclusion: In this large cohort of individuals with very high-risk of CVD, two common haplotypes in the GR gene explained a small proportion of the variation in CVD susceptibility among men but not among women.

Introduction

Glucocorticoids play an important role in the pathofysiology of atherosclerosis by their contribution to the development of hypertension, dyslipidemia, impaired glucose tolerance, and central adiposity.[1,2] The effects of glucocorticoids are known to be mediated primarily by binding to the intracellular glucocorticoid receptor (GR), which belongs to the nuclear receptor family of ligand-dependent transcription factors.[3] Upon ligand binding, the GR translocates to the nucleus where it results in a cascade of events, that eventually leads to the induction or repression of glucocorticoid responsive genes.

The sensitivity to glucocorticoids varies considerably among individuals.[4] The presence of functional DNA sequence variants within the GR gene has been shown to be partly responsible for variation in the sensitivity to glucocorticoids.[5] Mutations in the GR gene are extremely rare and result in generalized glucocorticoid resistance syndromes.[6] Several more common single nucleotide polymorphisms have been reported to alter glucocorticoid sensitivity more subtle and have been associated with cardiovascular disease (CVD) risk factors.[7]

Two polymorphisms in the GR gene, the N363S and *Bcl*I variants, have been associated with an increased sensitivity to glucocorticoids *in vivo*: carriers had lower cortisol levels after a dexamethasone suppression test.[4,8] In addition, the N363S variant has shown a relationship with increased insulin response to exogeneous dexamethasone, higher body mass index (BMI), elevated plasma total cholesterol and triglyceride concentrations, and a higher frequency of coronary artery disease.[4,9,10] The frequent *Bcl*I polymorphism has been linked to higher systolic blood pressure and increased abdominal visceral obesity.[11-14]

Opposite effects were found of the ER22/23EK polymorphism. Carriers of this polymorphism were relatively resistant to the effects of glucocorticoids, because they had higher cortisol levels after dexamethasone suppression test.[15] Furthermore, they had lower plasma total and low-density lipoprotein (LDL) cholesterol concentrations, an increased insulin sensitivity, a beneficial body composition at young-adult age, lower plasma C-reactive protein levels, and a better overall survival in the elderly.[15-17] Recently, we found subtle effects of this polymorphism on CVD risk that varied significantly by gender.[18]

The ER22/23EK variant has been previously linked to a fourth polymorphism in the GR gene, the GR-9β variant: the rare 22/23EK allele was only present in combination with the rare GR-9β G-allele.[19] Due to alternative splicing, two isoforms of the GR gene exists: GRα and GRβ.[20] These two forms differ in their 3'end (exon 9); GRα mRNA contains exon 9α and GRβ mRNA contains exon 9β. GRα can bind glucocorticoids and alter gene transcription, whereas GRβ does not bind hormone and is transcriptionally inactive. The GR-9β polymorphism was not associated with altered cortisol response to dexamethasone,[19] but has been suggested to influence the immune system. The GR variant was significantly related with a higher frequency of rheumatoid arthritis and a decreased susceptibility to nasal colonization by *Staphylococcus aureus*, suggesting a reduced immune suppression in carriers of the GR-9β polymorphism.[21,22]

The effects of glucocorticoids on inflammation are exerted via the transrepressing pathway. Recently, *ex vivo* experiments have shown a decreased glucocorticoid transrepression in persons carrying the GR-9β haplotype whereas glucocorticoid-induced transactivation was not significantly different.[19]

Although these four polymorphisms in the GR gene are associated with altered glucocorticoid sensitivity and cardiovascular risk factors, their combined influence on cardiovascular endpoints has thus far not been investigated. In the present study, we therefore determined the effect of haplotypes based on these four functional polymorphisms in the GR gene on CVD risk in patients with heterozygous FH who have a severely increased predisposition to premature cardiovascular events.

Materials and methods

Study design, population, and data collection

The design, population, and data collection of this multicenter cohort study have been described previously.[23] Briefly, DNA samples of clinically suspected FH patients in The Netherlands are submitted to a central laboratory for LDL receptor mutation analysis. From this DNA-bank database, we randomly selected 4,000 out of 9,300 patients whose DNA had been collected between 1989 and 2002. A total of 2,400 unrelated patients, aged 18 years and older, fulfilled the FH diagnostic criteria that are based on genetic and clinical information.[23] Over 99% were Caucasian. The Institutional Review Board of each participating hospital approved the study protocol and informed consent was obtained from all patients.

Information about age, gender, smoking, BMI, and the presence of hypertension and diabetes mellitus was collected from patients' medical records, general practitioners, hospital physicians, and with questionnaires.[23] Smoking was classified as ever having smoked. Body mass index (BMI) was calculated from height and length (kg/m^2). Hypertension was defined as a systolic blood pressure >140 mmHg or a diastolic blood pressure >90 mmHg at three consecutive office visits or patients with a documented diagnosis and using antihypertensive medication. Diabetes mellitus was defined as patients with fasting plasma glucose >6.9 mmol/L or patients using anti-diabetic medication. Lipid levels, as stated in the medical record, were determined in fasting patients not using lipid-lowering medication for at least 6 weeks. Total cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides were measured by standard methods. LDL cholesterol was calculated with the Friedewald formula.

CVD endpoints

Primary outcome of this study was fatal or non-fatal CVD defined as coronary, cerebral, and peripheral artery disease using internationally accepted criteria as published before.[23]

Molecular analysis

Genomic DNA was extracted from peripheral blood leukocytes according to a standard protocol.[24] Allelic discrimination was performed to genotype the patients, using Taqman Universal PCR master mix, primers, probes, and a Taqman ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, USA). Used primers and probes (Applied Biosystems) are shown in Table 1. Reaction components and amplification parameters were based on the manufacturer's instructions using an annealing temperature of 60°.

The ER22/23EK polymorphism comprises two linked, single nucleotide variations separated by one base pair in exon 2. The first mutation at nucleotide position 198 is silent, changing codon 22 from GAG to GAA, both coding for a glutamic acid (E). The second mutation changes codon 23 at nucleotide position 200 from AGG to AAG, causing a change from arginine (R) to lysine (K). The N363S polymorphism changes codon 363 of exon 2 at nucleotide position 1220 from AAT to AGT and results in an aspargine (N) to serine (S) amino acid change. The very common *Bcl*I intronic restriction fragment length polymorphism results in fragments of 2.3 kb and 4.5 kb due to a C to G nucleotide alteration, 646 nucleotides downstream from exon 2. Finally, the GR-9*β* polymorphism is located in the 3' untranslated region of exon 9β at nucleotide position 3669 and results in an A to G mutation. Successful DNA genotyping of the GR gene polymorphisms was possible in 2,024 patients for the ER22/23EK (G/A), 2,032 patients for the N363S (A/G), 2,063 patients for the *Bcl*I (C/G), and 2,025 patients for the GR-9*β* (A/G). The results of the remaining patients were missing due to lack of DNA or inconclusive

Table 1. Primer and probe sequences for polymorphisms in glucocorticoid receptor gene used in Taqman ABI Prism 7900 Sequence Detection System

Polymorphism		Sequence (5'–3')
ER22/23EK (G/A)	Forward primer	AGAAGAAAACCCCAGCAGTGT
	Reverse primer	CAGTAGCTCCTCCTCTTAGGGTTTTA
	Probes	VIC–CACATCTCCC**C**TCTCCTGA
		FAM–CACATCTCCC**T**TTTCCTGA
N363S (A/G)	Forward primer	GTCATTCCACCAATTCCCGTTG
	Reverse primer	GTCAAGTTGTCATCTCCAGATCCTT
	Probes	VIC–ACCTATTCCAA**T**TTTCGG
		FAM–CCTATTCCAA**C**TTTCGG
*Bcl*I (C/G)	Forward primer	CAGGGTTCTTGCCATAAAGTAGACA
	Reverse primer	GCACCATGTTGACACCAATTCC
	Probes	VIC–CTCTTAAAGAGATT**G**ATCAGC
		FAM–CTCTTAAAGAGATT**C**ATCAGC
GR-9*β* (A/G)	Forward primer	TCAGACTGTAAAACCTTGTGTGGAA
	Reverse primer	CCAATTCGGTACAAATGTGTGGTT
	Probes	VIC–CTTTTATTTTTTC**A**TTTAAATTT
		FAM–TTTATTTTTTC**G**TTTAAATTT

genotyping. A total number of 1,830 patients had complete GR genotypes and were used in haplotype analysis. There were no differences in CVD risk between subjects with total GR genotypes and excluded individuals.

