Towards Genetic Prediction of Coronary Heart Disease in Familial Hypercholesterolemia

Jeroen van der Net

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Towards Genetic Prediction of Coronary Heart Disease in Familial Hypercholesterolemia

Op weg naar genetische voorspelling van coronaire hartziekten bij patiënten met familiaire hypercholesterolemie

Proefschrift

ter verkrijging van de graad van doctor aan de Erasmus Universiteit Rotterdam

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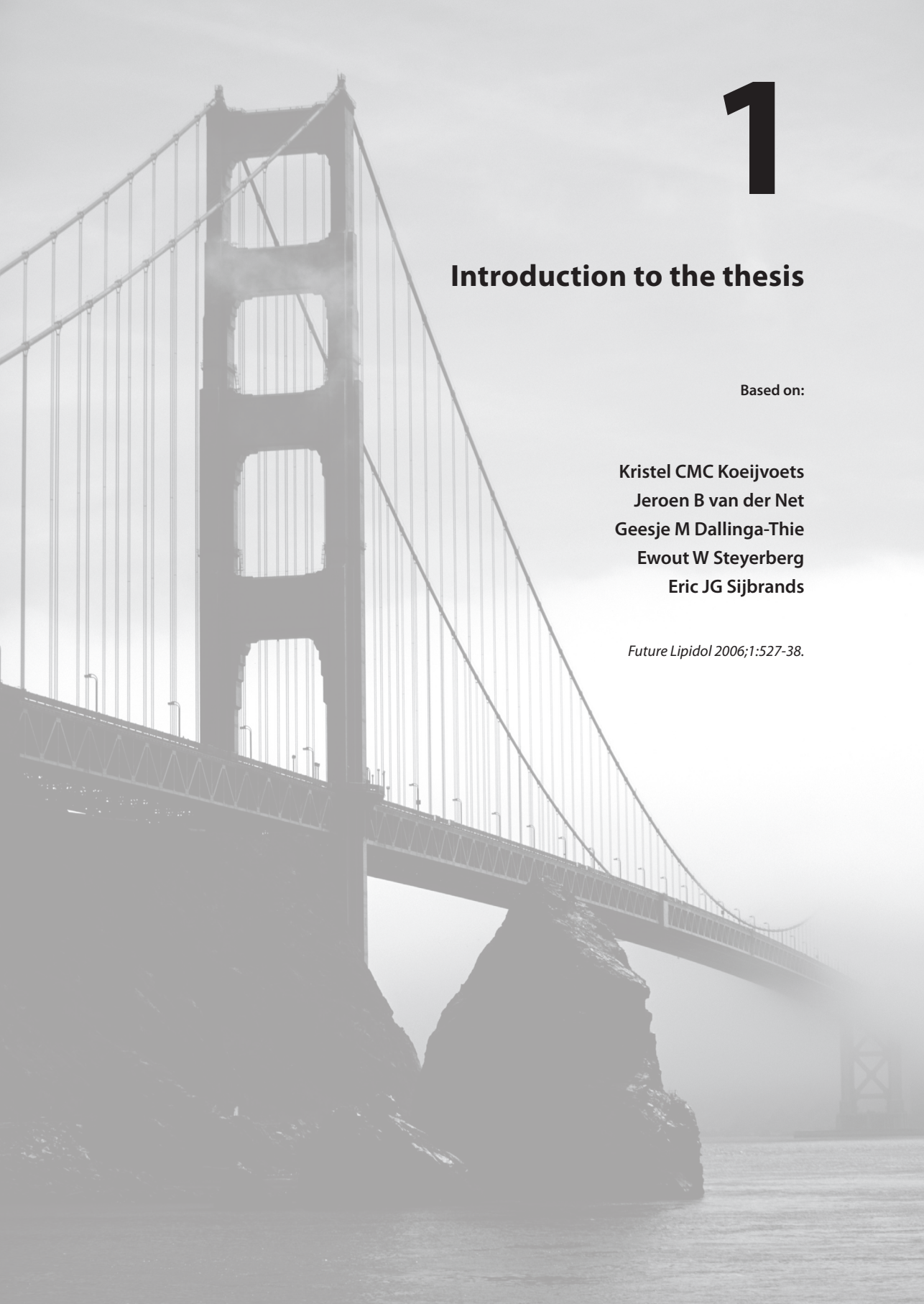

1

Introduction to the thesis

Based on:

Kristel CMC Koeijvoets
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Geesje M Dallinga-Thie
Ewout W Steyerberg
Eric JG Sijbrands

Future Lipidol 2006;1:527-38.

FAMILIAL HYPERCHOLESTEROLEMIA

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.[1] The LDL receptor is a transmembrane protein that regulates plasma cholesterol levels by uptake of LDL particles from the blood circulation (Figure). Mutations in the LDL receptor gene cause insufficient uptake of circulating LDL particles, which raises the endogenous cholesterol production by the hepatocytes, resulting in twofold increased plasma concentrations of LDL cholesterol in patients with the heterozygous form of FH.[2] The rare (1/million) homozygous FH patients have severely reduced or completely absent residual function of the LDL receptor causing extremely raised plasma LDL cholesterol concentrations. These patients develop tendon xanthomas in childhood and massive atherosclerosis occurs frequently at a very young age. This thesis, however, focuses on patients with heterozygous FH, which is more common with a prevalence of 1/500 in Western societies.[3] The typical heterozygous FH patients develop tendon xanthomas and have accelerated atherosclerosis and coronary heart disease (CHD) at a young age.[2] Nevertheless, substantial variation is seen in the age of onset of CHD among patients with heterozygous FH.[4, 5]

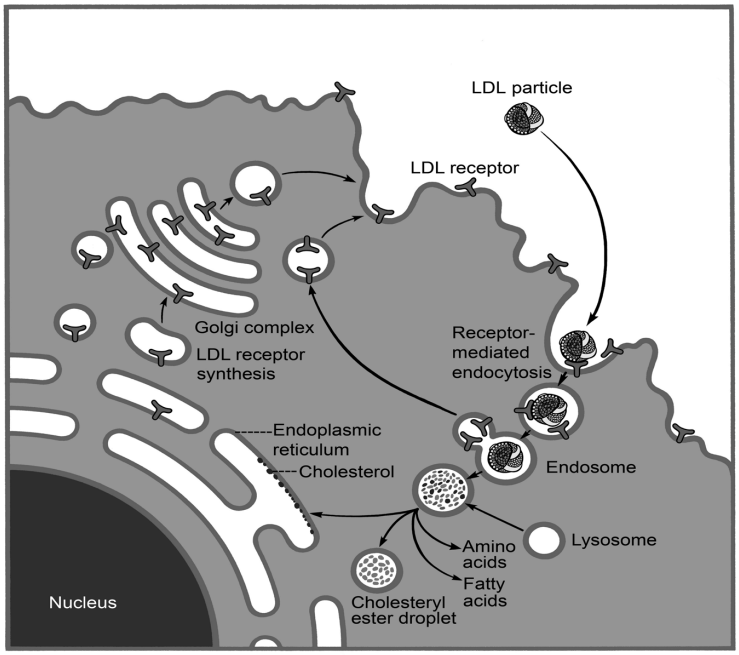


Figure. Schematic overview of LDL receptor cycle in the hepatocyte.

ETIOLOGY OF CORONARY HEART DISEASE

CHD is mainly caused by atherosclerosis of the coronary arteries. Atherosclerosis involves the slow formation of fatty atherosclerotic deposits called 'plaques' in the arteries. The etiology of atherosclerosis is complex and can be described as an inflammatory cascade marked by endothelial dysfunction, leukocyte recruitment, and proliferation of smooth muscle cells, ultimately culminating in plaque formation.[6] The addition of plaque rupture, thrombosis, or haemorrhage can lead to acute CHD (myocardial infarction, instable angina pectoris, or sudden cardiac death). A plaque that is susceptible to such complications is called a vulnerable plaque.[7] The susceptibility of an individual to develop acute CHD is not only determined by the vulnerable plaque, but also by factors related to plaque inflammation, blood coagulability, and myocardial susceptibility to develop fatal arrhythmia.[7, 8]

CORONARY HEART DISEASE AND MORTALITY IN FAMILIAL HYPERCHOLESTEROLEMIA

Before the widespread use of statins for treatment of hypercholesterolemia, approximately 52-85% of men with FH and 32-58% of women with FH had CHD by the age of 60 years.[9] Typically, the mean age of CHD onset in untreated men is between 40 and 45 years and in women about ten years later. In order to prevent the development of this premature CHD, FH patients receive lifelong treatment with ß-hydroxy-ß-methylglutaryl coenzyme A reductase inhibitors (statins), resulting in a lower incidence of CHD in the present-day, statin-treated patients.[10, 11] A mortality study in a large family-tree provided insight in the natural course of FH, as the persons in the pedigree were not selected on the basis of clinical manifestations of FH.[4] The excess mortality from untreated 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.[4] In multiple pedigrees with untreated FH patients, it was estimated that 40% of the patients had a normal life expectancy, whereas 60% suffered from premature death.[4, 12] This emphasizes that this monogenic disorder has a complex burden, which is modified by other genes, conventional risk factors, and unknown environmental factors.[13]

THE GIRAFH STUDY POPULATION

The major part of the studies presented in this thesis was conducted in the GIRaFH cohort (Genetic Identification of Risk factors in Familial Hypercholesterolemia), which is a retrospective multicenter cohort of heterozygous FH patients. The DNA of suspected FH individuals

from Dutch lipid clinics is routinely submitted to a central laboratory at the Academic Medical Center in Amsterdam for LDL receptor mutation analysis. We randomly selected 4000 hypercholesterolemic individuals (from 27 Dutch lipid clinics) from this central DNA database. A total of 2400 unrelated FH individuals (1180 men and 1220 women), aged 18 years and older, fulfilled the internationally established FH diagnostic criteria (see below).[14] The study population consists mainly of Caucasians (99%). The mean age at the last visit to the lipid clinic was 49.8 (± 13.2) years. All patients gave informed consent, and the Ethics Institutional Review Board of each participating hospital approved the protocol.[14]

During the observation period, with a median follow-up of 3.8 years, phenotypic data were acquired by reviewing medical records using a previously described protocol.[15] The medical records were used to acquire information on age, sex, smoking, body mass index (BMI), diabetes mellitus (documented diagnosis with medication (insulin or oral antidiabetics), or fasting plasma glucose >6.9 mmol/L), hypertension (documented diagnosis with antihypertensive medication, or systolic blood pressure >140 mmHg and/or diastolic blood pressure >90 mmHg at three consecutive office visits), and CHD status.

Plasma total cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides were measured by standard methods in fasting patients withdrawn from lipid-lowering medication at least 6 weeks prior to blood collection. Plasma LDL cholesterol was calculated with the Friedewald formula.[14]

The clinical characteristics of the GIRaFH study population are presented in Table 1.

Table 1. Characteristics of 2400 patients with FH

Characteristic	
Age at first lipid clinic visit (years)	44.7 (± 12.7)
Age at last lipid clinic visit (years)	49.7 (± 13.2)
Males (%)	49.2
Smoking, ever (%)	73.8
Hypertension (%)	9.7
Diabetes mellitus (%)	5.8
Body mass index (kg/m^2)	25.1 (± 3.5)
Total cholesterol (mmol/L)	9.53 (± 2.00)
LDL cholesterol (mmol/L)	7.39 (± 1.94)
HDL cholesterol (mmol/L)	1.21 (± 0.35)
Triglycerides (mmol/L)	1.80 (± 1.03)

Values are given as percentage or as mean ± standard deviation (SD).

HDL = high-density lipoprotein, LDL = low-density lipoprotein

DEFINITION OF FAMILIAL HYPERCHOLESTEROLEMIA

Throughout this thesis, FH is defined as follows: *(i)* the presence of a documented LDL receptor mutation, or *(ii)* a plasma LDL cholesterol level above the 95th percentile for sex and age, in combination with at least one of the following: *(a)* the presence of typical tendon xanthomas in the patient or in a first-degree relative, *(b)* a plasma LDL cholesterol level above the 95th percentile for age and sex in a first-degree relative, or *(c)* proven CHD in the patient or in a first-degree relative under the age of 60 years. Patients with mutations in the apolipoprotein B-100 gene were excluded from our study population.

DEFINITION OF CORONARY HEART DISEASE

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

CONVENTIONAL RISK FACTORS

The GIRaFH cohort provides extensive data on the influence of conventional risk factors on the development of CHD in FH. Sex, smoking, BMI, plasma triglycerides, plasma total, LDL, and HDL cholesterol are associated with cumulative CHD risk (Table 2). Although raised plasma LDL cholesterol levels are the hallmark of FH, variation in these levels generally does not predict an increased CHD risk among FH patients.[16-18] In the GIRaFH cohort, higher levels of plasma total and LDL cholesterol were associated with a lower cumulative CHD risk (Table 2). This paradoxical effect could be partly explained by the fact that FH patients with plasma LDL cholesterol levels above the median received statin therapy at a younger age than patients with levels below the median (42.2 vs.45.0 years, respectively, $p<0.001$).

GENETIC RISK FACTORS

Genetic risk factors may provide important clues to disease pathophysiology and, therefore, may suggest new opportunities for therapeutic intervention. Investigators can use several strategies when searching for genetic risk factors for CHD: the candidate gene approach or a genomic approach (e.g. genome-wide association or linkage analyses).[19] In this thesis we focus on the candidate gene approach.

Candidate gene association studies are characterized by limitation of scope and selection of genes; they have a specific a priori hypothesis. The variation in genes consists mainly of single nucleotide polymorphisms (SNPs), i.e. variations of single DNA base pairs that have a frequency of greater than 1% in the general population. Essentially, candidate SNPs can be chosen based on: *(a)* prior likelihood of being functional, *(b)* correlation of the SNP with a potentially but yet unknown causal genetic variant (linkage disequilibrium), *(c)* missense variants detected by sequencing, and *(d)* technological considerations including the availability of high-throughput and lower costs pre-selected SNPs.[20] SNPs are not inherited randomly, but are linked to eachother in various degrees (linkage disequilibrium). A set of closely linked SNPs is called a haplotype. In other words, a haplotype is a combination of SNPs that occurs

Table 2. Cumulative CHD risks according to conventional risk factors in 2400 patients with FH

Characteristic		Total N	N of events	Cumulative CHD risk		
				40 yr (%)	50 yr (%)	60 yr (%)
Sex*	Female	1220	248	2.8	10.8	26.9
	Male	1180	445	12.4	35.2	62.8
Smoking*	Never	568	110	4.5	11.9	26.8
	Ever	1598	524	8.5	27.0	51.1
Hypertension	No	2146	564	7.5	23.3	42.5
	Yes	230	122	8.8	20.6	47.2
Diabetes mellitus	No	2262	615	7.7	22.7	42.8
	Yes	138	78	6.7	22.8	47.9
Body mass index**	≤25	1092	243	7.8	21.7	38.4
	25<BMI≤30	784	245	8.2	22.5	41.8
	>30	181	68	9.3	28.4	57.6
Total cholesterol**	≤9.20 mmol/l	1101	302	7.4	25.5	46.0
	>9.20 mmol/l	1051	292	6.5	19.4	39.9
LDL cholesterol**	≤6.99 mmol/l	961	259	6.9	23.4	43.9
	>6.99 mmol/l	967	228	6.1	18.2	36.9
HDL cholesterol*	>1.16 mmol/l	958	199	3.4	13.4	30.6
	≤1.16 mmol/l	1002	304	9.6	28.3	52.4
Triglycerides**	≤1.58 mmol/l	1018	204	5.4	18.6	36.5
	>1.58 mmol/l	1008	334	8.2	24.4	45.6
Total		2400	693	7.6	22.7	43.2

For total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides we used the median to split the total population in two subpopulations. CHD = coronary heart disease, LDL = low-density lipoprotein, HDL = high-density lipoprotein. *p<0.001, **p<0.05.

together more often than you would expect by chance. Therefore, candidate gene association studies can also be performed using haplotypes to obtain more detailed information about the genetic variation of the locus in relation with CHD.

In general, the specific LDL receptor mutation does not provide prognostic information on CHD risk over and above that of the untreated plasma LDL cholesterol level and cardiovascular risk profile.[4, 12] Rare and mild LDL receptor mutations are an exception to this and are associated with a lower CHD risk compared to the other LDL receptor mutations.[5, 21] A number of genetic variants outside the LDL receptor gene have been implicated in the development of CHD in FH populations other than the GIRaFH (Table 3).[22-38] Bertolini *et al.* assessed the

Table 3. Candidate genes studied in FH populations other than the GIRaFH between 1995 and 2008.