Statistical analysis

All data were analyzed using SPSS for Windows software package version 11.5.0 (SPSS Inc., Chicago, IL, USA). Recently, we have reported opposite effects of the ER22/23EK polymorphism on CVD risk in men and women.[18] Hence, we stratified all present analyses by gender. Differences between men and women were tested with chi-square statistics for dichotomous variables and independent sample t-test for continuous variables. Statistical testing of triglyceride levels was performed after logarithmic transformation to normalize the distribution. Multiple logistic regression analysis was used to adjust statistical tests for age. Deviations of the genotype distribution from that expected for a population in Hardy-Weinberg equilibrium were tested using the chi-square test with one degree of freedom.

First, we analyzed the combined effect of all GR variants using haplotypes to obtain more detailed information about the genetic variation of the locus in relation with CVD. Haplotypes of the GR were estimated with the PHASE program (version 2.1), which implements a Bayesian statistical method for reconstructing haplotypes from population genotype data.[25] In over 99% of the individuals, the alleles of the haplotypes could be inferred with 96-100% certainty.

The association between GR haplotype and CVD was evaluated using a Cox proportional hazards regression model. Follow-up started at birth and ended at the first occurrence of established fatal or non-fatal CVD. Patients without CVD were censored at the date of the last lipid clinic visit or at the date of death attributable to other causes than CVD. The multiple Cox regression model was initially adjusted for covariates that were independent of the GR gene haplotypes: year of birth and smoking. In addition, we performed analyses to assess the effect of possible confounders or intermediates in the relationship between the GR polymorphism and CVD by adjusting for covariates which had significant effects on CVD risk in univariate Cox regression analyses. In the Cox regression analyses, persons with the haplotype of interest were compared to the wild type haplotype. We repeated the haplotype estimation and analyses, weighted for the posterior uncertainty in the haplotype assignments, using the haplo.glm function of haplo.stats.[26,27] The haplo.glm function applied a general linear model to investigate the association between the haplotypes and CVD with adjustment for age and smoking status.

Secondly, we assessed the relationship between each GR polymorphism and the risk of CVD separately. All polymorphisms were assessed using a dominant, additive, or recessive genetic model without adjustment for covariates and the model with the highest log-likelihood was used in multivariable Cox regression analysis. To examine gender-specific effects of GR variants on cardiovascular outcomes, we tested the interaction between each GR variant

Table 2. Characteristics of 1,830 individuals with familial hypercholesterolemia

	Men (n=879)	Women (n=951)	p-value*
Age at first lipid clinic visit (years)	43.7 (± 0.4)	45.9 (± 0.4)	<0.001
Age at last lipid clinic visit (years)	48.8 (± 0.4)	50.8 (± 0.5)	0.001
Smoking, ever (%)	79.1 (± 1.4)	67.2 (± 1.6)	<0.001
Hypertension (%)	8.6 (± 1.0)	9.4 (± 1.0)	0.9
Diabetes mellitus (%)	5.2 (± 0.8)	6.5 (± 0.8)	0.6
Body mass index (kg/m^2)	25.6 (± 0.1)	24.8 (± 0.1)	<0.001
Total cholesterol (mmol/L)	9.37 (± 0.07)	9.63 (± 0.07)	0.03
LDL cholesterol (mmol/L)	7.23 (± 0.07)	7.43 (± 0.07)	0.09
HDL cholesterol (mmol/L)	1.10 (± 0.01)	1.33 (± 0.01)	<0.001
Triglycerides (mmol/L)	1.96 (± 0.04)	1.64 (± 0.03)	<0.001†

Abbreviations: LDL indicates low-density lipoprotein; HDL, high-density lipoprotein.

Values are given as means ± standard error of the mean (SEM).

SI conversion factors: To convert total cholesterol, LDL cholesterol, and HDL cholesterol to mg/dL, multiply by 38.67; for triglycerides, multiply by 89.15.

*Additional adjustment for age.

†Statistical testing after logarithmic transformation.

and gender in the total population using Cox regression analysis with adjustment for variables that were significantly different between genders. Differences in cardiovascular risk factors between genotypes were tested with chi-square statistics for dichotomous variables and independent sample t-test or one-way ANOVA for continuous variables. For all statistical analyses, a P value of less than 0.05 was considered statistically significant.

Results

Patient characteristics and genotype distributions

The general characteristics of 879 men and 951 women with complete GR genotypes are shown in Table 2. Men visited the lipid clinic 2.2 ± (SEM) 0.6 years earlier, were more often smokers (difference, 12.0 ± 2.2%), had a higher BMI (difference, 0.8 ± 0.2 kg/m^2), lower plasma total cholesterol (difference, 0.26 ± 0.10 mmol/L) and HDL cholesterol levels (difference, 0.23 ± 0.02 mmol/L), and higher plasma concentrations of triglycerides (difference, 0.33 ± 0.05 mmol/L) compared to women.

Table 3 presents the genotype distributions for all GR gene polymorphisms. The observed genotype distributions were in Hardy-Weinberg equilibrium in the total population and among individuals with and without CVD ($\chi^2 < 3.29$; $P > 0.07$). Only in the subgroup of women with CVD, the ER22/23EK variant deviated from Hardy-Weinberg equilibrium ($\chi^2 = 11.06$; $p = 0.0009$), which is probably the result of only 2 rare ER22/23EK homozygotes in our population. Due to this small number of rare ER22/23EK homozygotes, heterozygous and homozygous

Table 3. Genotype distributions for all glucocorticoid receptor gene polymorphisms

	Men		Women	
	CVD	No CVD	CVD	No CVD
	N (%)	N (%)	N (%)	N (%)
ER22/23EK				
Wild type	337 (93.9)	482 (92.7)	210 (93.8)	677 (93.1)
Heterozygous ER22/23EK	22 (6.1)	38 (7.3)	12 (5.4)	50 (6.9)
Homozygous ER22/23EK	0 (0.0)	0 (0.0)	2 (0.9)	0 (0.0)
N363S				
Wild type	327 (91.1)	491 (94.4)	205 (91.5)	678 (93.3)
Heterozygous N363S	32 (8.9)	29 (5.6)	19 (8.5)	49 (6.7)
Homozygous N363S	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
*Bcl*I				
Wild type	139 (38.7)	232 (44.6)	87 (38.8)	299 (41.1)
Heterozygous *Bcl*I	173 (48.2)	238 (45.8)	111 (49.6)	336 (46.2)
Homozygous *Bcl*I	47 (13.1)	50 (9.6)	26 (11.6)	92 (12.7)
GR-9*β*				
Wild type	254 (70.8)	367 (70.6)	158 (70.5)	492 (67.7)
Heterozygous GR-9*β*	98 (27.3)	138 (26.5)	60 (26.8)	215 (29.6)
Homozygous GR-9*β*	7 (1.9)	15 (2.9)	6 (2.7)	20 (2.8)

Abbreviations: CVD, history of cardiovascular disease.

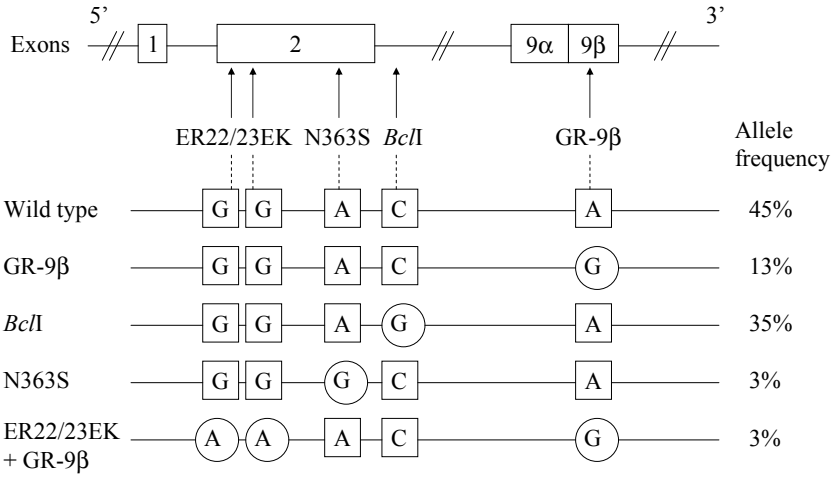

Figure 1. Schematic overview of the five most frequent haplotype alleles of the glucocorticoid receptor gene based on the four polymorphisms in 1,830 patients with familial hypercholesterolemia. The haplotype frequencies in the study population are depicted on the right side. Squares indicate the wild type alleles of the polymorphisms; circles indicate the variant alleles of the polymorphisms.

carriers of the minor allele were combined for further analyses. We did not find homozygote N363S carriers in our population.

GR haplotype and CVD

Frequencies of the five most common haplotypes of the GR gene based on the ER22/23EK, N363S, *Bcl*I, and GR-9*β* polymorphisms are presented in Figure 1. These five haplotypes captured more than 99% of the variation in the GR gene accounted for by the four polymorphisms. The most frequent haplotype (45%) was the haplotype with the wild type alleles of all four polymorphisms.

During 39,701 person years, 359 (40.8%) men had onset of CVD and a total of 224 (23.6%) women had their first cardiovascular event during 46,570 person years. The mean age of onset of CVD (± SD) was 45.8 ± 9.2 years in men and 52.9 ± 11.5 years in women. Separate analyses of a number of variables, which may act as confounder or intermediate trait in the relationship between the GR haplotype and CVD risk, are shown in Table 4. In men and women, year of birth, smoking, and lower plasma HDL cholesterol concentrations were significantly associated with increased CVD risk. The presence of diabetes mellitus and higher BMI also contributed to an increased risk of CVD in women but not in men.

Table 4. Risk factors of cardiovascular disease in individuals with familial hypercholesterolemia

Variables	Men			Women		
	Relative Risk (95% CI)		p-value	Relative Risk (95% CI)		p-value
Year of birth	1.05	(1.04 to 1.07)	<0.001	1.08	(1.06 to 1.10)	<0.001
Smoking	1.79	(1.25 to 2.55)	0.001	1.60	(1.17 to 2.18)	0.003
Diabetes mellitus	1.10	(0.77 to 1.57)	0.6	1.80	(1.27 to 2.57)	0.001
Hypertension	1.23	(0.91 to 1.66)	0.2	1.11	(0.78 to 1.56)	0.6
Body mass index	1.00	(0.96 to 1.04)	0.9	1.05	(1.01 to 1.10)	0.03
Total cholesterol	0.98	(0.91 to 1.06)	0.6	0.99	(0.92 to 1.07)	0.8
LDL cholesterol	0.98	(0.91 to 1.06)	0.6	0.99	(0.92 to 1.07)	0.8
HDL cholesterol	0.49	(0.32 to 0.76)	0.001	0.48	(0.30 to 0.78)	0.003
Triglycerides	1.16	(0.98 to 1.36)	0.08*	1.05	(0.85 to 1.30)	0.6*

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

*Statistical testing after logarithmic transformation.

Table 5. Relative risk of cardiovascular disease in men and women with familial hypercholesterolemia according to glucocorticoid receptor haplotype

GR haplotype	Men			Women		
	Relative Risk (95% CI)		p-value	Relative Risk (95% CI)		p-value
wild type	1.00		ref	1.00		ref
GR-9*β*	1.41	(1.02 to 1.94)	0.04	1.02	(0.69 to 1.51)	0.9
*Bcl*I	1.34	(1.02 to 1.76)	0.03	1.08	(0.76 to 1.54)	0.7
N363S	1.20	(0.71 to 2.02)	0.5	1.05	(0.53 to 2.07)	0.9
ER22/23EK+GR-9*β*	1.19	(0.70 to 2.00)	0.5	1.03	(0.45 to 2.34)	1.0

Abbreviations: GR, glucocorticoid receptor.

Relative risks were estimated with Cox proportional hazards regression and adjusted for year of birth and smoking.

Table 5 shows the effect of the GR gene haplotypes on CVD risk after adjustment for year of birth and smoking and stratified by gender. Compared to the wild type haplotype, the GR-9*β* haplotype, consisting of the mutant allele of the GR-9*β* polymorphism and the wild type of alleles of the other three polymorphisms, was associated with a 41% higher CVD risk in men only (p=0.04). A similar association in men was found for the *Bcl*I haplotype that consists of the mutant allele of the *Bcl*I polymorphism and wild types of all other polymorphisms: this haplotype was associated with a 34% higher risk of CVD in men (p=0.03). In women, none of the haplotypes was significantly related with CVD. Additional adjustment of covariates that had significant effects on CVD risk in univariate Cox regression analyses (plasma HDL cholesterol concentrations in men and plasma HDL cholesterol concentrations, BMI, and diabetes mellitus in women) did not influence the results. The results of the analyses with haplo.stats were concordant.

GR polymorphisms and CVD

Table 6 shows the effect of the individual GR gene polymorphism on CVD susceptibility after adjustment for year of birth and smoking and stratified by gender. We found no significant association between any of the GR variants and risk of CVD in men or women. Additional adjustment of covariates did not change the results (data not shown).

Table 6. Relative risk of cardiovascular disease in 1,830 individuals with familial hypercholesterolemia

GR polymorphism	Mode of inheritance	Men			Women		
		Relative Risk (95% CI)		p-value	Relative Risk (95% CI)		p-value
ER22/23EK	Dominant	0.74	(0.46 to 1.18)	0.2	1.50	(0.86 to 2.60)	0.2
N363S	Dominant	1.14	(0.79 to 1.64)	0.5	1.16	(0.70 to 1.91)	0.6
*Bcl*I	Recessive	1.28	(0.93 to 1.76)	0.1	0.82	(0.52 to 1.28)	0.4
GR-9*β*	Recessive	1.19	(0.56 to 2.52)	0.7	0.95	(0.39 to 2.31)	0.9

Abbreviations: GR, glucocorticoid receptor.

Relative risks were estimated with Cox proportional hazards regression and adjusted for year of birth and smoking.

As reported previously, the effect of the ER22/23EK variant on CVD risk was opposite in men and women.[18] The statistical interaction term between this polymorphism and gender was statistically significant (p = 0.02) in the total population using Cox regression analysis with adjustment for variables that were significantly different between genders (age, smoking, BMI, plasma LDL cholesterol, HDL cholesterol, and triglyceride levels). The influence of other GR variants on CVD susceptibility was not significantly different for men and women (N363S, p=0.7; *Bcl*I, p=0.2; GR-9*β*, p=0.7).

No statistical significant differences in general characteristics were observed between the GR genotype groups (data not shown), but for the following two exceptions: women with the ER22/23EK polymorphism visited the lipid clinic 4.7 ± (SEM) 1.8 years earlier and men with the N363S variant visited the lipid clinic 4.0 ± 1.4 years later.

Discussion

In this large cohort of FH individuals with severely increased predisposition to CVD, we found that two common GR gene haplotypes were associated with CVD susceptibility, but only among men. Men with the GR-9β haplotype had a 41% higher CVD risk and men with the *Bcl*I haplotype had a 34% higher CVD risk compared to male carriers of the wild type haplotype. We did not find an association between any of the single functional GR gene polymorphisms with CVD risk nor with cardiovascular risk factors such as BMI, plasma lipid levels, or hypertension.

The strength of our study is determined by the accessibility of a large, well-documented cohort of individuals with a monogenic cause of hypercholesterolemia who have a 8.5 times increased CVD risk.[28] Patients with heterozygous FH have mutations in the LDL receptor gene leading to plasma LDL cholesterol levels above the 95th percentile of the general population.[29] Although all individuals have severe hypercholesterolemia, CVD risk and mortality varies considerably and depends on both environmental and genetic factors.[30,31] The effect of any single gene on the susceptibility for a complex disorder such as CVD is a priori expected to be modest. Therefore, we prefer to study the association between genetic variants with CVD in a population at increased risk of CVD with the resulting high power to detect potential small effects of the genetic variant.