Gene	Polymorphism	Risk variant	N	Freq.	Phenotype	Effect
ATP binding cassette A1	Arg219Lys	Lys	374	0.29	CHD	OR 0.63; p<0.05[23]
Angiotensin-converting enzyme	Insertion/deletion	DD	213	0.33	MI	OR 2.57; p=0.02[31]
					CHD	OR 2.21; p=0.02[31]
		D	112	0.55	CHD	NS[32]
		DD	228	0.31	CHD	NS[25]
Angiotensinogen	Met235Thr	Thr	112	0.25	CHD	NS[32]
Angiotensin II type I receptor	A1166C	C	112	-	CHD	OR 3.10; p=0.04[32]
Apolipoprotein E	E2/E3/E4	E4	706	0.09	CVD	NS[34]
		E4	236	-	CHD	NS[24]
		E4	93	0.09	CHD	NS[35]
Cholesteryl ester transfer protein	Taq1 RFLP	B2/B2	300	0.20	CVD	NS[33]
Estrogen receptor alpha	T–1989G	GG	295	0.17	CHD	OR 4.5; p=0.04[29]
Lipoprotein lipase	Asn291Ser	AsnSer + SerSer	1045	0.07	CVD	OR 3.89; p=0.003[37]
	Asp9Asn	AspAsn + AsnAsn	2091	0.05	CVD	OR 2.2; p=0.04[38]
Methylenetetrahydrofolate reductase	C677T	TT	199	0.11	Age CHD	p<0.05[26]
		TT	249	0.25	CHD	NS[30]
Paraoxonase1	Leu55Met	LeuLeu	187	0.46	IMT	NS[27]
		LeuLeu	197	0.47	CVD	NS[28]
	Gln192Arg	GlnGln	187	0.48	IMT	NS[27]
		GlnGln	197	0.48	CVD	NS[28]
	Haplotype	LeuLeu/ GlnGln	187	-	IMT	p=0.002[27]
	G-824A	AA	181	0.10	IMT	p=0.03[36]
Paraoxonase2	Cys311Ser	CysCys + CysSer	197	0.37	CVD	p=0.01[28]
Platelet glycoprotein IIIa	C1565T	T	80	0.23	CHD	NS[22]

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

effects of 11 SNPs in 8 candidate genes involved in lipid metabolism on CHD in 221 unrelated FH index cases and 349 FH relatives.[39] In these Italian FH patients, the fatty acid binding protein-2 54TT variant was an independent predictor of increased CHD risk after adjustment for clinically relevant risk factors. The 219RK and KK variants of the ATP binding cassette A1 were independently associated with decreased CHD risk.

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 cohort described above.[40] In 1940 patients (80.1%) complete genotypes for all 65 polymorphisms could be obtained. The 20210A variant in the prothrombin gene was strongly associated with an increased CVD risk. Furthermore, the 235Thr variant of the angiotensinogen gene and the 347Ser variant of the apolipoprotein A4 gene were associated with increased CVD risk, whereas the 311Cys variant of the paraoxonase-2 gene and the 1100T variant of the apolipoprotein C3 gene were associated with decreased CVD risk.

In addition to genetic association studies on CHD and CVD risk, numerous studies reported an association between a plethora of polymorphisms and variability in plasma lipid concentrations among FH patients. A complete summary of these genetic variants is outside the scope of the present thesis, although we will consider the associations between polymorphisms in the ABCG8 gene and plasma lipid concentrations in Chapter 3.

GENETIC ASSOCIATION STUDIES OF CORONARY HEART DISEASE

Basically, the primary aim of genetic association studies of CHD is to identify genetic variants that play a role in the etiology of CHD. The identification of such genetic variants serves 2 purposes. First, the genetic risk factors for CHD may be useful to predict CHD risk of an individual. This could ultimately lead to a more personalized approach of medical care, in which therapeutic decisions are based on an individual's risk of CHD. The second is to develop pharmacological therapies based on the novel basic disease mechanisms. Although the genome cannot be changed yet with gene therapies, the consequence of genetic variants at the mRNA or protein levels, or further downstream, can potentially be specifically targeted with new pharmacological compounds.

AIMS AND OUTLINE OF THESIS

The primary aim of the studies described in this thesis is to identify genetic risk factors for CHD in FH, and to investigate whether or not these genetic risk factors could be helpful in distinguishing FH patients who will develop CHD from those who will not.

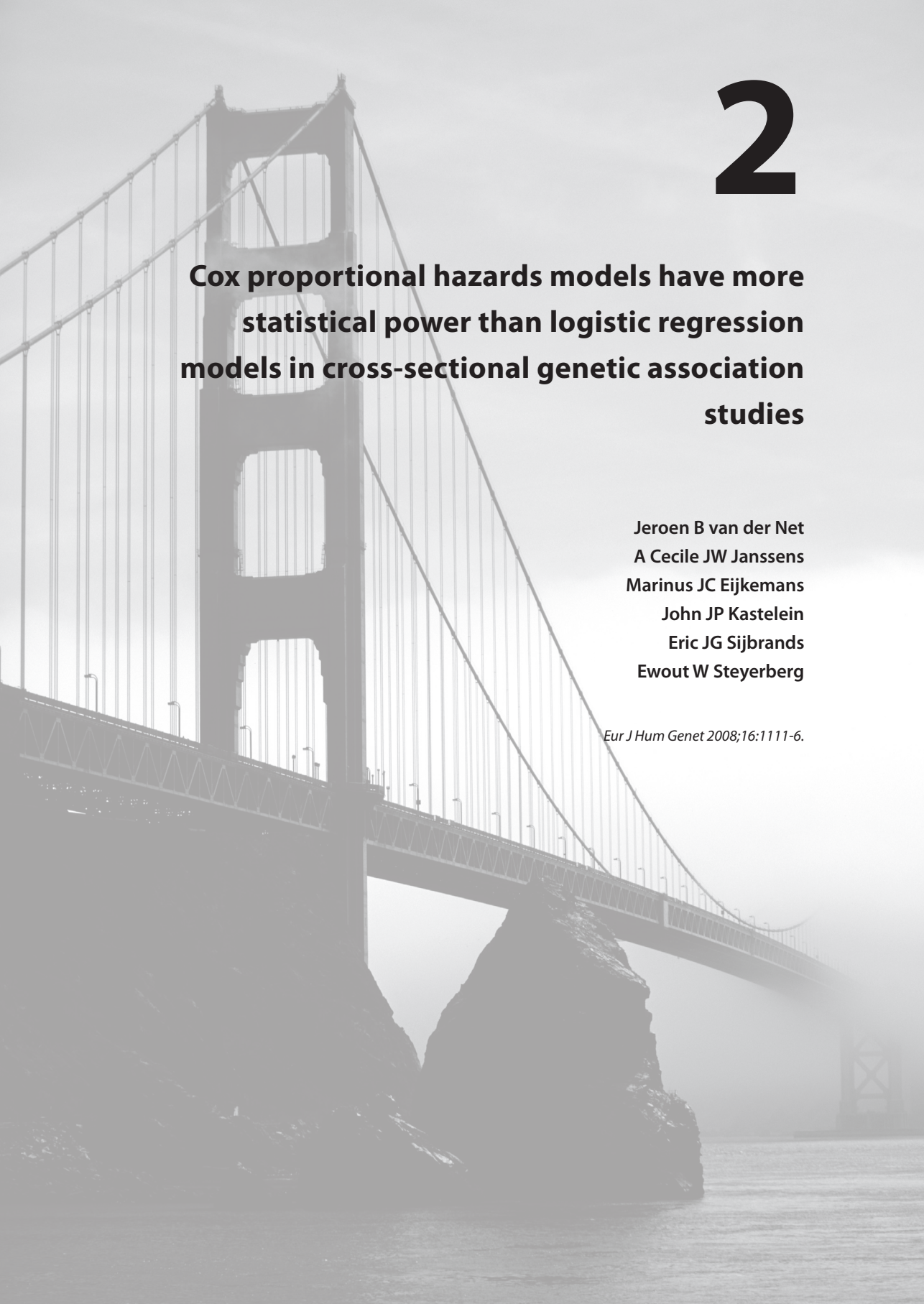
In Chapter 2, we investigate which statistical regression model should be used in cross-sectional genetic association studies. We compare the statistical power of the Cox proportional hazards model and the logistic regression model in cross-sectional genetic association studies. The statistical regression model with the highest statistical power is used in all candidate gene associations studies that we describe in Chapters 3-8.

In Chapters 3-7 we analyze candidate genes that are chosen on the basis of their relation to conventional CHD risk factors (Chapters 3, 4, 6, and 7), or their involvement in pathophysiological processes underlying CHD (Chapter 5). Furthermore, we investigate the contribution to CHD risk in FH of the most recently identified genetic variants with mostly unknown pathophysiology (Chapter 8).

In Chapters 9 and 10, we study whether genetic variants can be used to predict CHD risk in our FH population and the general population. Finally, we discuss the findings of this thesis in the context of ongoing research, the limitations and methodological issues relevant to our studies and directions for future research in Chapter 11.

PART II

Cox proportional hazards models in genetic association studies



2

Cox proportional hazards models have more statistical power than logistic regression models in cross-sectional genetic association studies

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ABSTRACT

Objectives

Cross-sectional genetic association studies can be analyzed using Cox proportional hazards models with age as time scale if age at onset of disease is known for the cases and age at data collection is known for the controls. We assessed to what degree and under what conditions Cox proportional hazards models have more statistical power than logistic regression models in cross-sectional genetic association analyses.

Methods

Analyses were conducted in an empirical study on the association of 65 polymorphisms and risk of coronary heart disease among 2400 Familial Hypercholesterolemia patients, and in a simulation study that considered various combinations of sample size, genotype frequency, and strength of association between the genotype and coronary heart disease. We applied Cox proportional hazards models and logistic regression models, and compared effect estimates (hazard ratios and odds ratios) and statistical power.

Results

In the empirical study, Cox proportional hazards models generally showed lower p-values for polymorphisms than logistic regression models. In the simulation study, Cox proportional hazards models had higher statistical power in all scenarios. Absolute differences in power did depend on the effect estimate, genotype frequency and sample size, and were most prominent for genotypes with minor effects. E.g. when the genotype frequency was 30% in a sample with size n=2000 individuals, the absolute differences were largest for effect estimates between 1.1 and 1.5.

Conclusion

Cox proportional hazards models can increase statistical power in cross-sectional genetic association studies, especially in the range of effect estimates that are expected for genetic associations in common diseases.

INTRODUCTION

Epidemiologic association studies are often analyzed using logistic regression models or Cox proportional hazards models. The choice between the two models is primarily based on the design of the study. Logistic regression models are used in cross-sectional and case-control studies, whereas Cox proportional hazards models are usually applied to prospective studies that have a follow-up period during which the occurrence of events is observed.[41] If follow-up data are available, Cox proportional hazards models are the recommended model because they have more statistical power than logistic regression models.[42, 43] This is due to the fact that the Cox proportional hazards models take account of the time until events occur.[44] However, these models have not been compared in cross-sectional genetic association studies.

Genetic association studies that do not have follow-up time are generally analyzed using logistic regression models. However, since genotype status does not change over time and hence also represents genotype status at birth, age at event can be considered as follow-up time. If the age at event is known, genetic association studies could be analyzed with Cox proportional hazards models, even in the absence of prospectively studying follow-up. In the literature, there are various examples of studies in which logistic regression models were used, where Cox proportional hazards models could have been applied.[45-47]

We aimed to compare the statistical power of Cox proportional hazards models with that of logistic regression models in cross-sectional genetic association studies. We hereto performed an empirical study on the risk of coronary heart disease (CHD) in patients with Familial Hypercholesterolemia (FH), and a simulation study in which we examined the conditions under which additional statistical power can be achieved.

METHODS

Study population

1. Empirical study

We analyzed a retrospective, multicentre cohort study of patients with heterozygous FH who were recruited from 27 lipid clinics in the Netherlands between 1989 and 2002. Details on the study design and the study population are given in Chapter 1. In total, 693 (29%) FH patients had proven CHD: 466 (19%) patients had a verified CHD event before study entry, and 227 (10%) incident CHD cases were observed during follow-up (median follow-up time without CHD 3.1 years).

A previous association study considered 65 polymorphisms located in candidate genes for cardiovascular disease in our FH population.[40] Three polymorphisms had only wild type alleles in our population and were therefore excluded from the present analyses. All patients

gave informed consent and the ethics institutional review board of each participating hospital approved the protocol.

2. Simulation study

We constructed a population of FH patients with sex, age at first visit, and age at last visit randomly sampled from the empirical data set. We simulated genotype status (for a single hypothetical polymorphism), age at event, and CHD status.

Genotype status was randomly assigned according to specified genotype frequencies. Although we recognize that individuals have one of three genotypes, we simulated only an at-risk genotype ('carrier') and another with the referent or baseline risk ('non-carrier') in our primary analyses. These two genotypes can be interpreted as dominant and/or recessive models of inheritance. In a secondary analysis, we repeated the simulations in the more complex setting of three genotypes. This yielded virtually identical results (data not shown).

Age at event was randomly drawn from distributions of age-, sex-, and genotype-specific CHD incidence rates for patients with FH. These distributions were obtained in three steps. First, we fitted Weibull distributions on age-specific CHD incidence rates in the general Dutch population, for men and women separately. The incidence rates were obtained from the National Institute for Public Health and the Environment (RIVM) [www.rivm.nl/vtv/object document/o1320n17964]. Second, these distributions were adjusted to fit the age-specific CHD incidence in the FH patients of the empirical study, resulting in a cumulative CHD incidence of 29%. Finally, separate distributions were constructed for carriers and non-carriers by changing the average hazard according to the strength of association of the risk genotype, and assuming proportional hazards. CHD status was considered present when the simulated age at event was lower than the age at the last visit, and considered absent when the simulated age at event was higher.

Statistical analysis

Cox proportional hazards models and logistic regression models were fitted in the empirical and simulated data sets. With the term 'effect estimate' we refer to hazard ratios in the Cox proportional hazards model and odds ratios in the logistic regression model. All analyses were adjusted for sex and the logistic regression models were additionally adjusted for age (as a linear term), which was age at event or age at last visit to the lipid clinic in the case of no event. For the Cox proportional hazards model we used age as time variable, thereby assuming that follow-up time started at birth and ended at the date of the first occurrence of established CHD, or at the last visit to the lipid clinic. In the empirical data set, we assumed that each polymorphic allele had an additive contribution to the log-hazard/log-odds scale (additive genetic mode of inheritance). We compared the effect estimates and the p-values of the two models and calculated Spearman's rank correlation coefficient for the p-values of the two models.

In the simulation study, we varied the size of the population (n=500; n=2000; n=5000), the frequency of the risk genotype (10%; 30%; 50%; 70%), and the strength of association between the polymorphism and risk of CHD (hazard ratio 1.0-2.0 with increments of 0.1) in separate scenarios. The hazard ratio of 1.0 was also simulated to check if Type 1 error rates of the two approaches were as simulated, namely 0.05, and this was confirmed for all scenarios. The simulated and observed hazard ratios were slightly lower than the observed age-adjusted odds ratios in all scenarios (data not shown). Prospective data collection is assumed when Cox proportional hazards models are used. In retrospective data collection early cases could be missed. To investigate whether missing data might influence our results, we simulated scenarios in which all prevalent cases (with an event before the date of first visit to the lipid clinic) were missed, thereby excluding the prevalent cases from the analysis and considering incident cases only. Each scenario was repeated 5000 times. The statistical power of the two models was defined as the percentage of statistically significant ($p<0.05$) associations between the polymorphism and CHD status found for that regression model in the 5000 repeated scenarios. The gain in statistical power with the Cox proportional hazards model was expressed as the absolute difference in power and as the potential reduction in required sample size that can be obtained when the most powerful model would have the same power as the least powerful model.[48] Percentage reduction in required sample size was calculated as: $100\text{-}100*(Z_2/Z_1)^2$ where Z_1 and Z_2 are the Wald statistics of the most and least powerful model, respectively. These measures are independent of the effect estimate, the α-value and sample size.[48] Therefore, we calculated the mean percentage reduction in required sample size for each genotype frequency. All statistical analyses and simulations were performed using the R statistical package (version 2.5.1).[49]

RESULTS

Empirical study

Figure 1 shows the effect estimates and p-values for the association between 62 polymorphisms and the risk of CHD obtained by Cox proportional hazards and logistic regression analyses. The effect estimates tended to be more extreme for the logistic regression models than for the Cox proportional hazards models (Figure 1a). The rank correlation coefficient for the p-values was 0.54. Logistic regression analyses showed statistical significance for two polymorphisms, whereas four polymorphisms were statistically significant using the Cox proportional hazards model.

Simulation study

Figure 2 shows that Cox proportional hazards models had more statistical power than logistic regression models in all scenarios. The absolute difference in power was determined by the

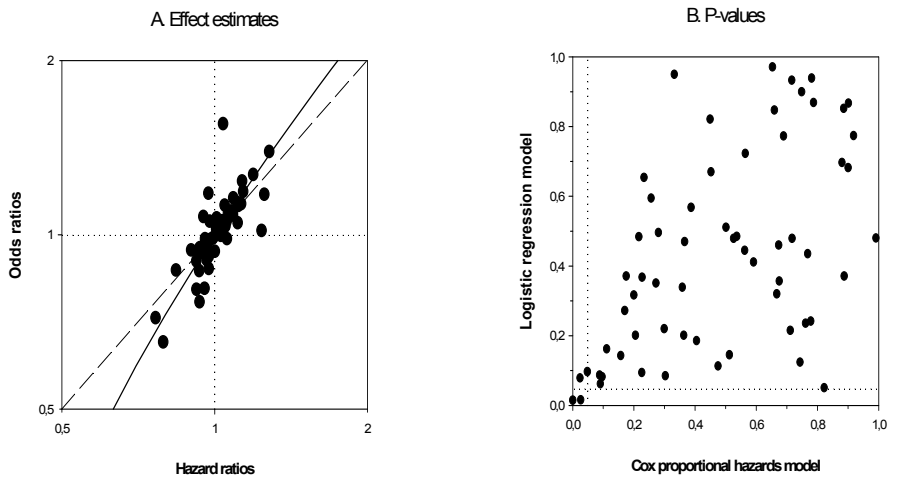


Figure 1: Effect estimates and p-v alues for 62 SNPs obtained by the Cox proportional hazards model and logistic regression model in the empirical study. Effect estimates are hazard ratios for the Cox proportional hazards models and odds ratios for the logistic regression models. Two outliers with effect estimates above 2.0 are not shown in these figures. A: dashed line represents the reference line for which the hazard ratio is equal to the odds ratio, solid line represents the linear regression line through the data points. B: dotted lines represent the significance threshold (p=0.05).