This is the first study that investigated the combined influence of GR gene variants on CVD susceptibility by using haplotypes. Previous research focused on single nucleotide polymorphisms (SNPs) in the GR gene and their effects on glucocorticoid sensitivity and cardiovascular risk factors.[7] Across the GR gene, there is strong linkage disequilibrium of SNPs with neighboring polymorphisms. Genetic variation in the majority of the population can then be defined into a limited number of haplotypes. The advantage of a haplotype-based approach is its ability to detect multiple effects within the same genetic region. Surprisingly, we found relationships between two GR haplotypes on CVD risk, but none of the single polymorphisms associated with cardiovascular endpoints. These findings suggest that the effects of the *Bcl*I haplotype and the GR-9β haplotype on CVD susceptibility cannot be explained by the individual *Bcl*I and GR-9β polymorphisms. Hence, it is possible that these genetic variants are in linkage disequilibrium with functional but yet unidentified polymorphisms that account for the haplotype effects.

One previous genetic study performed haplotype-analysis of the GR gene and determined association with postdexamethasone cortisol production.[32] This study included all GR polymorphisms that were tested in our analyses, together with other validated SNPs spaced across the gene. Stevens and co-workers found a three-point haplotype of intron B polymorphisms that segregates with increased sensitivity to glucocorticoids, as evidenced by lower cortisol levels after a 0·25 mg dexamethasone suppression test.[32] This three-marker haplotype includes the G allele of the *Bcl*I polymorphism. Since the *Bcl*I polymorphism was not associated

with postdexamethasone cortisol production in Stevens' study and with CVD susceptibility in our study, we could speculate that one of the two other polymorphisms in intron B (the A allele of the intron B 33389 and the T allele of the intron B 33388) may explain the relationship with increased CVD risk in our population.

The association between the GR-9β haplotype and the *Bcl*I haplotype and increased CVD risk was only significant in men. Previously, we already described opposite effects of the ER22/23EK polymorphism on CVD in men and women.[18] Obviously, the hormone dynamics and the risk of CVD differ considerably among males and females, but the exact reason for these differences of GR haplotypes between gender are yet unknown. The response of the hypothalamic-pituitary-adrenal axis to variations in circulating sex steroid concentrations differ between sexes: estrogen primarily exerts stimulatory effects on stress-induced ACTH and glucocorticoid release, whereas testosterone inhibits stress-related hypothalamic-pituitary-adrenal axis activity.[33] Otherwise, high variability in the expression of genes located at the X-chromosome between men and women has been reported and may also account for these gender differences.[34] Obviously, further research is warranted to acquire more information about the mechanisms underlying gender-specific differences in the expression of GR target genes but our observations support that separate analyses of men and women are indicated for studies on GR variants.

Our study has a number of limitations. First, data were obtained from medical records and some information of interest, such as body composition, plasma insulin levels and inflammation markers were not known. The influence of these factors on the relationship between the GR variants and CVD risk could therefore not be measured. In addition, the strength of any association study is highly increased by confirmation of the results in a second independent population. However, to our knowledge, this study is the only large FH cohort with well-defined cardiovascular endpoints. Obviously, our findings in heterozygous FH cannot be extrapolated to other populations and future research should establish the effect of GR haplotypes in other populations with increased CVD risk and in population-based studies.

In conclusion, in this large cohort study of high-risk patients with severe hypercholesterolemia, the GR-9β and the *Bcl*I haplotype were significantly associated with increased CVD risk in men. This suggests that variations at the GR gene modify CVD susceptibility among male FH patients.

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CHAPTER 8
General discussion

Introduction

Heterozygous familial hypercholesterolemia (FH) is an inherited disorder of lipid metabolism, leading to severely elevated low-density lipoprotein (LDL) cholesterol levels and increased risk of cardiovascular disease (CVD).[1] Despite the monogenic cause of FH, CVD susceptibility varies considerably and traditional risk factors account for only a minority of this variation.[2-4] In this thesis, we focussed on the identification of genetic variations at the LDL receptor locus as well as candidate genes outside the LDL receptor locus that could improve risk prediction in FH in order to tailor preventive strategies.

Variation at the LDL receptor locus

Previously, several studies in adult FH patients have assessed whether variation at the LDL receptor locus influenced lipoprotein metabolism and, consequently, cardiovascular risk. These studies, however, have yielded conflicting results.[3,5-13] We proposed that FH children might be more suitable to perform genotype-phenotype studies in because of a lower chronic exposure to known environmental factors that alter lipid levels and they are not selected on CVD. In the present thesis we tested this hypothesis by calculating the intra-class correlation (i.e. the variance in LDL cholesterol levels introduced by familial factors) in a pediatric and adult FH cohort with identified LDL receptor mutations. Familial factors explained 50.4% of the variance in LDL cholesterol levels among children with FH, whereas only 9.5% of the variance in LDL cholesterol levels was explained by family ties in untreated adult FH patients. This shows that children indeed share a more homogeneous environment. It supports that analyses of effects of LDL receptor genotypes in pediatric cohorts are more accurate. Hence, we examined the assumed relationship between LDL receptor genotypes and lipoproteins in a large pediatric cohort and the relation with the occurrence of parental CVD. The double-mutation named N543H/2393del9 was the most frequent mutation in the cohort and belongs to the receptor-defective group. Interestingly, children carrying this mutation had a less deteriorated lipid profile and CVD was expressed to a lesser extent in their parents relative to parents of children with other mutations. In general, however, the variation in lipid profile of the children poorly explained the differences in CVD risk among the parents. Plasma LDL cholesterol concentrations of children with null alleles were higher compared to children with receptor-defective mutations, but we found no significant differences in parental CVD risk between carriers of null alleles and receptor-defective mutations. It seems that during life other factors become more important modulators of CVD risk in FH than the LDL receptor genotype.

Another topic we addressed in this thesis was the response to statin therapy and LDL receptor genotype. After diagnosis, FH patients are treated lifelong with statins to prevent CVD.[14,15]

The lipid lowering response to statin therapy shows considerable interindividual variation and specific LDL receptor mutations have been suggested to affect statin response.[16-23] Results of previous genotype-phenotype studies, however, were conflicting and difficult to compare due to selection on specific founder mutations, limited numbers of patients, different classifications of the LDL receptor mutation types, and a variety of treatment strategies *without randomization*. Therefore, we examined in the present thesis whether the type of LDL receptor mutation influenced the response to statin treatment in a subgroup (only children with genetically confirmed FH) of a randomized, placebo-controlled two-year clinical trial with pravastatin in FH children, aged between 8 and 18 years. The randomization was used to reduce the influence of confounding factors. In addition, our observations were controlled for placebo effects, which enabled us to determine the natural course of the specific LDL receptor mutations during the two-year follow-up. Carotid intima-media thickness (IMT) was used to measure efficacy. IMT can be visualized with non-invasive B-mode ultrasound imaging. The edges of the lumen-intima and media-adventitia interfaces of the posterior artery wall represent the boundaries of the intima-media complex. The distance between the interfaces is called IMT. Carotid IMT has been accepted as a validated marker for atherosclerosis and future cardiovascular outcome.[24-27] In this thesis, we showed that before treatment children with null alleles had already a significantly higher carotid IMT than receptor-defective mutations, independent of their more elevated LDL cholesterol levels. Although the reduction of LDL cholesterol concentrations by statin treatment tended to be less in carriers of null alleles, we observed no significant difference in change of carotid IMT during the trial between the two LDL receptor genotype groups. Hence, after two-year treatment carotid IMT and lipid profile remained more unfavorable in children with null alleles. Our data suggest that the greater carotid IMT in carriers of null alleles was not solely the result of higher LDL cholesterol levels. Knowledge of LDL receptor genotype may therefore improve clinical decision-making: untreated *and* treated children carrying null alleles may have more pronounced atherogenesis that may be partially independent of their more increased LDL cholesterol levels. The risk of CVD is, however, a more complex endpoint.

Candidate genes of cardiovascular disease in FH

With the completion of the Human Genome Project and the availability of high throughput molecular methods, insight into the role of genetic variations in the pathogenesis of atherosclerosis has increased enormously. Several candidate genes have been suggested to influence CVD risk in FH patients already.[28-43] In this thesis we examined three additional candidate genes in 2,400 individuals of the GIRaFH cohort.

The first candidate gene is complement factor H (CFH). Recently, three independent genome-based studies identified the Y402H polymorphism of CFH as an important risk factor

of age-related macular degeneration.[44-46] This disorder is a progressive late-onset disease affecting central vision and results in progressive damage to the macula, ultimately leading to blindness.[47] Epidemiologic, genetic, and pathological evidence suggest a role for atherosclerosis in the pathophysiology of macular degeneration.[48] We therefore assessed the relationship between this common CFH variant and CVD risk. We observed that homozygous carriers of the Y402H polymorphism in CFH were protected against CVD in our FH population. This finding emphasizes the involvement of the complement system in the pathophysiology of atherogenesis. Atherosclerosis is characterized by a strong inflammatory component and activation of the complement system could be an important link between inflammation and atherosclerosis.[49,50] CFH plays a critical role in the protection of the host cells and tissues from damage by complement activation.[51] The localization of CFH, C-reactive protein, and proteoglycans in the superficial layer of the arterial intima suggests that CFH may protect the arterial wall from excess complement activation.[52] The Y402H polymorphism in the CFH gene is located within binding sites for heparin and C-reactive protein. The replacement of tyrosine for histidine at position 402 in exon 9 may alter the binding properties and could affect the level of inflammation, thereby influencing atherosclerosis.[53] Our finding is in total contrast with observations in macular degeneration that found increased risk of this disease in carriers of the CFH variant. We hypothesized that differences in angiogenesis may be a possible explanation for the opposite effects of the Y402H polymorphism in CVD and macular degeneration. Insufficient collateral blood vessel formation increases the burden of CVD, whereas neovascularisation clearly increases the severity of macular degeneration. Although speculative, CFH could be involved in the process of neovascularisation. To date, three studies have been conducted on potential associations between the Y402H polymorphism and CVD but with conflicting results.[54-56] This inconsistency may be partly due to heterogeneity in the cardiovascular phenotype, variations in population and study design, and because previous studies were underpowered. However, the CFH variant was associated with an increased risk of myocardial infarction in participants of the Rotterdam Study, a prospective population-based study among men and women aged 55 years and over.[56] We could not entirely explain the discrepancy in their and our results by differences in cardiovascular phenotype, age, and lipid concentrations. Obviously, future research is warranted to acquire more detailed characterization of the CFH gene and to establish the functional consequences of the CFH Y402H variant and its impact on complement activation, neovascularisation, and complex diseases such as macular degeneration and atherosclerosis.

The second candidate gene is the adenosine triphosphate binding cassette (ABC) G8, which is involved in plant sterol metabolism. The important role of plant sterols in human is illustrated by sitosterolemia in which plasma levels of plant sterols are increased >50-fold due to mutations in the ABCG8 gene, leading to severe atherosclerotic arterial disease at a young age.[57,58] Elevated plant sterol concentrations were also associated with cardiovascular events in several small studies among nonsitosterolemic individuals, thereby suggesting that

plant sterols are atherogenic.[59-63] ABCG8 actively prevents accumulation of plant sterols by effluxing sterols from enterocytes to small intestinal lumen and promoting the excretion of sterols from the liver into the bile.[64] Two polymorphisms in ABCG8 (D19H and T400K) have been shown to influence both plant sterols and cholesterol levels but their relation with CVD susceptibility was never assessed.[65-67] In the GIRaFH study, we found that the D19H polymorphism was not associated with plasma cholesterol levels nor with total CVD risk, but there was an association with higher risk of coronary heart disease. Based on the association with lower plant sterol concentrations, we had a priori expected that carriers of the D19H variant would have had a decreased risk of cardiovascular events.[65,66] Plasma plant sterol levels were not available in our cohort and could not be obtained because of its retrospective design. There is, however, controversy about the influence of plant sterols on atherosclerosis, because a high plant sterol content of the diet has been previously related with atheroprotection.[68,69] In the Asian diet, for example, the main protein is soy (which is rich in plant sterols), and this has been postulated as one of the possible explanations for the lower incidence of CVD in some Asian populations.[68] In addition, adding plant sterols to margarine and other foods is currently a therapy to optimize cardiovascular risk profile by decreasing plasma cholesterol concentrations.[69] The unfavorable effect of the D19H polymorphism could be restricted to coronary heart disease in our study since this is probably a more homogeneous phenotype than total CVD. The T400K polymorphism resulted in significant higher plasma HDL cholesterol levels, but we found no association with cardiovascular endpoints. These data suggest that the two genetic variations in ABCG8 analyzed in this thesis are not major contributors for differences in CVD susceptibility among FH patients.

The third candidate gene is the glucocorticoid receptor (GR). The GR mediates the effects of glucocorticoids.[70] Glucocorticoids play an important role in the pathofysiology of atherosclerosis by their contribution to the development of hypertension, dylipidemia, impaired glucose tolerance, and central adiposity.[71] The sensitivity to glucocorticoids, however, varies considerably among individuals.[72] Several functional genetic variants within the GR gene have been associated with variation in glucocorticoid sensitivity and cardiovascular risk factors.[73] In this thesis, we first studied the ER22/23EK polymorphism of GR. Carriers of the ER22/23EK variant were suggested to be relative resistant to glucocorticoids, because they had higher serum cortisol concentrations and a smaller decrease in cortisol levels after dexamethasone suppression testing.[74] In previous studies, this polymorphism has been associated with a beneficial cardiovascular risk profile defined by lower plasma total and LDL cholesterol levels, increased insulin sensitivity, a beneficial body composition, lower C-reactive protein levels, and a better overall survival.[74-76] Hence, we hypothesized that this functional ER22/23EK variant in the GR gene might decrease CVD risk. We found, however, that the association between the ER22/23EK variant and the occurrence of CVD was not significant overall. In separate analyses of men and women, however, we found opposite effects of the polymorphism on CVD. This variant has been preferentially analyzed in male individuals and most association

studies did not analyze men and women separately. In a cohort of young-adults followed from the age of 13 to 36 years, the body composition in carriers of the ER22/23EK variant was different between sexes: only male carriers were taller, leaner, and had more muscle strength.[75] The reason for these differential effects of the ER22/23EK variant between men and women is yet unknown. Differing responses of the hypothalamic-pituitary-adrenal axis to variations in circulating sex steroid concentrations have been proposed as a possible explanation. Soon after the publication of these findings, other investigators reported that the ER22/23EK polymorphism was only present in combination with another polymorphism: the GR-9*β* variant.[77] This observation prompted us to examine the effect of this GR variant in our FH population. In addition, we analyzed the N363S and *Bcl*I polymorphisms in GR, which have been previously associated with an increased sensitivity to glucocorticoids and an increased cardiovascular risk profile.[78-84] Individual analyses of these functional polymorphisms in the GIRaFH cohort revealed no significant association with CVD in men nor women. In this thesis, we also analyzed the combined effect of the GR variants using haplotypes to obtain more detailed information about the genetic variation at the locus in relation with CVD. As already observed by other research groups, we found that the ER22/23EK, N363S, and *Bcl*I polymorphisms never occurred on the same allele and ER22/23EK carriers always carried the GR-9*β* as well. Furthermore, the GR-9*β* also never occurred with the N363S and *Bcl*I polymorphisms on one allele. In women, none of the haplotypes was significantly related with CVD. In men, the GR-9*β* haplotype (consisting of the mutant allele of the GR-9*β* polymorphism and the wild type alleles of the other three polymorphisms) was associated with an increased CVD risk. A similar increased association with CVD in men was found for the *Bcl*I haplotype (that consists of the mutant allele of the *Bcl*I polymorphism and wild types of all other polymorphisms). We concluded that common functional variants in the GR gene explained a small proportion of the variation in CVD risk among FH men.

Limitations of genetic association studies

The initial enthusiasm about genetic association studies is partially tempered because published positive associations could frequently not be replicated in subsequent studies. This inconsistency among genetic association studies may be due to false positive results, false negative results or true variability in association among different populations (Box 1).[85-89] Several limitations of genetic association studies will be discussed in more detail.

The most likely reason for failure of replication is a false-positive result due to multiple testing (type I error), publication bias, and small sample size.[85,89] When none of the genetic variants are related to CVD, 1 out of 20 will show significant association at a p-value equal to or below 0.05 by chance alone. There are a number of methods used to deal with multiple testing problems. In the classical approach for correcting p-values, the nominal p-value is

Box 1. Causes of irreproducibility of genetic association studies.