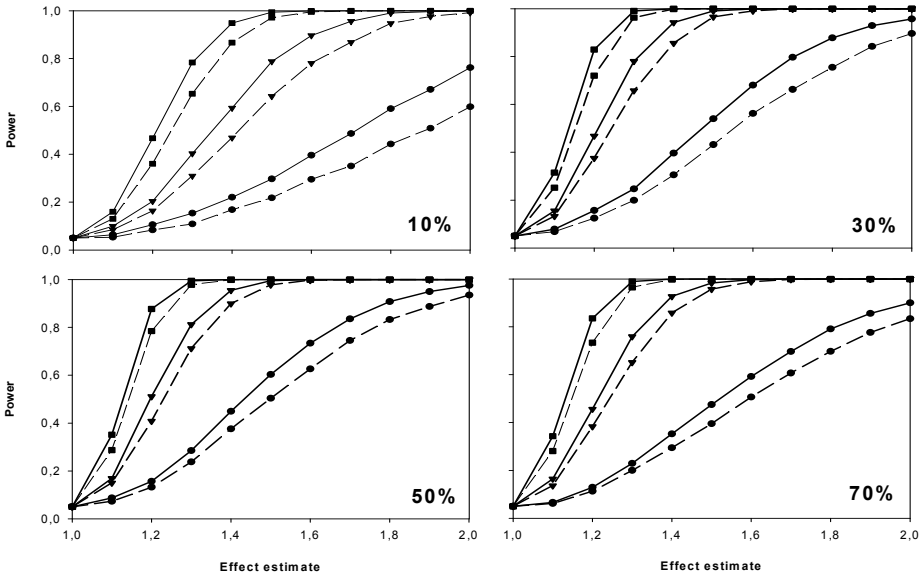


Figure 2: Statistical power of the Cox proportional hazards model and logistic regression model as a function of genotype frequency, effect estimate and sample size. Solid lines indicate power estimates of the Cox proportional hazards model and dashed lines of the logistic regression models. Power estimates are presented for sample sizes of 500 (●), 2,000 (▼) and 5,000 (■) patients.

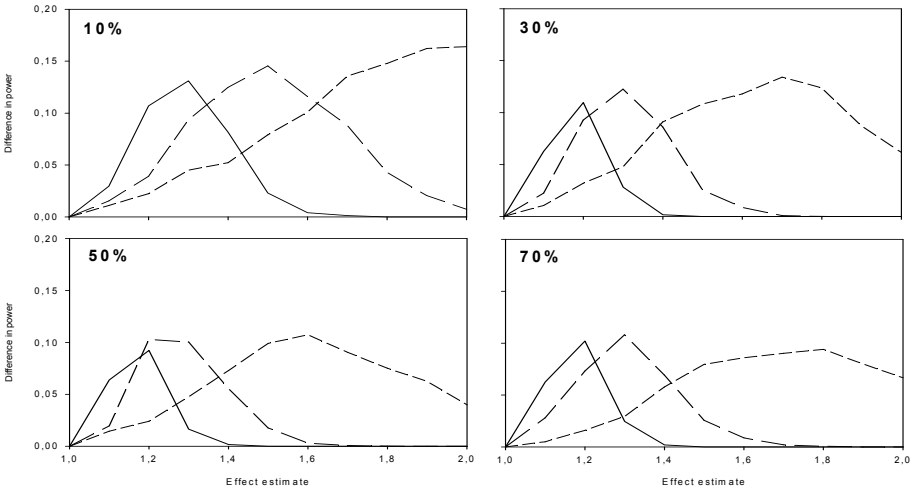


Figure 3: Absolute differences in statistica l power between the Cox proportional hazards model and logistic regression model. Differences in power estimates were obtained by subtracting the power estimates of the logistic regression models from the estimates of the Cox proportional hazards models. Solid lines (n=5,000), long-dashed lines (n=2,000), small-dashed lines (n=500).

effect estimate, genotype frequency and sample size (Figure 3). The absolute difference in power was larger when sample size was low, the risk genotype was infrequent or the risk associated with the genotype was low. For example, when the genotype frequency was 30% and sample size was 2000, the absolute differences in power were most prominent for effect estimates between 1.1 and 1.5. The differences in power equalled to a reduction in required sample size ranging from 33% when the genotype frequency was 10%, to 18% when the genotype frequency was 70%. Risk sizes and reduction in sample sizes were similar when analyses were restricted to incident cases. However, the statistical power was lower in both models because of a lower number of events (data not shown).

DISCUSSION

This study shows that Cox proportional hazards models may yield more statistical power than logistic regression models in cross-sectional genetic association studies. Differences in statistical power were most prominent for genotypes with minor effects in a range in which most genetic associations are expected.

The observation that Cox proportional hazards models have more statistical power than logistic regression models in association studies has been described previously.[42, 43] For example, the effect estimates will diverge when follow-up time is longer,[43, 44] and effect estimates of logistic regression models are less precise, especially when the event is more common or when there is a strong relative risk.[50] This is in line with our findings in the empirical study,

which showed that odds ratios tended to be more extreme than the hazard ratios. It has been previously shown that the Cox proportional hazards model gives more conservative effect estimates than the logistic regression model, especially when the incidence of the disease is high,[42] as is the case in FH.

An explanation for the higher power of Cox proportional hazards models is that these models take the time until events occur into account, thereby changing the unit of analysis from persons to person-years. Therefore, the interpretation of the results of the Cox proportional hazards model differs from those of the logistic regression model. Whereas the logistic regression model tests whether a risk factor affects the odds of disease, the Cox proportional hazards model tests whether a risk factor affects the age of onset of a disease. Logistic regression models do not take into account the time until events occur, but give 'early' events and 'late' events the same weight in the analysis.[41, 44] Young individuals who have had no event (yet) are classified as 'no event', while some would have experienced the event at an older age. This is a form of misclassification in terms of outcome. The superiority of the Cox proportional hazards model over the logistic regression model in analyzing longitudinal data has been mathematically proven for models which consider one dichotomous covariate[42] and models with multiple covariates.[43]

A number of considerations regarding the generalizability of our results merit discussion. First, we simulated populations with a high risk of CHD, which implies that the absolute estimates of the statistical power of the different scenarios only apply to populations with similar disease risks. Statistical power and differences in statistical power were lower when the disease risks were lower, but still in favour of the Cox proportional hazards model (data not shown); a relative measure such as the potential percentage reduction in required sample size did not depend on the incidence of the disease. Second, analysis of a cross-sectional genetic association study with Cox proportional hazards models assumes that follow-up time starts at birth. In a retrospective design, Cox proportional hazards models only yield valid estimates compared to prospective studies if there is no selective loss-of-follow-up. Our study only included patients who at least survived until a first visit to the lipid clinic, and early CHD cases could have been missed. Although these early cases might have been rare as demonstrated in a previous study,[4] we cannot exclude this possibility. We investigated the extreme scenario in which all prevalent cases were missed. This did not influence the effect estimates, because the analysis included all characteristics related to missingness of these cases (age, genotype status). Third, in our simulations we did not adjust for covariables other than age. In cross-sectional genetic association studies, the use of Cox proportional hazards models is formally only valid when no adjustment is needed, or when adjustment is needed only for covariables that can be reliably assessed in retrospect, such as sex and education.

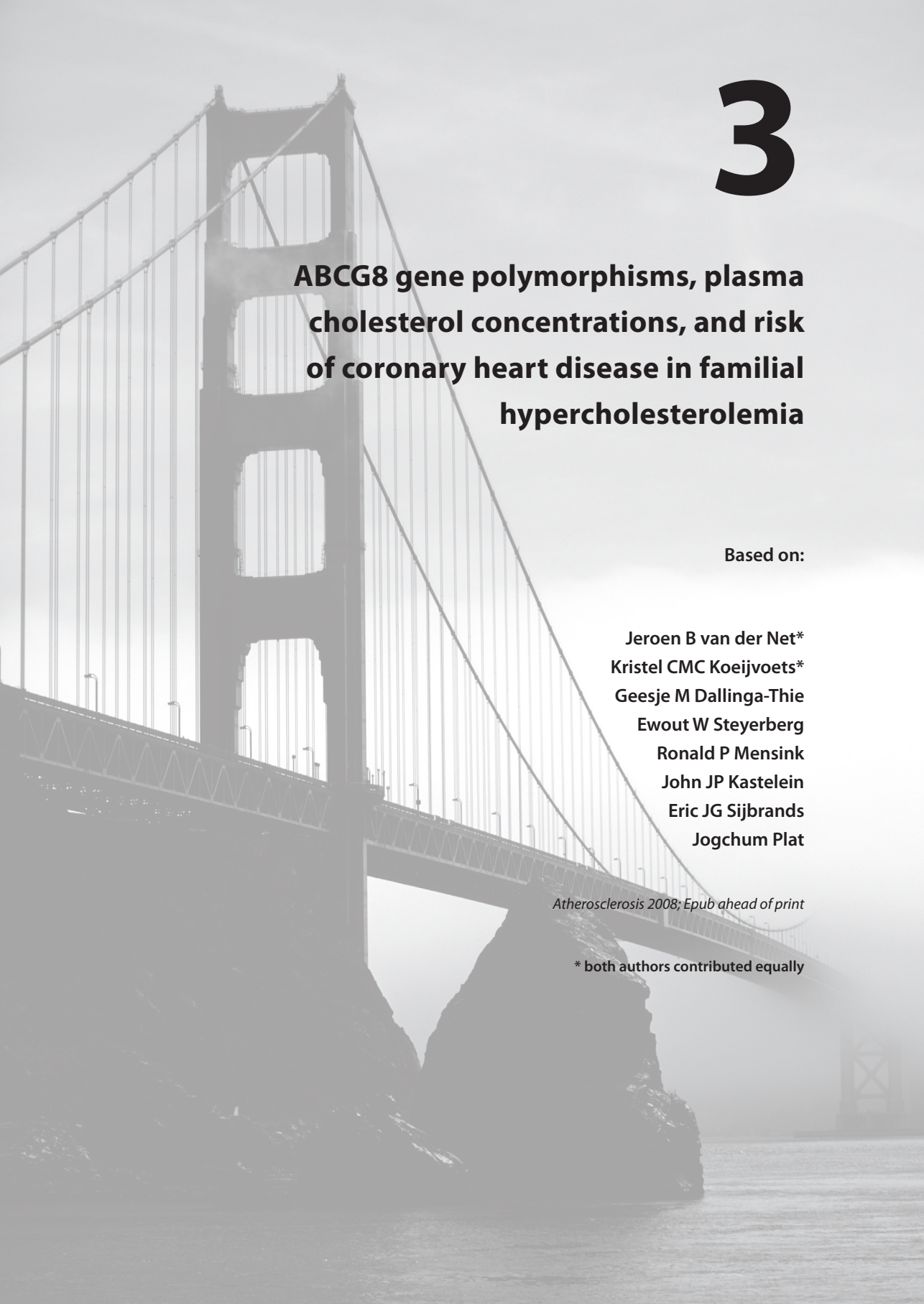
When there is no reason to expect selective loss of follow-up and no other variables than age and sex need to be adjusted for, Cox proportional hazards models are the preferred strategy for the analyses of genetic association studies. As the increase in power is indepen-

dent of the type 1 error rate, Cox proportional hazards models may not only be preferred in association studies of candidate genes, but also in the statistical analysis of genome-wide association (GWA) studies, which generally consider lower type 1 error rates. Current GWA studies most often make simple and less powerful comparisons of genotype counts between cases and controls. Application of Cox proportional hazards models may lead to the additional identification of susceptibility genes with weaker effects that will remain undetected otherwise. In the case of cross-sectional genetic association studies in which adjustment for other variables than age and sex is needed, it is not immediately clear which is the model of choice. Additional variables, such as blood pressure and cholesterol levels, are more difficult to assess in retrospect. Ideally, these additional variables should be treated as time-dependent variables.[51] Since the levels of these variables at the time of the event are often unknown in cross-sectional studies, the levels at the time of study conduction are frequently used as surrogates. Whether this will introduce a different size of bias in the two models is not clear. Yet, this potential bias is of lesser importance in gene-finding studies (as described in the present study) than in risk prediction studies in which it is more important to accurately estimate the effect.[52]

We conclude that the advantage in terms of statistical power of the Cox proportional hazards model in comparison with the logistic regression model was most prominent for the range of effect estimates that are expected for most genetic associations. We recommend to consider the use of the Cox proportional hazards model in both cross-sectional genetic association studies and GWA studies.

PART III

Candidate gene association studies in familial hypercholesterolemia



3

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

Based on:

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Atherosclerosis 2008; Epub ahead of print

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ABSTRACT

Objectives

Elevated plasma plant sterol concentrations may be a risk factor for coronary heart disease (CHD). 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 between ABCG8 gene polymorphisms and CHD are lacking.

Methods and Results

We examined associations between the D19H and T400K polymorphisms in the ABCG8 gene and CHD in the GIRaFH population, a large cohort study of patients with heterozygous familial hypercholesterolemia (FH). A total of 244 individuals carried one or two alleles of the D19H variant and 568 individuals the T400K variant. During 100212 person years, 579 (28.8%) individuals developed at least one CHD event. In a Cox proportional hazard regression model adjusted for relevant cardiovascular risk factors, the D19H polymorphism was associated with risk of CHD (HR 1.42, CI 1.04–1.95; $p = 0.03$). We observed no relationship between the T400K polymorphism and CHD ($p > 0.1$). However, FH individuals carrying the risk genotype for both ABCG8 variants, defined by at least one H-allele and two T-alleles, had an increased risk of CHD (HR 1.72, 95% CI 1.23 – 2.41; $p = 0.002$).

Conclusion

Our data suggest that genetic variation in the ABCG8 gene may influence the burden of atherosclerosis.

INTRODUCTION

Increased plasma cholesterol levels are among the most important risk factors of coronary heart disease (CHD). Cholesterol is the most common sterol and its levels are mainly regulated by cholesterol absorption, synthesis, and secretion. Sterols of plant origin are structurally similar to cholesterol but they cannot be synthesized endogenously and are thus 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.[53]

The accumulation of plant sterols is actively prevented by two members of the adenosine triphosphate binding cassette (ABC) transporter family, ABCG5 and ABCG8.[54, 55] 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 into the lumen of the small intestine and to 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, which is a rare autosomal recessive disorder characterized by increased intestinal absorption and markedly impaired biliary secretion of plant sterols.[56] Patients with sitosterolemia have plasma and tissue levels of plant sterols that are >50-fold increased and they develop CHD already at a young age.[57]

Moderately elevated plasma concentrations of plant sterols have been associated with a personal or family history of CHD in both hypercholesterolemic and normocholesterolemic individuals.[58-61] Moreover, a higher ratio of sitosterol and campesterol to cholesterol in serum was associated with a higher ratio in the carotid artery wall of individuals undergoing carotid endarterectomy and in stenotic aortic valves of subjects undergoing valve surgery.[62, 63] These data are however not consistent since other large epidemiological studies have not been able to find an association between elevated plasma plant sterol concentrations and CHD, [64, 65] or even demonstrated a protective effect on CHD.[66]

Plasma plant sterol concentrations vary over a 5- to 10-fold range among individuals, but are very stable within individuals and are highly heritable.[67] It was found that common DNA sequence variations in the ABCG8 gene contributed to the variation in plasma concentrations of plant sterols such as sitosterol and campesterol.[67-69] In addition, ABCG8 gene variants were also associated with plasma total cholesterol and low-density lipoprotein (LDL) cholesterol levels in some studies,[68, 70, 71] but again associations were not consistent since this was not confirmed by others.[67, 69] Although plant sterol concentrations have been related to CHD 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 CHD is a priori expected to be modest.[72] The association between ABCG8 variants with

CHD should, therefore, preferably be examined in a population at increased risk of CHD with an associated high power to detect potential small effects of the genetic variant. Patients with heterozygous familial hypercholesterolemia (FH) have severely elevated plasma LDL cholesterol levels, leading to accelerated atherosclerosis and an increased risk of premature CHD.[1] Despite the monogenic cause, the cardiovascular morbidity and mortality of FH shows considerable variation related to both environmental and genetic factors.[12] The disorder is considered to be an exemplary model to analyze secondary (or modifier) genes involved in CHD.[73]

We hypothesized that the presence of common genetic variants in the ABCG8 gene may affect variation in CHD risk. We therefore examined the relationship between two polymorphisms in the ABCG8 gene and plasma cholesterol concentrations as well as susceptibility to CHD in patients heterozygous for FH. Since the D19H and T400K polymorphisms in the ABCG8 gene showed the strongest relationship with cholesterol standardized serum plant sterol concentrations,[67, 68] we decided to evaluate associations with these ABCG8 variants.

METHODS

Study design and study population

A description of the GIRaFH study population and the definition of FH and CHD are given in Chapter 1.