- **False positive studies**
  multiple testing
  publication bias
  small sample size
  population stratification
  genotyping error
- **False negative studies**
  small sample size
  failure to consider that initial effect size is overestimation of true effect size
  inconsistent phenotype
- **True variation in association between populations**
  variation in linkage disequilibrium between the SNP being studied and the true causal variant among populations
  population-specific gene-gene or gene-environmental interactions
  population admixture

multiplied by the number of hypotheses tested (Bonferroni correction). However, this correction is too conservative, because many true positive associations between genetic variants and phenotypes may not become significant. In the Bayesian method, the threshold p-value for declaring success takes into account the a priori probability that a particular genetic variant is truly associated with the phenotype. This approach allows calculation of the posterior probability that an association is genuine based on pre-test estimates, but the method gives rise to analytic problems. There is clear consensus that no available statistical method can effectively distinguish between true-positives and false-positives in genetic association studies.

Another potential source of variable findings among genetic association studies is false negative results because subsequent studies are underpowered (type II error). This difficulty is heightened by the winner's curse phenomenon, in which the initial reported effect size is almost always an overestimation of the true effect of the genetic marker.[90,91] Therefore, replication studies should be powered to detect effect sizes that are a bit smaller than the initial effect size reported and thus need to include larger numbers of patients to achieve statistical significance.

Of course true variation in association between the populations of two different studies may exist. If a genetic variant's influence on CVD risk is only manifest on a certain genetic or environmental background, then differences in genetic and environmental factors could account for irreproducibility. The strong genetic background in FH patients (leading to severe hypercholesterolemia) could modify the association between a genetic marker and CVD susceptibility; this may explain discrepant results in FH populations and population-based

studies. True heterogeneity between the populations may also exist if there is variation in linkage disequilibrium between the SNP being studied and the true causal variant among populations. Historical recombination between the tested and the causal variant could be different in populations leading to variation in the strength of an association between populations.

Finally, the success of genetic studies highly depends on the characterization of the phenotype of interest.[89] For example, myocardial infarction can be arbitrarily defined as a history of myocardial infarction based on questionnaires or can be assessed according to rigorous and reproducible criteria such as classical symptoms >15 min with specific electrocardiographic abnormalities and/or elevated cardiac enzymes >2 times the upper limit of normal as we did in the GIRaFH study. The first phenotype is likely to include heterogeneity in cause and disease course and may confound genetic analyses. Therefore, precision of the study phenotype is of key importance in genetic association studies.

Future perspectives: genetic association studies

In general, to detect a significant correlation between a specific genetic marker and CVD susceptibility effectively, the association must be strong and the allele of interest must occur with relatively high frequency. Rare genetic markers with strong effects on CVD susceptibility and common genetic markers with weaker associations to the phenotype are both difficult to identify and polygenic involvement may cause inconsistent results among genetic studies.[92] Candidate gene studies comprise mainly common genetic variants with a relatively small effect on the phenotype. Simple association studies of SNPs in the general population are not likely to identify new genes in which rare variants have a strong effect on the phenotype in a few individuals; case-control studies in high-risk populations such as FH may be a powerful alternative to detect these. Genotyping FH individuals at the extremes of the phenotype distribution can increase statistical power of a genetic association study. For example, the genotypes of FH patients with severe premature onset of CVD symptoms can be compared with older FH patients who did not experience CVD during life. This study design will improve the power to detect rare variants with relatively large effects.

Highly significant associations with a plausible biological mechanism are most convincing. A reported association will gain evidence by replication of the association with the same allele, the same phenotype, and the same direction of the effect in independent population samples. The genes that determine the susceptibility to CVD in FH should be tested in other high-risk populations (diabetes mellitus, hypertension) and in the general population also. This type of replication in populations without FH serves another purpose: generalization of the findings. On the one hand, a monogenic background and large variation of CVD risk in FH offer a unique opportunity to identify genes that are generally involved in CVD. On the other

hand, their expression may be context dependent and restricted to severe hypercholesterolemia and other high-risk disorders.

Ultimately, results could be combined in a meta-analysis to give a reliable estimation of the effect of gene variation on CVD susceptibility in FH.[91] Meta-analyses, however, are not devoid of potential biases, because most are conducted on highly heterogeneous studies according to size and phenotype and because publication bias against studies with negative results may lead to overestimation of the true effect. Another possibility to increase statistical power to detect candidate genes is multicenter, international collaborations for collecting large FH populations. Investigators can routinely bank DNA and clinical data of well-phenotyped FH patients in anticipation of large-scale genomic analyses to determine the association of gene(s) with CVD risk.

Future perspectives: genome wide association studies

Multiple genes generally act through complex networks involving gene-gene and gene-environment interactions. Accordingly, each gene explains only a limited proportion of the CVD risk, thereby making it difficult to explicitly study one gene. A genomic strategy that analyzes genes on the entire genome may be more informative and more efficiently than the candidate gene approach that focuses on selected genes.[86] Two main strategies are used to identify potential candidate genes in genomic studies: genetic-linkage analyses and genome wide association studies.[89] Linkage studies in families are useful in analyzing the entire genome to identify chromosomal regions that may harbor genes responsible for CVD. The linkage method has been extremely successful in dissecting Mendelian disorders, but analytic problems occur when 3 or more loci are involved. Genome wide association studies can be used to determine the effect of thousands of genetic variants simultaneously on occurrence of CVD. Such genomic studies are based on hypothesis-free methods in which no pre-selection of genes is made based on inference about a potential relationship with CVD.

In a recent gene centric study, a large collection of SNPs that were selected on potentially affecting protein function of expression were tested in multiple populations.[93] A total of 4 gene variants were associated with myocardial infarction. These 4 genes were not related before with myocardial infarction. This illustrates the potential for genomic association studies in the search for novel insights into CVD pathogenesis. Especially, because the genomic coverage of novel gene-chips, containing 500-550K SNPs, is much better. False positive findings were largely eliminated by the multistage design; replication in multiple independently recruited, large populations was performed to reduce the false discovery rate.

No genome wide association studies on CVD risk in FH have been reported so far. As a consequence of the recent progress in genome wide association scans and with the lower-

ing of genotyping costs, abundant genetic association studies and eventually genome wide association studies will be conducted among FH populations in the next years.

Prognostic model

The incorporation of new genetic risk factors in a prognostic model that gives an accurate estimation of a FH individual's CVD risk will be the next challenging step towards more personalized preventive medicine. Prognostic models in FH can be developed using multiple regression analysis of a data set containing genetic and clinical information of individual FH patients. The development of such a prognostic model would ideally take place in a very large database to accurately estimate regression coefficients. In practice, this ideal is seldom achieved because the data sets of individual patient data are often relatively small. Recently, a new statistical method was described that combines univariable regression results from the medical literature (for example from meta-analyses) with univariable and multivariable results from the data set containing individual patient data.[94] This 'adaptation method' was shown to benefit from the incorporation of literature data evidenced by a better model performance and less variability of the regression coefficients when compared with models that use individual patient data only. This method offers interesting opportunities to optimally combine the results of a large number of studies in the development of a prognostic model. Finally, the prognostic model should be validated in independent FH populations to assess its overall performance and optimize the model when needed. Although findings may be generalized to other populations, relevant post-test probabilities will only be attained when genetic panels have extremely high discriminative ability or when pre-test probabilities are high.

Conclusion

In conclusion, children with FH provide a better model to perform genotype-phenotype analyses than FH adults. LDL receptor genotype may guide treatment strategies in future, because selection of null alleles identifies children with FH who may have more pronounced atherogenesis and who may benefit by more aggressive as well as earlier lipid lowering treatment. The risk of CVD among adults with FH is, however, a more complex endpoint. With the exception of rare mild LDL receptor mutations, the specific LDL receptor mutation provides limited prognostic information over and above the untreated LDL cholesterol level and general CVD risk profile. The Y402H polymorphism of CFH is strongly related with decreased CVD risk in FH whereas variation at the GR locus was associated with increased CVD susceptibility among FH men. At present, however, there is no strong evidence to test individual

polymorphisms in routine clinical practice to improve risk prediction in FH in order to tailor preventive strategies to the individual risk level. With the availability of large-scale sequencing and the ability to measure genotypes at thousands of loci simultaneously, however, we expect that the creation of a genetic fingerprint of CVD risk in FH is feasible in the coming years. The challenge remains to link genetic data and clinical information to refine individualized approaches to CVD care in FH patients.