Molecular analysis

The DNA was available of 2145 patients. The DNA of the remaining patients was missing, because it was contaminated, severely fragmented, or fully used in previous analyses. Genomic DNA was extracted from peripheral blood leukocytes according to a standard protocol.[74] We decided to analyze 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.[67-71] 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 was C_26135643_10 for the D19H and C_375061_10 for the T400K polymorphism (Applied Biosystems). Reaction components and amplification parameters were based on the manufacturer's instructions using an annealing temperature of 60º Celsius. Results were scored blinded for CHD status. The genotyping was successful in 2053 (95.7%) patients for the T400K polymorphism, and in 2065 (96.3%)

patients for the D19H polymorphism. Finally, 2012 (93.8%) patients were fully genotyped for both polymorphisms.

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 an exact test.[75] 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 CHD. Differences in plasma cholesterol concentrations and patient characteristics among ABCG8 genotypes were analyzed with t-tests and 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, sex, and smoking.

The association between the D19H and T400K polymorphisms in ABCG8 and the occurrence of CHD 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 CHD. Patients without CHD were censored at the date of the last lipid clinic visit or at the date of death attributable to other causes than CHD. 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 CHD traits, we additionally adjusted for variables that were significant risk factors for CHD in the Cox regression analysis. To investigate whether statin therapy might have influenced our results, we additionally adjusted for statin therapy in our models. 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 2400 individuals with heterozygous FH are shown in Table 1 of Chapter 1. Table 1 shows ABCG8 genotype and allele frequencies. For the D19H polymorphism, we observed homozygosity (HH) in 7 (0.3%) patients, heterozygosity (DH) in 237 (11.8%) patients, and 1768 (87.9%) patients were homozygous for the wild type allele (DD). For the T400K polymorphism, 45 (2.2%) patients were homozygous (KK), 523 (26.0%) patients were heterozygous (TK), and in 1444 (71.8%) patients the homozygous wild type allele (TT) was detected. Genotype distributions in the total cohort and in patients without and with a his-

Table 1. Genotype and allele distributions for D19H and T400K polymorphisms in ABCG8

		D19H			T400K	
Genotypes	Total N (%)	CHD – N (%)	CHD + N (%)	Total N (%)	CHD – N (%)	CHD + N (%)
Wild type (DD or TT)	1768 (87.9)	1264 (88.2)	504 (87.0)	1444 (71.8)	1015 (70.8)	429 (74.1)
Heterozygous (DH or TK)	237 (11.8)	163 (11.4)	74 (12.8)	523 (26.0)	390 (27.2)	133 (23.0)
Homozygous (HH or KK)	7 (0.3)	6 (0.4)	1 (0.2)	45 (2.2)	28 (2.0)	17 (2.9)
Total	2012 (100.0)	1433 (100.0)	579 (100.0)	2012 (100.0)	1433 (100.0)	579 (100.0)

CHD+ indicates history of coronary heart disease, CHD- indicates no coronary heart disease

tory of CHD were in Hardy-Weinberg equilibrium for both ABCG8 genotypes ($p > 0.1$). Using chi-square analyses, ABCG8 genotype distributions were similar between individuals without and with a history of CHD (D19H, $p = 0.5$; T400K, $p = 0.1$)

ABCG8 genotypes and plasma cholesterol concentrations

Plasma cholesterol concentrations according to D19H and T400K genotypes are presented in Table 2. No significant differences in plasma lipid concentrations among D19H and T400K genotypes were found. Additional adjustment for year of birth, sex, and smoking did not change the results. Patients homozygous or heterozygous for the T400K polymorphism had significantly less hypertension compared to carriers of the wild type alleles (7.0% vs. 9.8%; $p = 0.03$). In addition, age, sex, body mass index (BMI), the presence of smokers and diabetes mellitus were comparable among the ABCG8 genotype groups (data not shown).

During the observation period, a total of 1692 (84.1%) patients were being treated with a statin. The mean (±SD) plasma lipid levels on treatment were 6.04 (±1.41) mmol/L for total cholesterol, 4.10 (± 1.37) mmol/L for LDL cholesterol, 1.32 (±0.37) mmol/L for HDL cholesterol, and 1.41 (±0.87) mmol/L for triglycerides. The percentage of D19H and T400K carriers that were treated was similar to non-carriers. The LDL cholesterol levels on treatment were comparable and there was no difference in this respect between patients with or without CHD (data not shown).

Table 2. Plasma cholesterol concentrations according to D19H and T400K genotypes

Polymorphism	Genotype	Total cholesterol	LDL cholesterol	HDL cholesterol	Triglycerides
D19H	DD	9.50 (±1.99)	7.32 (±1.91)	1.22 (±0.36)	1.81 (±1.04)
	DH/HH	9.50 (±1.92)	7.40 (±1.87)	1.22 (±0.36)	1.75 (±1.04)
T400K	TT	9.50 (±2.00)	7.32 (±1.91)	1.21 (±0.34)	1.82 (±1.04)
	TK/KK	9.51 (±1.94)	7.36 (±1.92)	1.24 (±0.39)	1.78 (±1.07)

Values are given as means (mmol/l) with standard deviations in brackets.

Table 3. Association between ABCG8 polymorphisms and coronary heart disease in 2012 FH individuals

	Model 1		Model 2		Model 3	
Polymorphism	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
D19H	1.27 (0.98 – 1.64)	0.07	1.42 (1.04-1.95)	0.03	1.42 (1.04 – 1.95)	0.03
T400K	0.87 (0.72 – 1.06)	0.2	0.84 (0.66-1.06)	0.1	0.83 (0.65 – 1.05)	0.1

Model 1: adjusted for year of birth, gender, and smoking, model 2: additionally adjusted for diabetes, plasma HDL cholesterol and triglyceride concentrations, model 3: additionally adjusted for statin use.

ABCG8 genotypes and risk of CHD

During 100212 person years, 579 (28.8%) FH individuals had their first CHD event. Mean age of onset of CHD was 48.7 ± 10.8 years. Year of birth, male sex, smoking, diabetes, lower plasma HDL cholesterol concentrations, and higher plasma triglyceride levels were significantly associated with increased CHD risk (data not shown). As shown in Table 3, the D19H polymorphism was associated with increased risk of CHD, but only after adjustment for year of birth, sex, smoking, diabetes, HDL cholesterol, and triglyceride levels in multiple Cox regression analyses (model 2, Table 3). Further adjustment for statin therapy did not change this result (model 3, Table 3). We found no significant relationship of the T400K polymorphism with susceptibility of CHD.

The average (± SD) age at end of follow-up of patients without CHD was 46.9 ± 12.7 years and this was significantly lower than the mean age (48.7 ± 10.8 years) at onset of CHD 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).

Although we found no significant relationship between the T400K variant and CHD, the effect estimates seemed opposite for the D19H and T400K polymorphisms (Table 3). Therefore, we investigated whether individuals with the risk genotypes of both polymorphisms had an increased CHD risk. The risk genotype for the D19H variant was the DH/HH genotype ("carriers" of the D19H polymorphism) and the risk genotype for the T400K variant was the frequent TT genotype ("non-carriers" of the T400K polymorphism). A total of 170 (8.4%) individuals carried the combination of the ABCG8 risk genotypes. After adjustment for year of birth, sex, and smoking, this combined ABCG8 risk genotype was significantly associated with increased risk of CHD compared to individuals that carried any other combination of genotypes (HR 1.51, 95% CI 1.13 – 2.01; p = 0.005). Further adjustment for diabetes, plasma HDL cholesterol and triglyceride concentrations showed a hazard ratio of 1.72 (95% CI 1.23 – 2.41; p = 0.002). Additional adjustment for statin therapy yielded a similar hazard ratio (HR 1.68, 95% CI 1.19 – 2.36; p = 0.003).

DISCUSSION

In this large cohort study of FH patients with a high-risk of CHD, we found that the D19H polymorphism was not significantly associated with plasma cholesterol levels, but there was evidence of an association with higher risk of CHD. We observed no significant association between the T400K polymorphism and plasma cholesterol levels or CHD. Nonetheless, individuals with the risk genotypes of both ABCG8 polymorphisms (DH/HH and TT) had an increased risk of CHD.

Our present analysis is the first study that examined the association between polymorphisms in the ABCG8 gene and risk of CHD. 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 dysfunction of either the ABCG5 or ABCG8 protein, leading to severe atherosclerotic arterial disease at a young age.[57] 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.[67] In addition, several studies have reported that also in nonsitosterolemic individuals elevated plasma concentrations of plant sterols are associated with cardiovascular events.[58-61]

Heterozygous and homozygous carriers of the D19H polymorphism had a slightly higher risk of CHD than carriers of the wild type. Previous studies have consistently demonstrated associations of the 19H allele with lower plasma sitosterol and campesterol concentrations.[67, 68] 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 the lumen of the small intestine 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 has never been directly determined in an *in vitro* assay. Studies investigating 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,[68] whereas other genetic association studies have failed to confirm this relationship.[67, 70]

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 CHD. Recently, however, elevated plant sterol levels were associated with a reduced cardiovascular risk.[66] Moreover, a high plant sterol content of the diet has been linked 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 CHD in some Asian populations.[76] In addition, adding plant sterols to margarine and other foods is currently used to optimize cardiovascular risk profile by decreasing plasma cholesterol concentrations.[77] The question nowadays is whether the LDL cholesterol reduction outweighs the increase in serum plant sterol concentrations. This debate is among others based on data of the 4S study

in which those individuals with the largest increase in serum campesterol concentrations during simvastatin treatment did not benefit in terms of a lower cardiovascular mortality despite the same reduction in serum cholesterol concentrations as those individuals with the lowest campesterol increase.[78] Based on these data it is tempting to suggest that an elevation in serum plant sterols – or actually an increase in the cholesterol standardized serum plant sterol concentrations meaning that lipoprotein particles become selectively enriched in plant sterols - may overrule the anti-atherogenic effects of lowering serum cholesterol concentrations.[79]

We found no association between the T400K polymorphism and CHD or 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.[67-69] In line with our observations, none of these studies found a significant relationship between the T400K variant and plasma cholesterol levels.

The combination of the risk genotypes of both ABCG8 polymorphisms, present in 8.4% of the cohort, was associated with a more evidently increased susceptibility to CHD. It is tempting to suggest that differences in plant sterol concentration could explain the observed association with CHD risk, although we were unable to measure plasma plant sterol concentrations. We cannot rule out the possibility that other, yet unknown, factors are responsible for the observed increased risk in carriers of the ABCG8 risk genotype. Our observations should, therefore, be replicated in larger population-based studies.

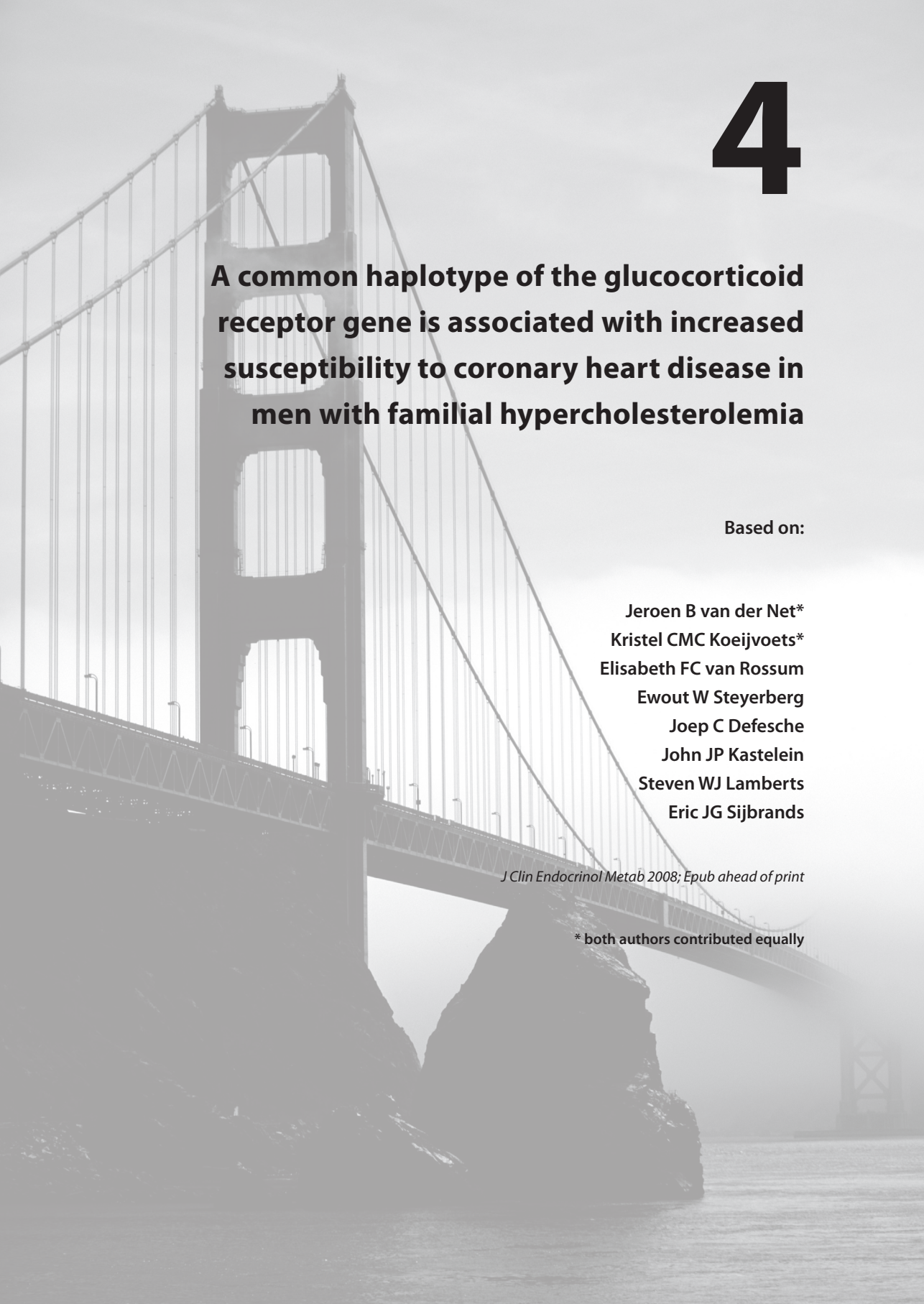
Because treatment with statins influences CHD risk and plasma plant sterol concentrations in opposite directions,[80] we investigated whether statin therapy might have been a confounder in our analysis. We were fortunate to have a relatively large proportion of untreated patients in our historical cohort, because nowadays almost all FH patients receive statin therapy. We adjusteded for statin therapy in our Cox proportional hazards models, but this did not change our results.

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 severely increased risk of CHD.[12] Although all FH patients have severe hypercholesterolemia, excess mortality caused by CHD occurs in 60% of the untreated patients whereas 40% reach a normal life span.[12] Clearly, secondary genetic factors and environmental factors determine CHD risk in FH.[12] The monogenic background and the large variation of CHD risk offer a unique opportunity to analyze secondary genes involved in CHD.[73] 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.[67-71]

Several potential limitations of the present study have to be mentioned. First, it depended on medical records, questionnaires, and information retrospectively obtained from physicians as the primary source of data. Some information of interest, such as plasma plant sterol

levels, was 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 CHD risk. In addition, we could not replicate our findings in a second independent study population, because to our knowledge this is the only large FH cohort with 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 CHD, but we did not observe a relationship between the ABCG8 T400K polymorphism and CHD. Carrying the risk genotype of both ABCG8 polymorphisms was associated with a more evidently increased risk of CHD. Our observations suggest that the ABCG8 gene may influence the burden of atherosclerosis.



4

A common haplotype of the glucocorticoid receptor gene is associated with increased susceptibility to coronary heart disease in men with familial hypercholesterolemia

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ABSTRACT

Objectives

Glucocorticoids contribute to the development of atherosclerosis. Four polymorphisms in the glucocorticoid receptor (GR) gene have been reported to alter glucocorticoid sensitivity and have been associated with cardiovascular risk factors. Studies on the relationship between these GR variants and coronary heart disease (CHD) risk, however, have yielded conflicting results**.** We sought to determine whether haplotypes based on functional polymorphisms in the GR gene influenced susceptibility to CHD in a high-risk population.

Methods

In a multicenter cohort study, 1830 patients with heterozygous familial hypercholesterolemia were genotyped for the functional ER22/23EK, N363S, *Bcl*I, and 9β variants. We analyzed the combined effect of all GR variants by constructing haplotypes and using a Cox proportional hazards regression model with adjustment for year of birth and smoking. The analyses were stratified for sex.

Results

A total of 333 (37.9%) men and 191 (20.1%) women had at least one CHD event. In men, the *Bcl*I haplotype was associated with a 36% higher CHD risk (CI, 1.02–1.80; p = 0.04). In women, none of the GR haplotypes was significantly related with CHD. We did not find differences in cardiovascular risk factors between GR haplotypes.

Conclusions

In this large cohort of high-risk individuals, one common haplotype in the GR gene modified CHD susceptibility among men.

INTRODUCTION

Glucocorticoids play an important role in the pathophysiology of atherosclerosis by their contribution to the development of hypertension, dyslipidemia, insulin resistance, glucose intolerance, and central adiposity.[81, 82] 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.[83] Upon ligand binding, the GR translocates to the nucleus where it results in a cascade of events, which eventually leads to the induction or repression of glucocorticoid responsive genes.

The sensitivity to glucocorticoids varies considerably among individuals.[84] 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.[85] Mutations in the GR gene are extremely rare and result in generalized glucocorticoid resistance syndromes.[86] Several more common polymorphisms moderately alter glucocorticoid sensitivity and have been associated with cardiovascular risk factors.[87]

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.[84, 88] In addition, the N363S variant has shown a relationship with increased insulin response to exogenous dexamethasone, higher body mass index (BMI), elevated plasma lipid levels, and a higher frequency of coronary heart disease (CHD).[84, 89, 90] The frequent *Bcl*I polymorphism has been linked to higher systolic blood pressure and increased abdominal visceral obesity.[91, 92] Opposite effects were found for the ER22/23EK polymorphism.[93-95] Carriers of this polymorphism were relatively resistant to the effects of glucocorticoids, because they had higher cortisol levels after a dexamethasone suppression test.[94] Furthermore, they had lower plasma total and low-density lipoprotein (LDL) cholesterol concentrations, an increased insulin sensitivity, and a beneficial body composition at young-adult age.[94, 95] We also found subtle effects of this polymorphism on CHD risk that varied significantly by sex.[93] The ER22/23EK variant has been previously linked to a fourth polymorphism in the GR gene, the 9β variant: the rare 22/23EK allele was only present in combination with the rare 9β G-allele.[96] Recently, the 9β haplotype was associated with increased risk of CHD in a cohort of elderly individuals.[97]

The effect of a genetic variant on susceptibility for a complex disorder such as CHD is a priori expected to be modest. Therefore, the association between GR variants with CHD should preferably be examined in a population at increased risk of CHD with an associated high power to detect potential small effects of the genetic variant.[73] In the present study, we therefore determined the effect of haplotypes based on these four functional polymorphisms in the GR gene on CHD risk in patients with heterozygous familial hypercholesterolemia (FH) who have a severely increased predisposition to premature cardiovascular events.

MATERIALS AND METHODS

Study design and study population

A description of the GIRaFH study population and the definition of FH and CHD are given in Chapter 1.

Molecular analysis

Genotypes were detected by allelic discrimination, 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° Celsius.

The ER22/23EK polymorphism comprises two linked, single nucleotide variations separated by one base pair in exon 2 (rs 6189 and rs 6190). 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 (rs 6195) 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 (no rs number) 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 9β polymorphism (rs

Table 1. Primer and probe sequences for polymorphisms in the glucocorticoid receptor gene

Polymorphism		Sequence (5′–3′)
ER22/23EK (G/A)	Forward primer	AGAAGAAAACCCCAGCAGTGT
	Reverse primer	CAGTAGCTCCTCCTCTTAGGGTTTTA
	Probes	VIC–CACATCTCCCCTCTCCTGA
		FAM–CACATCTCCCTTTTCCTGA
N363S (A/G)	Forward primer	GTCATTCCACCAATTCCCGTTG
	Reverse primer	GTCAAGTTGTCATCTCCAGATCCTT
	Probes	VIC–ACCTATTCCAATTTTCGG
		FAM–CCTATTCCAACTTTCGG
*Bcl*I (C/G)	Forward primer	CAGGGTTCTTGCCATAAAGTAGACA
	Reverse primer	GCACCATGTTGACACCAATTCC
	Probes	VIC–CTCTTAAAGAGATTGATCAGC
		FAM–CTCTTAAAGAGATTCATCAGC
GR-9β (A/G)	Forward primer	TCAGACTGTAAAACCTTGTGTGGAA
	Reverse primer	CCAATTCGGTACAAATGTGTGGTT
	Probes	VIC–CTTTTATTTTTTCATTTAAATTT
		FAM–TTTATTTTTTCGTTTAAATTT

6198) is located in the 3' untranslated region of exon 9β at nucleotide position 3669 and results in an A to G mutation. The DNA of the remaining patients was missing, because it was contaminated, severely fragmented, or fully used in previous analyses. Successful DNA genotyping of the GR gene polymorphisms was possible in 2024 (94.4%) patients for the ER22/23EK (G/A), 2032 (94.7%) patients for the N363S (A/G), 2063 (96.2%) patients for the *Bcl*I (C/G), and 2025 (94.4%) patients for the 9β (A/G). Analysis of 174 duplicates showed highly concordant results (99%). A total number of 1830 patients had complete GR genotypes and were used in haplotype analysis. There were no differences in CHD risk between individuals with total GR genotypes and excluded individuals (data not shown).

Statistical analysis

All data were analyzed using SPSS for Windows software package version 11.5.0 (SPSS Inc., Chicago, IL, USA). Previously, we have reported opposite effects of the ER22/23EK polymorphism on CHD risk in men and women.[93] Hence, we stratified all present analyses by sex. 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 an exact test.[75]

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 to CHD. The specific 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.[98] In over 99% of the individuals, the alleles of the haplotypes could be inferred with 96-100% certainty. Differences in cardiovascular risk factors between haplotypes were tested with chi-square statistics for dichotomous variables and independent sample t-test or one-way ANOVA for continuous variables.

The association between GR haplotype and CHD was evaluated using a Cox proportional hazards regression model.[99] Follow-up started at birth and ended at the first occurrence of established fatal or non-fatal CHD. Patients without CHD were censored at the date of the last lipid clinic visit or at the date of death attributable to other causes than CHD. 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 CHD by adjusting for covariates that had significant effects on CHD 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.[100] The haplo.glm function applied a general linear model to investigate the association between the haplotypes and CHD with adjustment for age and smoking status.

Unfortunately, we do not have information about the age of menopause in our cohort. Alternatively, we studied the presence of an age effect among women by additionally adjusting the Cox proportional hazards models for age tertiles, which were defined by cut-off values of 43.3 and 58.3 years. This adjustment did not change the results (data not shown).

For all statistical analyses, a P value of less than 0.05 was considered statistically significant.

RESULTS

Patient characteristics

The general characteristics of 879 men and 951 women with complete GR genotypes are shown in Table 2. Men visited the lipid clinic earlier, were more often smokers, had a higher BMI, lower plasma total and HDL cholesterol levels, and higher plasma concentrations of triglycerides compared to women.

Table 2. Characteristics of 1830 individuals with familial hypercholesterolemia

	Men	Women	P value*
	(n=879)	(n=951)	
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	67.2	<0.001
Hypertension (%)	8.6	9.4	0.9
Diabetes mellitus (%)	5.2	6.5	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†

Values are given as means ± standard deviation or percentages. Abbreviations: LDL = low-density lipoprotein, HDL = high-density lipoprotein.
*Additional adjustment for age. † Statistical testing after logarithmic transformation.

Table 3 presents the genotype distributions for all studied GR gene polymorphisms. The observed genotype distributions were in Hardy-Weinberg equilibrium ($p > 0.3$).

Table 3. Genotype distributions for all glucocorticoid receptor gene polymorphisms

Polymorphism	Genotype	Men		Women	
		CHD N (%)	No CHD N (%)	CHD N (%)	No CHD N (%)
ER22/23EK	Wild type	506 (92.7)	313 (94.0)	709 (93.3)	178 (20.1)
	Heterozygous	40 (7.3)	20 (6.0)	51 (6.7)	11 (17.7)
	Homozygous	0 (0)	0 (0)	0 (0)	0 (0)
N363S	Wild type	516 (94.5)	302 (90.7)	707 (93.0)	176 (92.1)
	Heterozygous	30 (5.5)	31 (9.3)	53 (7.0)	15 (7.9)
	Homozygous	0 (0)	0 (0)	0 (0)	0 (0)
*Bcl*1	Wild type	242 (44.3)	129 (38.7)	311 (40.9)	75 (39.3)
	Heterozygous	251 (46.0)	160 (48.0)	352 (46.3)	95 (49.7)
	Homozygous	53 (9.7)	44 (13.2)	97 (12.8)	21 (11.0)
GR-9β	Wild type	382 (70.0)	239 (71.8)	516 (67.9)	134 (70.2)
	Heterozygous	149 (27.3)	87 (26.1)	223 (29.3)	52 (27.2)
	Homozygous	15 (2.7)	7 (2.1)	21 (2.8)	5 (2.6)

CHD indicates history of coronary heart disease.

GR haplotype distributions

Frequencies of the five most common haplotypes of the GR gene based on the ER22/23EK, N363S, *Bcl*I, and 9β polymorphisms are presented in the Figure. The most frequent haplotype (haplotype 1, wild type haplotype; 45%) was the haplotype with the wild type alleles of all four polymorphisms. No statistical significant differences in cardiovascular risk factors were observed between any specific GR haplotype and the wild type haplotype (data not shown).

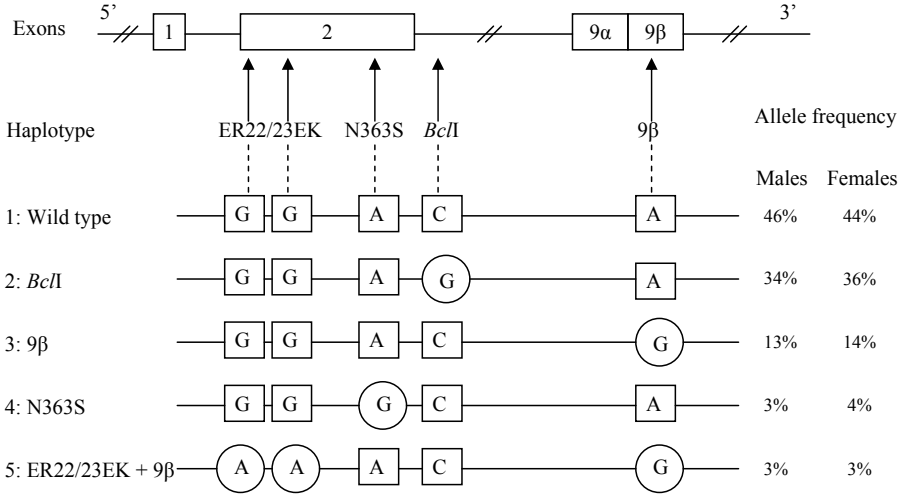


Figure. Schematic overview of the five most frequent haplotype alleles of the glucocorticoid receptor gene based on the four polymorphisms in 1830 patients with familial hypercholesterolemia. The haplotype frequencies in the study population are depicted on the right side. Squares indicate the wild type alleles of the polymorphisms; circles indicate the variant alleles of the polymorphisms.

Table 4. Association between glucocorticoid receptor haplotypes and CHD in men and women with FH

GR haplotype	Men			Women		
	HR	(95% CI)	P value	HR	(95% CI)	P value
Wild type	1.00		reference	1.00		reference
GR-9β	1.37	(0.98 to 1.92)	0.06	0.95	(0.62 to 1.47)	0.8
*Bcl*I	1.36	(1.02 to 1.80)	0.04	0.97	(0.66 to 1.44)	0.9
N363S	1.31	(0.77 to 2.22)	0.3	0.90	(0.42 to 1.94)	0.8
ER22/23EK + GR-9β	1.11	(0.63 to 1.95)	0.7	1.26	(0.55 to 2.90)	0.6

Abbreviations: GR indicates glucocorticoid receptor. FH = familial hypercholesterolemia, HR = hazard ratio, CHD = coronary heart disease

GR haplotype and CHD

During 42868 person years, 333 (37.9%) men had onset of CHD and a total of 191 (20.1%) women had their first CHD event during 48294 person years. The mean age of onset of CHD (± SD) was 46.0 ± 9.1 years in men and 53.7 ± 11.1 years in women. In men and women, year of birth, smoking, and lower plasma HDL cholesterol concentrations were significantly associated with increased CHD risk (data not shown). The presence of diabetes mellitus and higher BMI also contributed to an increased risk of CHD in women but not in men (data not shown).

Table 4 shows the effect of the GR gene haplotypes on CHD risk after adjustment for year of birth and smoking and stratified by sex. Compared to the wild type haplotype, the *Bcl*I haplotype (haplotype 2), consisting of the mutant allele of the *Bcl*I polymorphism and the wild type alleles of the other polymorphisms, was associated with a 36% higher CHD risk in men only (p = 0.04, Table 4). A similar association in men was found for the 9β haplotype (haplotype 3) that consists of the mutant allele of the 9β polymorphism and wild type alleles of all other polymorphisms: this haplotype was associated with a 37% higher risk of CHD in men, but this association was only marginally significant (p = 0.06, Table 4). In women, none of the haplotypes was significantly related to CHD. Additional adjustment for covariates that had significant effects on CHD risk in univariate Cox regression analyses did not influence the results. The interaction terms between the haplotypes and sex were not statistically significant (data not shown). The results of the analyses with haplo.stats were concordant.

DISCUSSION

In this large cohort of FH individuals with severely increased predisposition to CHD, we found that one common GR gene haplotype was associated with CHD susceptibility in men. Men carrying the *Bcl*I haplotype had a 36% higher CHD risk compared to men who carried the wild type haplotype. We did not find differences in cardiovascular risk factors such as BMI, plasma lipid levels, or hypertension between this GR haplotype and the wild type haplotype. This suggests that the increased risk for CHD might be due to until now unknown risk factors

for CHD. An alternative explanation may be that mild alterations, not yielding statistically significant differences, can ultimately lead to increased CHD risk, because of the lifelong effect of this genetic variant.

The strength of our study is determined by the availability of a large, well-documented cohort of individuals with a monogenic cause of hypercholesterolemia who generally have a severely increased CHD risk.[1] 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. Although all individuals have severe hypercholesterolemia, CHD risk and mortality varies considerably and depend on both environmental and additional genetic factors.[4] The disorder is therefore an exemplary model to analyze modifier genes involved in CHD.[73]

This is the first study that showed a relationship between the *Bcl*I haplotype and CHD susceptibility. The increased susceptibility to CHD in carriers of the *Bcl*I haplotype is in line with previous studies in middle-aged individuals that found an association between this GR polymorphism with an unfavorable cardiovascular risk profile.[91, 92] After a dexamethasone suppression test, heterozygous and homozygous G allele carriers showed a greater suppression of cortisol levels, suggesting a hypersensitivity to glucocorticoids,[88] which was confirmed in a study by Stevens et al.[101] Contrasting data have been reported about the relationship between this polymorphism and body composition, probably due to differences in age and BMI.[87] Studies restricted to middle-aged, non-obese individuals as in our population showed, however, clearly negative effects on body composition: the G allele of the *Bcl*I polymorphism was consistently associated with increased abdominal visceral obesity.[91, 102] Interestingly, these studies showed no relationship of the *Bcl*I polymorphism with general obesity and these observations are in concordance with the lack of an association between this GR polymorphism and BMI in the present study. Unfortunately, we did not have extended data on body composition to confirm the association with increased abdominal fat.

At present it is not known whether this polymorphic *Bcl*I site is functionally related to the previously observed features, ultimately leading to an elevated CHD risk. Since this is an intronic polymorphism, functionality is difficult to test in vitro. To present knowledge, it seems not to be located in a splice enhancer or silencer site nor in a coding or regulatory region of the GR gene. We cannot exclude that this polymorphism may be linked to another functional polymorphism in the promoter region or in the transactivating, ligand binding or DNA binding domains.

The association between the GR-9β haplotype and CHD was marginally significant. The 9β polymorphism is located in an "ATTTA" motif and results in an increased stability of the GRβ mRNA and in an increased GRβ protein expression.[103, 104] Enhanced GRβ expression may cause greater inhibition of GRα transcriptional activity, resulting in relative glucocorticoid resistance. It may seem paradoxically that two genetic variants with opposite effects on glucocorticoid sensitivity are both associated with an increased risk of CHD in FH patients. However, glucocorticoids affect the process of atherosclerosis through several mechanisms.[82]

It is important to distinguish between the transactivational effects on the one hand and transrepressional effects of glucocorticoids on target genes on the other hand. Transactivational effects mediated by the GR e.g. result in increase of visceral fat accumulation,[105] and regulate lipid and glucose metabolism,[106] acting through binding of the GR as a dimer to the DNA of target genes.[107] In contrast, effects on the immune system and inflammatory system are predominantly mediated by transrepressional action of the GR, whereby the GR acts as a transcription factor, interacting as a monomer with other proteins to repress target genes.[108] In this way, the GR acts on the immune system and inflammation, through effects on e.g. many interleukins, nuclear factor (NF)-κB, toll-like receptors and numerous other factors involved in these signalling pathways. Therefore, hypersensitivity to the transactivational GR effects, as previously has been found to be present in carriers of the *Bcl*I polymorphism,[88, 101] as well as hyposensitivity to the transrepressional effects of the GR, as has been demonstrated for the GR-9β polymorphism [96] can both lead to enhancement of the atherosclerotic process.

In our study, the association between the *Bcl*I haplotype with increased CHD risk was restricted to men. The reason for the differences of GR variants between men and women is yet unknown. The response of the hypothalamic-pituitary-adrenal axis to variations in circulating sex steroid concentrations differs -at least in rats- between sexes: estrogen primarily exerts stimulatory effects on stress-induced adrenocorticotropic hormone (ACTH) and glucocorticoid release, whereas testosterone inhibits stress-related hypothalamic-pituitary-adrenal axis activity.[109] 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 sex differences.[110] Obviously, further research is warranted to acquire more information about the mechanisms underlying sex-specific differences in the expression of GR target genes.