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CHAPTER 9

Summary

Nederlandse samenvatting

Dankwoord

Curriculum vitae

Summary

Familial hypercholesterolemia (FH) is a common inherited disorder of lipid metabolism caused by mutations in the low-density lipoprotein (LDL) receptor gene. FH is characterized by elevated plasma LDL cholesterol levels, tendinous xanthomas, and premature cardiovascular disease (CVD).

As introduced in **chapter 1**, CVD susceptibility in heterozygous FH varies considerably and depends only partially on the traditional cardiovascular risk factors. Modifier genes have been suggested to influence the susceptibility to CVD in FH. The aim of this thesis was to identify genetic factors that could potentially enhance the ability of clinicians to identify FH individuals with severely increased CVD risk, who will most likely benefit from targeted therapeutic intervention. First, we focused on genetic variants in the LDL receptor gene (chapter 2 and 3). Secondly, we analyzed the effects of candidate genes outside the LDL receptor locus (chapter 4 to 7).

In **chapter 2** we first established that children with FH provide a better model than adults to analyze the relation between the LDL receptor genotype and lipid levels. Subsequently, we investigated the role of variations at the LDL receptor locus on lipid levels and parental CVD risk in 645 children with FH. In these children, null alleles were clearly associated with more elevated LDL cholesterol levels compared to receptor-defective mutations. However, CVD risk in the parents was not significantly different between carriers of null alleles and receptor-defective mutations. Hence, the variation in lipid profile of the children poorly explained the differences in CVD risk among parents. The N543H/2393del9 mutation was the most frequent LDL receptor variant in the cohort. Interestingly, this specific mutation was clearly associated with a less deteriorated lipid profile and the parents had less often CVD relative to parents with other mutations.

In **chapter 3** we assessed the relationship between LDL receptor genotype and response to statin treatment in children with genetically confirmed FH using carotid intima-media thickness (IMT) to measure efficacy. All children participated in a randomized, placebo-controlled, double-blind, two-year trial with pravastatin. At baseline, children with null alleles had higher LDL cholesterol levels and a greater carotid IMT compared to children with receptor-defective mutations. Although the reduction of LDL cholesterol levels by pravastatin treatment tended to be less in carriers of null alleles, we observed no significant difference in change of carotid IMT during the trial between the two LDL receptor genotype groups. After two-year treatment, however, carotid IMT and lipid profile continued to be more unfavorable in children with null alleles. Selection of null alleles identifies children with more pronounced atherogenesis who may benefit by more aggressive as well as earlier lipid lowering treatment.

In **chapter 4** we describe the role of the Y402H polymorphism of complement factor H (CFH) in CVD. This polymorphism has been strongly and consistently related with increased risk of age-related macular degeneration, an atherosclerosis-related phenotype. We hypothesized

that the Y402H variant is also related to CVD susceptibility. Therefore we investigated this polymorphism in the GIRaFH-study (**G**enetic **I**dentification of **R**isk F**a**ctors in **F**amilial **H**ypercholesterolemia), a Dutch cohort of 2400 unrelated FH patients, aged 18 years and older, who were identified at lipid clinics throughout the Netherlands between 1989 and 2002. Homozygous carriers of the Y402H polymorphism had a 2-fold decreased risk of CVD relative to heterozygous carriers and noncarriers. Adjustment for clinically relevant cardiovascular risk factors and age effects did not influence this association. We also analyzed the risk of coronary, cerebral, and peripheral events separately and found similar decreased relative risks in homozygous Y402H carriers. These results suggest that the CFH gene is a modifier gene of CVD among patients with FH.

In **chapter 5** we investigated a second candidate gene named ABCG8 because of its association with plasma plant sterol levels. High plasma concentrations of plant sterols have been suggested to increase CVD risk. In the GIRaFH-study, we examined associations between the D19H and T400K polymorphisms in the ABCG8 gene and cardiovascular events. The D19H variant was not associated with plasma cholesterol levels nor with total CVD risk, but there was evidence of an association with higher risk of coronary heart disease after adjustment for all cardiovascular risk factors. We observed no significant relationship between the T400K polymorphism and plasma cholesterol levels or cardiovascular endpoints. These data suggest that these two genetic variations in the ABCG8 gene are not major contributors for differences in CVD risk among patients with FH.

In **chapter 6** we describe a third candidate gene of CVD: the glucocorticoid receptor (GR) gene. Glucocorticoids influence atherosclerosis by their contribution to the development of hypertension, dylipidemia, impaired glucose tolerance, and central adiposity. Glucocorticoids exert their function primarily via binding to the GR. In this chapter we focused on the functional ER22/23EK polymorphism in the GR gene. Individuals with this polymorphism are relatively resistant to the downstream consequences of glucocorticoids and they have a more favorable cardiovascular risk profile. We determined whether carriership of the ER22/23EK improves CVD suceptibility in the GIRaFH-study. In contrast to expected results, we observed no significant association of the ER22/23EK variant with CVD risk. In addition, no differences existed in cardiovascular risk factors between patients with and without ER22/23EK. However, in a post hoc analysis we found opposite effects of the polymorphism on CVD in men and women.

In **chapter 7** we studied the combined effect of the ER22/23EK variant and several other functional polymorphisms within the GR gene (N363S, *Bcl*I, and GR-9β) on CVD risk in the same FH population. We analyzed the combined effect of all GR variants by reconstructing haplotypes. Men with the GR-9β haplotype (only variation at the GR-9β site) and the *Bcl*I haplotype (only variation at the *Bcl*I site) had a higher CVD risk compared to male carriers of the wild type haplotype (wild type alleles of all four polymorphisms). In women, none of the haplotypes was significantly related with CVD. We did not find an association between

any of the single functional GR gene polymorphisms with CVD risk nor with cardiovascular risk factors such as body mass index, plasma lipid levels, or hypertension. Thus two common haplotypes in the GR gene explained a small proportion of the variation in CVD susceptibility among FH men.

Chapter 8 contains a general discussion in which the main findings of this thesis are put into a broader perspective. We also discuss the limitations and potential pitfalls when conducting candidate gene studies and developing individual prognostic models. Finally, we conclude that LDL receptor genotype may guide treatment strategies in future, but it is not a major determinant of CVD risk in patients with FH. Although there is no strong evidence to test individual polymorphisms in routine clinical practice to improve risk prediction in FH in order to tailor preventive strategies to the individual risk level, the CFH gene and GR gene appear modifier genes of CVD in FH individuals.

Nederlandse samenvatting

Familiaire hypercholesterolemie (FH) is een vaak voorkomende erfelijke aandoening van het lipiden metabolisme welke wordt veroorzaakt door mutaties in de low-density lipoprotein (LDL) receptor gen. FH wordt gekenmerkt door verhoogde plasma LDL cholesterol waarden, peesxanthomen en premature hart-en vaatziekten (HVZ).

Zoals geïntroduceerd in **hoofdstuk 1** is het cardiovasculaire risico in patiënten met heterozygote FH zeer variabel en dit kan slechts gedeeltelijk worden verklaard door de traditionele risicofactoren. Zogenoemde modificerende genen zouden het cardiovasculaire risico in FH kunnen beïnvloeden. Het doel van dit proefschrift was het identificeren van genetische factoren welke de clinicus kunnen helpen om FH personen met het hoogste cardiovasculaire risico te onderscheiden die baat hebben bij agressieve, individuele therapeutische behandeling. In eerste instantie hebben we ons gericht op genetische varianten in het LDL receptor gen (hoofdstuk 2 en 3). Hierna hebben we de effecten van enkele kandidaat-genen buiten het LDL receptor locus onderzocht (hoofdstuk 4 tot 7).