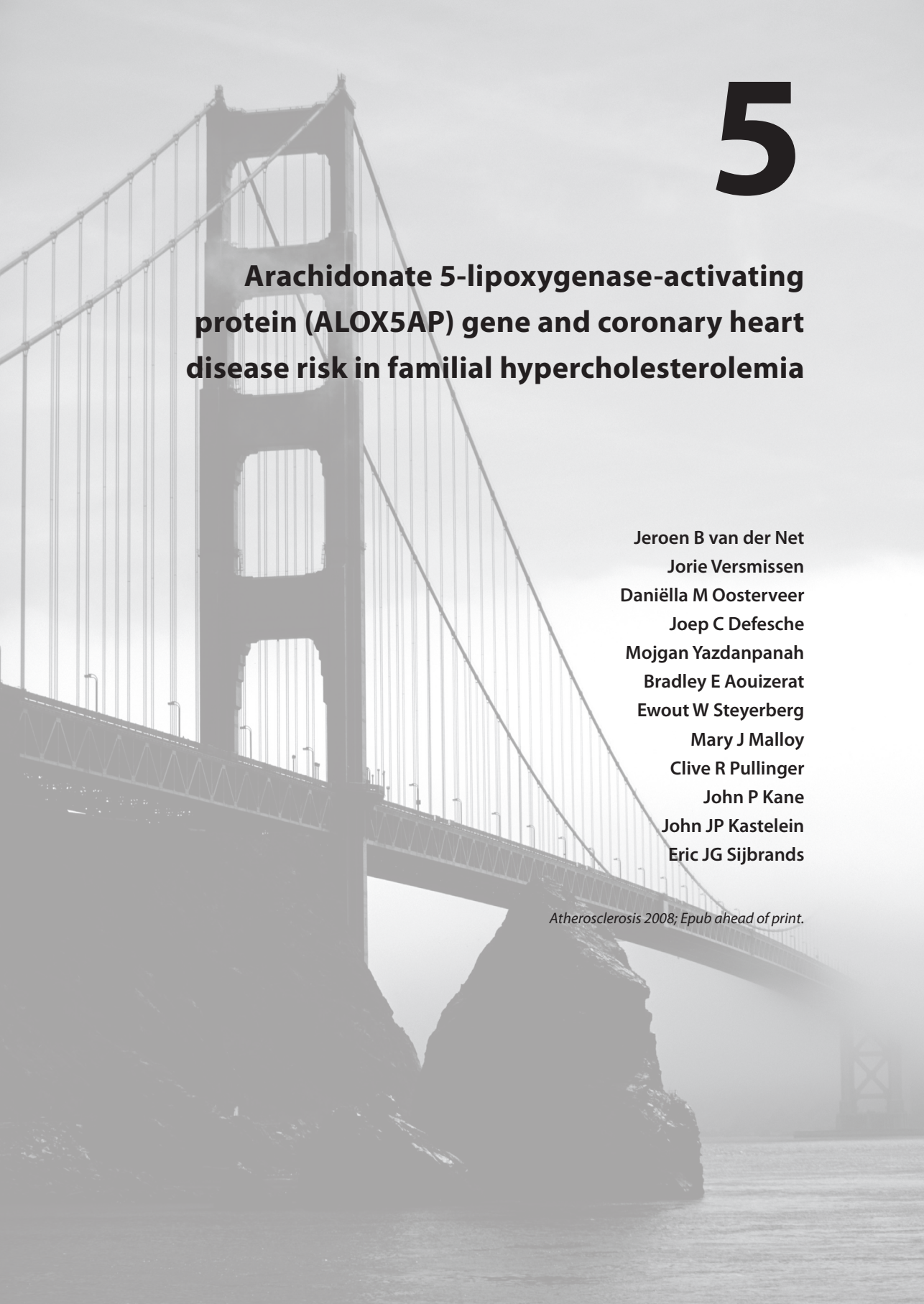
In the present cohort study, we did not find an association of the N363S and the ER22/23EK haplotypes with CHD susceptibility. A possible explanation for the lack of an association between these two GR variants and CHD susceptibility could be their relatively low frequency. Only 3% of the population carried the ER22/23EK and the N363S haplotype. The effect of such an infrequent haplotype on CHD susceptibility should be very strong to achieve adequate statistical power.

In heterozygous FH patients, the cholesterol uptake in the adrenal gland is impaired because of partially reduced LDL receptor function. However, despite this impaired cholesterol uptake, a normal glucocorticoid synthesis is maintained in FH patients.[111] This is partly due to an increased endogenous cholesterol synthesis as a consequence of the reduced availability of exogenous LDL cholesterol.[112] Therefore, we believe that our findings can be applied to other high-risk populations.

The most important limitation of our study is that data were obtained from medical records and some information of interest, such as body composition, plasma insulin levels, inflammation markers, and age of menopause were not known. The influence of these factors on the relationship between the GR variants and CHD risk could therefore not be measured. We

estimated that approximately half of the women had passed menopause at the end of follow-up. Among women, we adjusted for age tertiles in order to take this possible confounder into account, but this did not change our results (data not shown).

In conclusion, in this large cohort study of high-risk patients with severe hypercholesterolemia, the *Bcl*I haplotype was significantly associated with increased CHD risk in men. This suggests that the GR gene modifies CHD susceptibility among male FH patients. Our results provide insight in the pathophysiology underlying CHD, which could ultimately lead to novel pharmacological interventions. Furthermore, genotyping GR variants may help to identify subgroups of male FH patients with a particularly increased CHD risk.



5

Arachidonate 5-lipoxygenase-activating protein (ALOX5AP) gene and coronary heart disease risk in familial hypercholesterolemia

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ABSTRACT

Objectives

The ALOX5AP gene is required for the synthesis of leukotrienes, a protein family involved in inflammatory responses. Recently, genetic variation in this gene was shown to be associated with myocardial infarction in an Icelandic and British population. Since FH is characterized by severely increased levels of plasma low-density lipoprotein (LDL) cholesterol levels, chronic inflammation of the arterial wall, and subsequent premature CHD, the ALOX5AP gene could be an important modifier gene for CHD in FH.

Methods

In a cohort of 1817 FH patients, we reconstructed two four-marker haplotypes, previously defined in Icelandic (HapA) and British (HapB) individuals. The haplotypes were inferred with PHASE and the associations between the haplotypes and CHD were analyzed with a Cox proportional hazards model, adjusted for year of birth, sex, and smoking.

Results

HapB had a frequency of 6.9% and 8.2% in the group without and with CHD, respectively, conferring a hazard ratio of 1.48 (95% CI 1.17-1.89, p=0.001). This association was predominantly found in patients with LDL cholesterol levels above the median (HR 1.82, 95% CI 1.20-2.76, p=0.005). HapA was not associated with CHD.

Conclusion

We conclude that genetic variation in the ALOX5AP gene contributes to CHD risk in patients with FH. Our findings emphasize the important role of inflammation in the pathogenesis of early CHD in this disorder, particularly in patients with more severely raised LDL cholesterol levels.

INTRODUCTION

Familial hypercholesterolemia (FH) is an autosomal dominant disorder commonly caused by mutations in the low-density lipoprotein (LDL) receptor gene.[2] Coronary heart disease (CHD) is the major burden of FH,[113] but there is considerable variation in the onset of CHD and all-cause mortality among FH patients.[4, 5] In addition to classical risk factors, modifier genes may be of importance in determining the risk of CHD in FH.[12, 40]

The 5-lipoxygenase-activating protein (FLAP), encoded by the arachidonate 5-lipoxygenase-activating protein (ALOX5AP) gene, is an important mediator of the activity of 5-lipoxygenase, a key enzyme in the biosynthesis of leukotrienes.[114] Leukotrienes are proinflammatory eicosanoid signaling molecules that are produced by inflammatory cells from omega-3 and omega-6 fatty acid precursors.[115] Because it has been suggested that the arterial wall of hypercholesterolemic patients is continuously subjected to an inflammatory challenge,[116, 117] the ALOX5AP gene could be an important candidate gene for CHD in FH patients. The accumulating evidence that atherosclerosis, the main cause of CHD, is a chronic inflammatory disorder supports this notion.[118]

Previous studies have indicated that genetic variation in the ALOX5AP gene is associated with myocardial infarction (MI), the main complication of CHD.[119, 120] Haplotype HapA, which is defined by four single nucleotide polymorphisms and spans the ALOX5AP gene, was associated with MI and stroke in an Icelandic population, and another haplotype HapB (also based on four polymorphisms), was associated with MI in British cohorts.[119] In subsequent studies, HapB has been replicated in other Caucasian populations: a significant association was found with CHD in an Italian cohort [121] and with MI in a German case-control study.[122]

Because of the high prevalence of CHD and the proposed chronic inflammatory state of the arterial wall in individuals with FH, the ALOX5AP gene may influence the clinical consequences of FH. The aim of the present study was to investigate the association between CHD and genetic variation in this potential modifier gene of CHD risk in a large FH population.

METHODS

Study design and study population

A description of the GIRaFH study population and the definition of FH and CHD are given in Chapter 1.

Genetic analyses

Seven polymorphisms in the ALOX5AP gene were examined in the present study: SG13S25 (rs17222814), SG13S377 (rs17216473), SG13S114 (rs10507391), SG13S89 (rs4769874), SG13S32 (rs9551963), SG13S41 (rs9315050), and SG13S35 (rs17222842). All genotypes were

determined using fluorescence-based TaqMan allelic discrimination assays and analyzed on an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). Primer and probe sequences are presented in Table 1. Reaction components and amplification parameters were based on the manufacturer's instructions using an annealing temperature of 60° C. Results were scored blinded to CHD status. The genotyping of the polymorphisms had success rates between 92% and 96%. A total of 192 duplicate samples showed highly concordant results (>99%).

Statistical analyses

All statistical analyses were performed with the SPSS for Windows 14.0 statistics program and the R statistical package.[49] All data are provided as mean ± standard deviation, unless stated otherwise, and all reported p-values are based on two-sided tests of significance. A p-value of <0.05 was considered statistically significant. We used the χ^2-test, t-test or ANOVA to test for differences between groups. In order to assess for genotype assay failure, Hardy-Weinberg equilibrium of the polymorphisms was tested with an exact test.[75] Because of a skewed distribution, plasma triglycerides were analyzed after logarithmic transformation.

To investigate the effect of cardiovascular risk factors on the cumulative risk of CHD till the age of 40, 50, and 60 years, we used Kaplan-Meier curves and log-rank tests. We assessed

Table 1. Primer and probe sequences

Polymorphism		Sequence
SG13S25	Forward primer	GACAGCATCAGCTAGTCTCTTTCC
	Reverse primer	CAAATTGGCCTTGATGAGTGGAA
	Probe VIC	CAGCCACTGTTACCCA
	Probe FAM	AGCCACTGTTGCCCA
	rs number	rs17222814
SG13S377	Forward primer	AGTAGAGATAGGGTTTTGCCATTTTGG
	Reverse primer	GCTCATGCCTATAATCACAAAACTGT
	Probe VIC	CCTGCCTCAGCCTC
	Probe FAM	CTGCCTCGGCCTC
	rs number	rs17216473
SG13S114	Assay number	C__27330284_10
	rs number	rs10507391
SG13S89	Assay numer	C__27929140_10
	rs number	rs4769874
SG13S32	Assay number	C__30145896_10
	rs number	rs9551963
SG13S41	Assay number	C__29803126_10
	rs number	rs9315050
SG13S35	Forward primer	GCCTGGCATTGAGGAGTTTTC
	Reverse primer	ATGCACCCCACAAATACCTACAA
	Probe VIC	TTTTAAAAAACTGAAAGGACC
	Probe FAM	AAAACCGAAAGGACC
	rs number	rs17222842

the association between each polymorphism and CHD with a genotypic test (2-df) in Cox proportional hazards regression.[99] Patients without CHD were censored at the date of the last lipid clinic visit or at the date of death attributable to causes other than CHD. The proportional hazards assumption was tested by drawing log minus log plots of the survival function and was met for all Cox proportional hazard models. In the primary model, Cox regression analyses were applied to determine the association of polymorphisms with CHD adjusted for sex, year of birth, and smoking. For smoking we implemented a linearly decreasing risk effect for the 6 years after cessation.[51] Secondary models were constructed to investigate whether potential associations could be explained by possible intermediary variables, such as hypertension, diabetes mellitus, BMI, plasma LDL and HDL cholesterol, and plasma triglycerides. The following variables had missing values: smoking (9.1%), BMI (14.1%), plasma LDL (20.0%) and HDL cholesterol (18.5%), and plasma triglycerides (15.7%). Therefore, we applied the areglmpute function of the R statistical package to impute missing values in the analyses that were adjusted for these variables.[49]

Unfortunately, software that combines haplotype estimation with person-years regression analysis is not available to date. Haplotypes were estimated with the PHASE program (version 2.1), which implements a Bayesian statistical method for reconstructing haplotypes from population genotype data.[98] HapA was reconstructed by the SG13S25, SG13S114, SG13S89, and SG13S32 polymorphisms, whereas HapB was reconstructed by the SG13S377, SG13S114, SG13S41, and SG13S35 polymorphisms.[119] We performed power calculations based on a simulation method,[99] in which we assumed a haplotype frequency of 15.8% and a relative risk of 1.80 for HapA, and a haplotype frequency of 7.5% and a relative risk of 1.95 for HapB, as reported in the original study.[119] This resulted in sufficient power for both haplotypes (100 and 99.9%, respectively). We also inferred haplotypes based on all seven polymorphisms. In over 95.6% of the cases, the alleles of the haplotypes could be inferred with 91-100% certainty, whereas likelihoods between 81-90% were obtained in 3.7% of the cases. In 0.7%, the likelihoods were between 56-80%. We performed Cox regression analyses with the haplotypes estimated with PHASE, weighted for the posterior uncertainty in the haplotype assignments. In these analyses, the haplotype of interest was compared to all other haplotypes constructed from the same polymorphisms, and we adjusted for cardiovascular risk factors as described above for the polymorphisms.

We investigated whether there was an interaction between the haplotypes and LDL cholesterol on the risk of CHD by determining the statistical significance of the interactionterms of the haplotypes and LDL cholesterol in the Cox proportional hazards analyses. In addition, we repeated the Cox proportional hazards analyses in two subgroups based on whether the LDL cholesterol level was below or above the median.

During the observation period 84% of the patients received statin therapy with a mean duration of 4.7 ± 3.8 years. Therefore, we investigated the association between CHD risk and each polymorphism and haplotype adjusted for statin use. This analysis gave similar results.

RESULTS

Patient characteristics

The clinical characteristics of the 2400 patients are presented in Table 1 of Chapter 1 and the cumulative risk of CHD at the age of 40, 50, and 60 years in Table 2 of Chapter 1. Haplotyping was successful for 1817 FH patients, due to missing genotype data in the remaining patients. These remaining patients did not differ from the group in the analysis with regard to CHD or cardiovascular risk factors (data not shown). During a total of 90473 person years, 514 patients had at least one CHD event (mean incidence rate: 5.7 new cases per 1000 person years). The mean age of onset of the first CHD event was 48.6 ± 10.5 years. The following variables were associated with a higher cumulative CHD risk in the group of 1817 patients: male gender, smoking, plasma LDL and HDL cholesterol levels below the median, and plasma triglyceride levels above the median (data not shown).

Polymorphisms and CHD

All polymorphisms were in Hardy-Weinberg equilibrium (p>0.05). Table 2 shows the associations between the seven studied polymorphisms and CHD. Patients heterozygous for the

Table 2. Genotypic association between polymorphisms and CHD

Polymorphism		Genotype frequency	Primary model		Secondary model	
			HR (95% CI)	p-value	HR (95% CI)	p-value
SG13S25	GG	0.80	1.00 (Ref)		1.00 (Ref)	
	GA	0.19	1.03 (0.82-1.29)	0.78	1.01 (0.80-1.26)	0.95
	AA	0.01	1.20 (0.59-2.43)	0.61	1.06 (0.52-2.18)	0.86
SG13S377	GG	0.73	1.00 (Ref)		1.00 (Ref)	
	GA	0.24	1.19 (0.97-1.45)	0.10	1.24 (1.01-1.52)	0.04
	AA	0.03	1.06 (0.61-1.85)	0.83	1.08 (0.62-1.89)	0.78
SG13S114	TT	0.44	1.00 (Ref)		1.00 (Ref)	
	TA	0.44	1.25 (1.04-1.50)	0.02	1.31 (1.08-1.58)	0.006
	AA	0.12	1.21 (0.90-1.62)	0.21	1.18 (0.88-1.58)	0.26
SG13S89	GG	0.93	1.00 (Ref)		1.00 (Ref)	
	GA	0.07	1.11 (0.79-1.58)	0.55	1.14 (0.80-1.61)	0.47
	AA	0.003	1.29 (0.32-5.19)	0.72	1.03 (0.25-4.21)	0.96
SG13S32	CC	0.25	1.00 (Ref)		1.00 (Ref)	
	CA	0.51	1.04 (0.84-1.28)	0.72	1.01 (0.81-1.25)	0.93
	AA	0.24	1.07 (0.81-1.42)	0.62	1.07 (0.83-1.37)	0.59
SG13S41	AA	0.88	1.00 (Ref)		1.00 (Ref)	
	AG	0.12	1.13 (0.86-1.49)	0.37	1.16 (0.88-1.52)	0.28
	GG	0.01	1.43 (0.59-3.45)	0.43	1.35 (0.56-3.28)	0.51
SG13S35	GG	0.83	1.00 (Ref)		1.00 (Ref)	
	GA	0.17	1.00 (0.78-1.26)	0.97	1.03 (0.81-1.32)	0.78
	AA	0.01	0.60 (0.19-1.87)	0.38	0.65 (0.21-2.03)	0.45

95% CI = 95% confidence interval, CHD = coronary heart disease, HR = hazard ratio. Primary model adjusted for sex, year of birth and smoking. Secondary model additionally adjusted for hypertension, diabetes mellitus, BMI, plasma LDL and HDL cholesterol, and plasma triglycerides.

SG13S114 polymorphism had a 25% higher risk than patients homozygous for the T-allele (p=0.02, Table 2). Carriers of at least one A-allele of this polymorphism (AA/AT) had a 24% higher risk than carriers of two T-alleles (dominant model, 95% CI 1.04-1.48, p=0.02, primary model, data not shown). Additional adjustment for hypertension, diabetes mellitus, BMI, plasma LDL and HDL cholesterol, and plasma triglycerides yielded similar results in all analyses (Table 2).

Haplotypes and CHD

The frequencies of the most common haplotypes based on the seven polymorphisms are presented in Figure 1, together with the frequencies of HapA and HapB. HapA had a frequency of 14.0% in the control group and 13.0% in the CHD group. The hazard ratio (HR) for CHD of HapA was 0.89 compared to the other haplotypes constructed from the same four polymorphisms after adjustment for sex, year of birth and smoking (95% CI 0.74-1.07, p=0.2, Table 3). The frequency of HapB was 6.9% in the control group and 8.2% in the CHD group, conferring a hazard ratio of 1.48 compared to the other haplotypes constructed from the same four polymorphisms (95% CI 1.17-1.89, p=0.001, adjusted for sex, year of birth and smoking, Table 3).

The mean plasma LDL cholesterol level in carriers of HapB did not differ from that in non-carriers of HapB (7.25 ± 1.74 mmol/l and 7.32 ± 1.89 mmol/l, p=0.6). However, there was an interaction between HapB and LDL cholesterol on CHD risk (p=0.01, adjusted for sex,

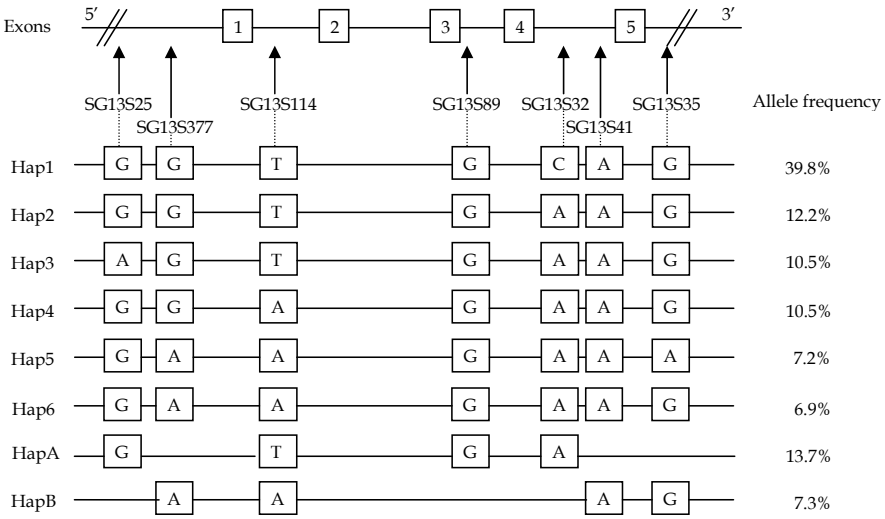


Figure 1: Schematic overview of the six most frequent haplotype alleles of the ALOX5AP gene based on the seven polymorphisms, together with HapA and HapB. Haplotype frequencies in the study population are depicted on the right side.

Table 3. Associations between haplotypes and CHD

			Primary model			Secondary model		
Haplotype	CHD (n)	Person Years	HR	95% CI	p-value	HR	95% CI	p-value
HapA-	390	67696	1.00			1.00		
HapA+	124	22777	0.89	0.74-1.07	0.23	0.90	0.74-1.08	0.26
HapB-	433	78057	1.00			1.00		
HapB+	81	12415	1.48	1.17-1.89	0.001	1.51	1.19-1.93	0.001
Hap6-	438	78549	1.00			1.00		
Hap6+	76	11924	1.45	1.13-1.86	0.003	1.47	1.14-1.89	0.003

95% CI = 95% confidence interval, CHD = coronary heart disease, HR = hazard ratio. HapA-/HapB-/Hap6- = patients without a HapA/HapB/Hap6 allele, HapA+/HapB+/Hap6+ = patients with at least one HapA/HapB/Hap6 allele. Primary model adjusted for sex, year of birth and smoking. Secondary model additionally adjusted for hypertension, diabetes mellitus, BMI, plasma LDL and HDL cholesterol, and plasma triglycerides.

year of birth and smoking). In patients with an LDL cholesterol level below the median of 6.97 mmol/l, the association between HapB and CHD was non-significant (HR 1.16, 95% CI 0.77-1.73, p=0.5), whereas this association was statistically significant in patients with an LDL cholesterol level above the median (HR 1.82, 95% CI 1.20-2.76, p=0.005). Figure 2 shows the Kaplan-Meier curves of the CHD-free survival stratified for HapB and LDL cholesterol levels.

The most frequent seven-marker haplotype (Hap1) was the haplotype with the wild-type alleles of the seven polymorphisms (Figure 1), and had a haplotype frequency of 39.9% in the control group, and 39.6% in the CHD group. Haplotype Hap6 had a frequency of 6.6% in the control group and 7.7% in the CHD group, and had a hazard ratio of 1.45 compared to the other haplotypes based on the seven polymorphisms (95% CI 1.13-1.86, p=0.003, adjusted for sex, year of birth, and smoking, Table 3). There was an interaction between Hap6 and LDL cholesterol on CHD risk, similar to the interaction described for HapB (data not shown).

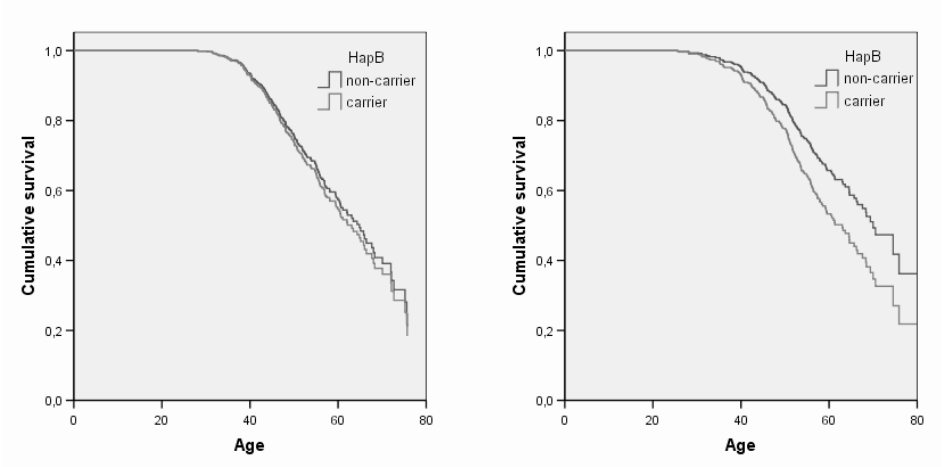


Figure 2: Cumulative CHD-free survival stratified for HapB. A: patients with LDL cholesterol levels below or equal to the median (≤ 6.97 mmol/l). B: patients with LDL cholesterol levels above the median (> 6.97 mmol/l).

Additional adjustment for hypertension, diabetes mellitus, BMI, plasma LDL and HDL cholesterol, and plasma triglycerides yielded similar results in all analyses (Table 3).

DISCUSSION

In the present study, we have shown that genetic variation within the ALOX5AP gene is associated with CHD in FH patients. Both HapB and Hap6 were associated with an increased risk of CHD in our FH population, and the patients with more severely raised LDL cholesterol levels contributed mainly to these associations.

With these findings we have validated HapB as a CHD risk factor. This haplotype was found to be associated with MI in a British cohort study with a haplotype frequency of 4.0% in the control group, and 7.5% in the MI cases.[119] However, subsequent studies showed conflicting results regarding the association of HapB with MI in Caucasian populations: a significant association was found with coronary artery disease (CAD) in an Italian cohort[121] and with MI in a German case-control study,[122] whereas no association was found with MI in the prospective Physician's Health Study[123] and another German case-control study.[124] The explanation for these inconsistent findings is not fully clear but may be the result of genetic heterogeneity across the different populations causing differences in the frequencies of the at-risk haplotypes.[119, 121, 123, 124] We also found an association between Hap6 and CHD. This association is not surprising, because Hap6 shares exactly the same alleles of the four polymorphisms on which HapB is based. Nevertheless, our findings suggest that there is no additional value of testing Hap6 over HapB, which means that the same information on the risk of CHD related to the ALOX5AP gene can be obtained by genotyping four instead of seven polymorphisms.

It remains unclear which underlying functional variant explains the association between HapB and CHD. We found an association between the A allele of the SG13S114 polymorphism and an increased CHD risk. It is not likely that the SG13S114 polymorphism itself is responsible for the association between HapB and CHD, because this polymorphism was not associated with MI/CAD in previous studies,[121, 123, 124] not even in the British cohort, in which the association between HapB and MI was first described.[119] Furthermore, the risk-associated allele of the SG13S114 polymorphism is also located on other haplotypes, which were not associated with CHD. Therefore, another yet unidentified genetic variant within HapB or within another gene in linkage disequilibrium with this haplotype is most likely responsible for the association between HapB and CHD.

It has been suggested that the association between ALOX5AP and CHD results from the influence of ALOX5AP on the leukotriene pathway.[115] FLAP, encoded by ALOX5AP, is an important mediator of the activity of 5-lipoxygenase, a key enzyme in the biosynthesis of leukotrienes.[114] Leukotrienes promote leukocyte chemotaxis and increase vascular permeability.[125]

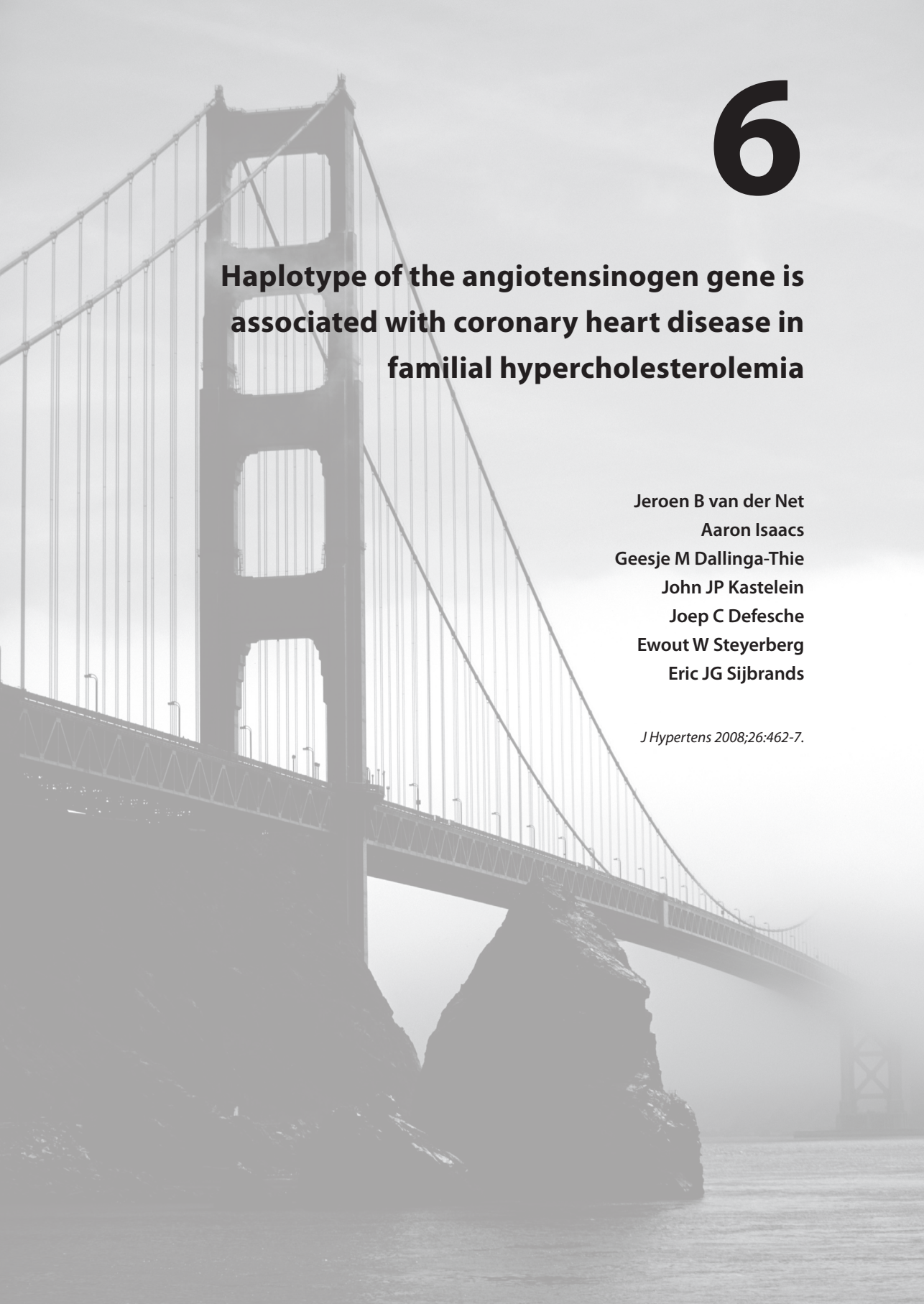
Interestingly, the original study by Helgadottir *et al.* showed that at-risk haplotypes were associated with an increased leukotriene B4 release by neutrophils.[119] A number of studies have illustrated the relevance of inflammation[118] and in particular of the leukotriene pathway in atherosclerosis.[126] In addition, other genes involved in inflammation, like the lipoprotein-associated phospholipase A2 (Lp-PLA2) gene and the toll-like receptor 4 (TLR4) gene, have been related to atherosclerotic phenotypes.[127, 128] Taken together, these data strengthen the hypothesis of involvement of this inflammatory pathway in the pathogenesis of atherosclerosis.

Recent studies have suggested that the arterial wall of hypercholesterolemic patients is subjected to a chronic inflammatory state.[116, 117] Our findings are in line with an interaction between hypercholesterolemia and inflammation on the risk of MI and CHD as observed in previous studies.[129] For instance, one study showed that infusion of C-reactive protein aggravated endothelial dysfunction in FH patients, whereas this was not observed in normocholesterolemic individuals.[129] This suggests that the consequences of the inflammatory state could be particularly deleterious in FH patients, and especially among those with more severely raised LDL cholesterol levels, as illustrated by the present study. The mean plasma LDL cholesterol level in carriers of HapB did not differ from that in non-carriers of HapB, and adjustment for plasma LDL cholesterol levels did not change the association between HapB and CHD in the total population (Table 3). An explanation for this modifying effect could be that patients with higher LDL cholesterol levels have higher levels of oxidized LDL, which has been shown to increase the 5-lipoxygenase activity by increasing the expression of ALOX5-AP.[130]

In the present population, higher levels of total and LDL cholesterol were associated with a lower cumulative CHD risk (Table 2 of Chapter 1). This could at least in part be explained by the fact that FH patients with total and/or LDL cholesterol levels above the median received statin therapy at a younger age than patients with levels below the median (mean age 43.5 ± 12.8 versus 44.9 ± 11.9 years, respectively, p=0.04, data not shown). Moreover, the mean number of years on statin treatment was 5.46 ± 3.8 and 3.5 ± 3.2 years for the patients with LDL cholesterol levels above and below the median, respectively ($p<0.001$).

There are a number of limitations to our study. First, plasma was not available to measure inflammatory parameters. Second, we did not have enough statistical power to study associations with MI or stroke, since only half of our patients with CHD had MI and only 3.5% of our total population had stroke. Third, we cannot rule out the possibility that some of the patients without clinically apparent CHD in fact had subclinical CHD. This could have caused underestimation of the associations. An important strength of the present study is that it represents a population with a severely increased CHD risk, different from other studied populations. The internal validity of the present study population is supported by the fact that the event rate in the present cohort is consistent with the literature on cardiovascular disease in FH patients.[2]

We conclude that genetic variation in the ALOX5AP gene is associated with CHD in our FH population. Our findings emphasize the important role of inflammation in the pathogenesis of CHD, particularly in FH patients with more severely raised LDL cholesterol levels. Determining the presence of HapB in these patients may be helpful in identifying a subgroup of FH patients particularly susceptible to develop CHD.



6

Haplotype of the angiotensinogen gene is associated with coronary heart disease in familial hypercholesterolemia

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ABSTRACT

Objectives

Familial hypercholesterolemia is characterized by high plasma low-density lipoprotein cholesterol levels and premature coronary heart disease. Despite the monogenetic origin of familial hypercholesterolemia, the incidence of coronary heart disease varies considerably among patients, which is only partly explained by classical risk factors. Hypertension is an important risk factor for coronary heart disease that is associated with angiotensinogen levels. Therefore, we analyzed the angiotensinogen gene as a modifier gene for coronary heart disease risk in patients with familial hypercholesterolemia.