In **hoofdstuk 2** hebben we eerst aangetoond dat kinderen met FH meer geschikt zijn dan volwassenen om fenotype studies van het lipidenmetabolisme in te verrichten. Vervolgens hebben we de rol van variaties in het LDL receptor locus onderzocht op cholesterol waarden van 645 kinderen met FH en op het cardiovasculaire risico van hun ouders. In deze kinderen waren nul allelen duidelijk geassocieerd met hogere LDL cholesterol waarden dan receptordefectieve mutaties. Echter het risico van HVZ in de ouders was niet significant verschillend tussen dragers van nul allelen en receptordefectieve mutaties. De variatie in het lipiden profiel bij de kinderen verklaart dus slecht de verschillen in HVZ risico bij de ouders. De N543H/2393del9 mutatie was de meest frequente LDL receptor mutatie in het cohort. Deze specifieke mutatie was duidelijk geassocieerd met een minder ongunstig lipiden profiel en de ouders hadden minder HVZ in vergelijking met de ouders van andere mutaties.

In **hoofdstuk 3** hebben we de relatie tussen het LDL receptor genotype en het effect van statine behandeling in kinderen met genetisch bewezen FH onderzocht door het meten van de intima-media thickness (IMT) in de carotiden. Alle kinderen hadden deelgenomen aan een gerandomiseerd, placebogecontroleerd, dubbelblind, tweejarig geneesmiddelen onderzoek met pravastatine. Bij de start van het onderzoek hadden kinderen met nul allelen hogere LDL cholesterol waarden en een dikkere IMT dan kinderen met receptordefectieve mutaties. Hoewel de LDL cholesterol daling door pravastatine minder leek in dragers van nul allelen vonden we geen significante verschillen in IMT verandering tijdens het onderzoek tussen de twee LDL receptor genotypes. Na twee jaar behandeling bleven IMT en het lipiden profiel echter meer ongunstig in kinderen met nul allelen. Selectie van nul allelen identificeert kinderen met meer uitgesproken atherosclerose en zij zouden baat kunnen hebben bij een agressievere en ook eerdere cholesterol verlagende behandeling.

In **hoofdstuk 4** beschrijven we de rol van het Y402H polymorfisme in complement factor H (CFH) in HVZ. Dit polymorfisme is sterk en consequent geassocieerd met een verhoogd risico op leeftijdsgerelateerde maculadegeneratie, een atherosclerose-gerelateerd fenotype. Wij veronderstelden dat de Y402H variant ook geassocieerd is met het cardiovasculaire risico. Vandaar dat we dit polymorfisme hebben onderzocht in de GIRaFH studie (Genetische Identificatie van Risicofactoren in Familiaire Hypercholesterolemie), een cohort van 2400 niet-gerelateerde FH patiënten, 18 jaar en ouder, die werden geïdentificeerd in lipiden klinieken uit geheel Nederland tussen 1989 en 2002. Homozygote dragers van het Y402H polymorfisme hadden juist een sterk verlaagd cardiovasculair risico in vergelijking met heterozygote dragers en niet-dragers. Correctie voor klinisch relevante risicofactoren en leeftijdseffecten kon deze associatie niet beïnvloeden. We hebben coronaire, cerebrale en perifere vasculaire ziekten ook apart geanalyseerd en vonden vergelijkbare verlaagde relatieve risico's in homozygote Y402H dragers. Deze bevindingen suggereren dat het CFH gen een modificerend gen is voor HVZ in patiënten met FH.

In **hoofdstuk 5** hebben we een tweede kandidaat gen, ABCG8, onderzocht vanwege de associatie van dit gen met plasma plantsterolen. Hoge plasma concentraties van plantsterolen zouden het cardiovasculaire risico mogelijk kunnen verhogen. In de GIRaFH studie hebben we de associatie tussen de D19H en T400K polymorfismes in het ABCG8 gen en HVZ onderzocht. De D19H variant was niet geassocieerd met cholesterol waarden noch met het totale cardiovasculaire risico, maar er waren aanwijzingen voor een hoger risico op coronaire hartziekten na correctie voor andere risicofactoren. We vonden echter geen significante relatie tussen het T400K polymorfisme en cholesterol waarden of cardiovasculaire eindpunten. Deze data suggereren dat de twee genetische variaties in het ABCG8 gen geen belangrijk aandeel hebben in het cardiovasculaire risico van patiënten met FH.

In **hoofdstuk 6** beschrijven we een derde kandidaat gen voor HVZ: het glucocorticoïd receptor (GR) gen. Glucocorticoïden beïnvloeden atherosclerose door hun bijdrage aan de ontwikkeling van hypertensie, dyslipidemie, gestoorde glucose tolerantie en centrale adipositas. Glucocorticoïden oefenen hun functie voornamelijk uit door binding aan de glucocorticoïd receptor. In dit hoofdstuk hebben we ons geconcentreerd op het functionele ER22/23EK polymorfisme in het GR gen. Individuen met dit polymorfisme zijn relatief resistent voor de nadelige effecten van glucocorticoïden en zij hebben een gunstiger cardiovasculair risicoprofiel. Wij hebben in de GIRaFH studie geanalyseerd of het ER22/23EK polymorfisme het cardiovasculaire risico in FH patiënten verlaagd. In tegenstelling tot de verwachte resultaten vonden we geen significante relatie tussen de ER22/23EK variant en HVZ. Ook waren er geen verschillen in cardiovasculaire risicofactoren tussen personen met en zonder het ER22/23EK polymorfisme. Echter in een post hoc analyse zagen we tegengestelde effecten van het polymorfisme op HVZ in mannen en vrouwen.

In **hoofdstuk 7** bestuderen we het gecombineerde effect van de ER22/223EK variant en enkele andere functionele polymorfismes in het GR gen (N363S, *Bcl*I en GR-9β) op het

cardiovasculaire risico in dezelfde FH populatie. We analyseerden het gecombineerde effect van alle GR varianten door haplotypes te reconstrueren. Mannen met het GR-9β haplotype (enige variatie op de GR-9β plaats) en het *Bcl*I haplotype (enige variatie op de *Bcl*I plaats) hadden een hoger risico op HVZ dan mannen met het wild type haplotype (wild type allelen van alle 4 de polymorfismes). In vrouwen was geen van de GR haplotypes significant geassocieerd met HVZ. We vonden geen relatie tussen één van de enkele functionele GR gen polymorfismes met HVZ risico noch met cardiovasculaire risicofactoren zoals body mass index, lipiden waarden of hypertensie. Wij concluderen dat twee veel voorkomende haplotypes in het GR gen een klein deel van de variatie in het cardiovasculaire risico in mannen met FH verklaren.

Hoofdstuk 8 bevat een algemene discussie waarin de belangrijkste bevindingen van dit proefschrift in een breder perspectief worden gezet. We bespreken ook de beperkingen en mogelijke valkuilen wanneer kandidaat gen studies worden verricht en individuele prognostische modellen worden gemaakt. Ten slotte concluderen we dat het LDL receptor genotype een leidraad kan zijn voor behandelingsstrategieën in de toekomst, maar dat het geen belangrijke determinant is van het cardiovasculaire risico in FH patiënten. Momenteel is er geen bewijs om individuele polymorfismes in de praktijk te testen om de risicovoorspelling in FH te verbeteren en preventieve strategieën naar een individueel niveau te tillen, maar het CFH en GR gen lijken modificerende genen van HVZ in patiënten met FH.

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Curriculum vitae

Kristel Koeijvoets werd op 30 januari 1976 te Roosendaal geboren. In 1994 behaalde zij haar VWO diploma aan het Norbertus College te Roosendaal. Aansluitend ging zij geneeskunde studeren aan de Erasmus Universiteit in Rotterdam. In 2000 behaalde zij haar artsexamen met lof. Zij werkte in 2001 als AGNIO interne geneeskunde in het Medisch Centrum Rijnmond-Zuid, locatie Clara te Rotterdam (Dr. A. Grootendorst). In 2002 begon zij haar promotieonderzoek en haar opleiding tot internist als AGIKO aan het Erasmus Medisch Centrum Rotterdam (Dr. E.J.G. Sijbrands, Prof.dr.H.A.P. Pols, Prof.dr.J.J.P. Kastelein). Tevens startte zij de opleiding tot Master of Science in de richting 'Clinical Epidemiology' aan the Netherlands Institute of Health Sciences, welke in 2006 succesvol werd afgerond. Momenteel werkt zij in het Medisch Centrum Rijnmond-Zuid, te Rotterdam in het kader van haar opleiding tot internist.