Methods

In a cohort of 1785 familial hypercholesterolemia patients, we reconstructed five frequent haplotypes of the angiotensinogen gene, based on four polymorphisms. The five haplotypes cover approximately 98% of the genetic diversity accounted for by these four polymorphisms. The associations between the haplotypes and coronary heart disease were analyzed with the haplo.stats program, adjusted for age, sex and smoking.

Results

Patients homozygous for the C allele of the 4072 T→C polymorphism had a 34% increased coronary heart disease risk (p=0.017), compared to patients homozygous for the T allele. Haplotype H3, consisting of the minor allele of the 4072T→C polymorphism and the major alleles of the other polymorphisms, had a frequency of 15% and was associated with a 45% increased coronary heart disease risk (p=0.006) compared to the wild type haplotype H1.

Conclusion

We conclude that genetic variation in the angiotensinogen gene contributes to coronary heart disease risk in patients with familial hypercholesterolemia.

INTRODUCTION

Familial hypercholesterolemia (FH) is characterized by high plasma low-density lipoprotein (LDL) cholesterol levels, tendon xanthomas and premature coronary heart disease (CHD). Mutations in the LDL receptor are the cause of this disorder. Despite its monogenetic nature, there is considerable variation in the onset of cardiovascular disease and all-cause mortality.[4] In FH patients, coronary heart disease (CHD) represents the main cardiovascular burden. The variation in CHD risk is only partly explained by classical risk factors for CHD, such as sex, age, smoking, body mass index (BMI), hypertension and diabetes mellitus.[14] Therefore, modifier genes are also expected to contribute to the development of CHD in FH.[12, 40, 131]

Hypertension is an important risk factor for CHD; this is also the case in FH patients.[14] Plasma angiotensinogen levels are associated with blood pressure [92, 132] and the risk of hypertension.[133] Genetic association studies have shown a relationship between variation in the angiotensinogen gene and blood pressure, risk of hypertension and ischemic heart disease.[134-138] A number of studies showed that haplotypes of the angiotensinogen gene were significantly associated with hypertension.[139-143] However, the association with CHD remains to be clarified.[144] We hypothesized that the angiotensinogen gene is a modifier gene for CHD in our large cohort of patients with heterozygous FH.

We studied the association between CHD risk and haplotypes of the angiotensinogen gene. These haplotypes were based on four single nucleotide polymorphisms: -217G→A, 3889C→T (Thr174Met), 4072T→C (Met235Thr) and 11535C→A. With these four polymorphisms, five frequent haplotypes of the angiotensinogen gene can be reconstructed in Caucasian populations.[139]

Methods

Study design, population and data collection

A description of the GIRaFH study population and the definition of FH and CHD are given in Chapter 1. Antihypertensive treatment was defined as the use of angiotensin-converting enzyme (ACE) inhibitors, β-blockers, calcium antagonists and/or diuretics. In the whole group, BP levels were based on three measurements during a single office visit.

Genetic analyses

Four polymorphisms in the angiotensinogen gene were examined in the present study: -217G→A (rs5049), 3889C→T (Thr174Met, rs4762), 4072T→C (Met235Thr, rs699) and 11535C→A (rs7079). –217, 3889, and 11535 genotypes were determined using fluorescence-based TaqMan allelic discrimination assays and analyzed on an ABI Prism 7900 Sequence Detection System (Applied Biosystems). Primer and probe sequences are presented in Table 1.

Table 1. Primer and probe sequences

Polymorphism		Sequence
-217G→A	Forward primer	TCCTGCAAACTTCGGTAAATGTGT
	Reverse primer	GAAGTCTTAGTGATCGATGCAGAGT
	Probe VIC	TGCACCAGCTCAC
	Probe FAM	TGCACCGGCTCAC
	Design strand	Forward
	rs number	rs5049
3889C→T	Forward primer	CAGGGCAGGGCTGATAGC
	Reverse primer	GCACAAACGGCTGCTTCAG
	Probe VIC	CGCCCACCACCGTG
	Probe FAM	CGCCCACCACCATG
	Design strand	Reverse
	rs number	rs4762
11535C→A	Forward primer	GCAAGCACCTGAATTTCTGTTTGAA
	Reverse primer	GCTTATTGTGGCAAGACGTTTATTACT
	Probe VIC	CAGCTATTGTTCCGC
	Probe FAM	CAGCTATGGTTCCGC
	Design strand	Reverse
	rs number	rs7079

Reaction components and amplification parameters were based on the manufacturer's instructions using an annealing temperature of 60° C. Results were scored blinded to CHD status. The -217G→A (rs5049), 3889C→T (Thr174Met, rs4762), and 11535C→A (rs7079) polymorphisms had success rates of 91%, 95%, and 94%, respectively. The 4072T→C polymorphism was analyzed with a multilocus genotyping assay based on a probe mismatch hybridization method that was published previously, and had a success rate of 86%.[40]

Statistical analyses

The statistical analyses were performed with the SPSS for Windows 12.0.1 statistics program and the R statistical package.[49] We used the χ^2-test or t-test to test for differences between groups. Hardy-Weinberg equilibrium of the polymorphisms was tested with an exact test.[75] Due to a skewed distribution, plasma triglycerides were used after logarithmic transformation. All data are provided as mean ± standard error of the mean, unless stated otherwise, and all reported p-values are based on two-sided tests of significance. A p-value of <0.05 was considered statistically significant.

We studied the association between each polymorphism and hypertension status with a genotypic test (2-df) in logistic regression (univariable as well as multivariable with adjustment for age, sex, smoking and use of antihypertensive medication).

We assessed the association between each polymorphism and CHD with a genotypic test (2-df) in Cox proportional hazards regression. The years before the first occurrence of established fatal or non-fatal CHD in the population were removed from the analyses. Patients without CHD were censored at the date of the last lipid clinic visit or at the date of death attributable to causes other than CHD. In the primary model, Cox regression analyses

were applied to determine the association of polymorphisms with CHD adjusted for year of birth, sex, and smoking. In this primary model, we did not adjust for hypertension since blood pressure is a major part of the pathophysiological pathway studied in this analysis. In a secondary model, we additionally adjusted for hypertension, to study whether the potential associations were dependent on hypertension status. A tertiary model was constructed to investigate whether potential associations could be explained by possible intermediate covariables, such as diabetes mellitus, BMI, plasma HDL cholesterol, and plasma triglycerides. The following variables had missing values: smoking (9.9%), BMI (13.7%), plasma HDL-cholesterol (18.3%), and plasma triglycerides (15.6%). Therefore, we applied the aregImpute function of the R statistical package as imputation method in the analyses with adjustment for these variables.[49]

We performed haplotype analyses, weighted for the posterior uncertainty in the haplotype assignments, with the haplo.glm function of haplo.stats.[100, 145] The haplo.glm function applies a general linear model to investigate the association between the haplotypes and hypertension, with and without adjustment for age, sex, smoking and use of antihypertensive medication. Subsequently, we investigated the association between the haplotypes and CHD with adjustment for age, sex, and smoking. In further analyses, we additionally adjusted for possible confounding variables as described above. In addition, we estimated the haplotypes with the PHASE program (version 2.1), which implements a Bayesian statistical method for reconstructing haplotypes from population genotype data.[98] We performed Cox regression analyses with the haplotypes estimated with PHASE; the haplotypes were compared with the wild type haplotype.

RESULTS

Patient characteristics

The clinical characteristics of the 2400 patients are presented in Table 1 of Chapter 1 and the cumulative risk of CHD at the age of 40, 50, and 60 years in Table 2 of Chapter 1. The haplotyping was successful for 1785 FH patients. The 405 remaining patients did not differ from the group in the analysis with regard to CHD and hypertension, although they were more frequently male (54% vs. 48% males respectively, p=0.023). Furthermore, the patients included in our analysis were slightly older (50.0 vs. 48.6 years of age respectively, p=0.041). During a total of 56960 person years, 508 (28%) patients had at least one CHD event. The mean age of onset of the first CHD event was 49.0 years.

Genotype frequencies of polymorphisms

Table 2 shows the genotype frequencies of the four polymorphisms. All polymorphisms were in Hardy-Weinberg equilibrium in the whole population (p>0.15 for all polymorphisms) and

Table 2. Frequency distributions of polymorphisms in the angiotensinogen gene

Polymorphism	Total	CHD-		CHD+		p-value
		N	Freq (%)	N	Freq (%)	
-217G→A (rs5049)						
GG	1341	961	79	380	79	0.78
GA	329	235	19	94	20	
AA	19	15	1	4	1	
3889C→T (Thr174Met, rs4762)						
CC	1355	983	77	372	74	0.42
CT	396	274	22	122	24	
TT	20	14	1	6	1	
4072T→C (Met235Thr, rs699)						
TT	672	496	41	176	37	0.018
TC	770	554	46	216	45	
CC	253	163	13	90	19	
11535C→A (rs7079)						
CC	657	461	36	196	39	0.59
CA	855	619	49	236	47	
AA	259	188	15	71	14	

CHD- = patients without coronary heart disease, CHD+ = indicates patients with coronary heart disease. P-value is for χ^2-testing the differences in genotype distribution between cases and controls.

CHD subgroups (p>0.11 and p>0.35 for the patients with and without CHD, respectively). Patients with CHD were more frequently homozygous for the 4072C allele than patients without CHD (Table 2). For the other polymorphisms, there were no differences in genotype frequencies between patients with and without CHD.

Polymorphisms and hypertension/CHD

There were no significant associations of the polymorphisms with hypertension status (data not shown). Patients homozygous for the 4072C allele had a 34% increased CHD risk compared to patients homozygous for the 4072T allele (Table 3, p=0.017, adjusted for year of birth, sex, and smoking). Additional adjustment for hypertension (Table 3), diabetes mellitus, BMI, plasma HDL cholesterol, and plasma triglycerides did not change this association (HR 1.35, 95% CI 1.06-1.71, p=0.016). The other polymorphisms were not associated with CHD (Table 3).

Haplotypes and hypertension/CHD

The frequencies of the five most common haplotypes based on the four polymorphisms are presented in Figure 1. The most frequent haplotype (H2, 37%) was the haplotype with the A-allele of the 11535C→A polymorphism and the wild type alleles of the other polymorphisms. The haplotype consisting of all wild type alleles (haplotype H1) had a frequency of

Table 3. Genotypic association between polymorphisms and CHD

Polymorphism	Primary model		Secondary model	
	HR (95% CI)	p-value	HR (95% CI)	p-value
-217G→A (rs5049)				
GG	1.00		1.00	
GA	0.99 (0.78-1.27)	0.97	0.97 (0.76-1.24)	0.83
AA	0.79 (0.29-2.15)	0.65	0.78 (0.29-2.13)	0.63
3889C→T (rs4762)				
CC	1.00		1.00	
CT	1.02 (0.83-1.26)	0.85	1.01 (0.82-1.26)	0.91
TT	1.50 (0.67-3.38)	0.33	1.57 (0.70-3.54)	0.28
4072T→C (rs699)				
TT	1.00		1.00	
TC	1.08 (0.88-1.33)	0.47	1.08 (0.87-1.32)	0.50
CC	1.34 (1.05-1.70)	0.017	1.34 (1.05-1.70)	0.017
11535C→A (rs7079)				
CC	1.00		1.00	
CA	0.98 (0.81-1.20)	0.86	0.98 (0.81-1.20)	0.86
AA	1.02 (0.76-1.36)	0.91	1.02 (0.76-1.35)	0.92

95% CI = 95% confidence interval, CHD = coronary heart disease, HR = hazard ratio. Primary model adjusted for sex, year of birth and smoking. Secondary model additionally adjusted for hypertension.

25%. None of the haplotypes was associated with hypertension status. Haplotype H3, consisting of the minor allele of the 4072T→C polymorphism and the major alleles of the other polymorphisms, was associated with a 45% increased CHD risk (p=0.006, Figure 2) compared

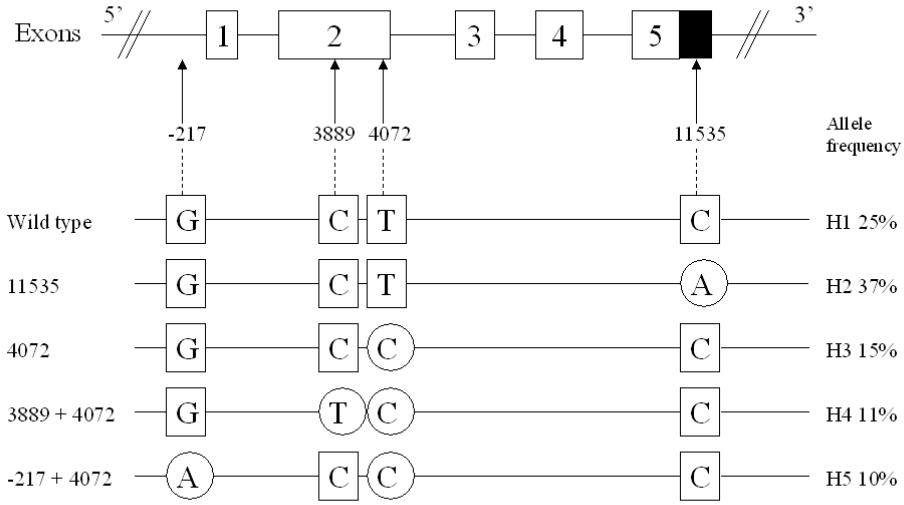


Figure 1: Schematic overview of the five most frequent haplotype alleles of the angiotensinogen gene based on the four polymorphisms. Haplotype frequencies in the study population are depicted on the right side. Squares indicate the wild type alleles of the polymorphisms; circles indicate the variant alleles of the polymorphisms. The black box represents the 5' untranslated region of exon 5.

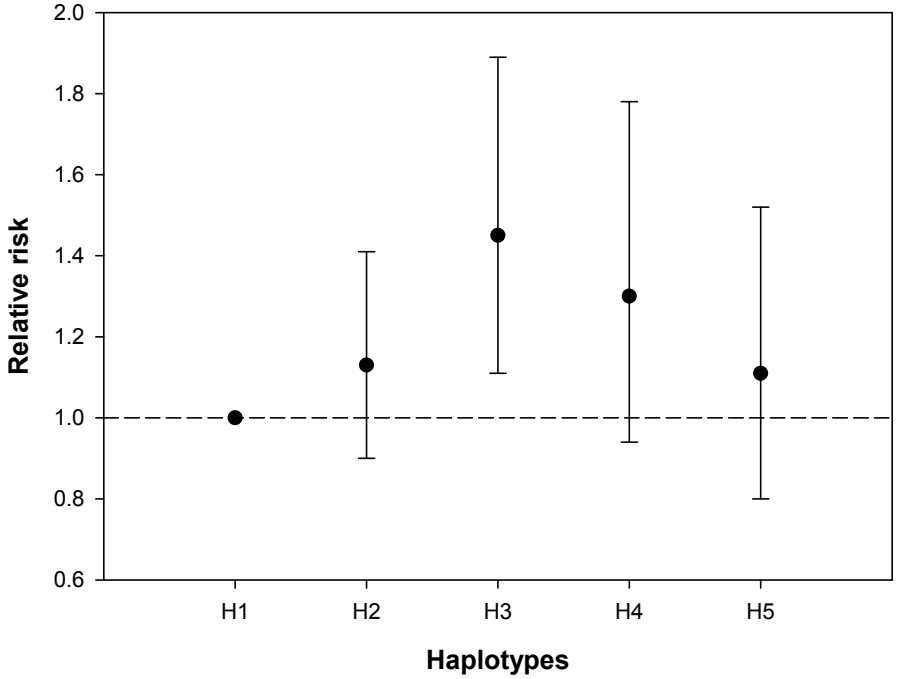


Figure 2: Associations between haplotypes of the angiotensinogen gene and CHD. Associations adjusted for sex, age and smoking. The haplotypes were compared with haplotype H1. Black squares represent relative risk estimates, vertical lines represent the 95% confidence intervals, CHD = coronary heart disease, RR = relative risk.

to the wild type haplotype H1, adjusted for age, sex, and smoking. The other haplotypes were not associated with CHD (Figure 2). Adjustment for hypertension did not change the association between haplotype H3 and CHD (RR 1.46, 95% CI 1.12-1.90, p=0.006). Additional adjustment for diabetes mellitus, BMI, plasma HDL cholesterol and plasma triglycerides had a marginal influence on the association between haplotype H3 and CHD (RR 1.40, 95% CI 1.10-1.77, p=0.007).

Using the PHASE program, the haplotypes could be inferred with 92-100% certainty in 99.3% of subjects, whereas in 0.7% the certainties were between 65% and 92%. The results of the Cox proportional hazards models with the haplotypes estimated by PHASE were identical to those with haplo.stats (HR 1.46, 95% CI 1.15-1.84, p=0.002, adjusted for year of birth, sex, and smoking).

DISCUSSION

We found that genetic variation in the angiotensinogen gene is associated with CHD risk in patients with FH. Haplotype H3, defined by the minor allele of the 4072T→C (Met235Thr) polymorphism, is associated with an increased CHD risk.

The 4072T→C polymorphism itself was also associated with an increased CHD risk, which is in line with previous studies.[134-138] Furthermore, this polymorphism has been associated with elevated angiotensinogen levels and an increase in risk of hypertension in white subjects.[133, 137, 146, 147] These findings might suggest that this polymorphism is the functional variant responsible for these associations. However, in the present study, the association between the 4072C allele and CHD was weaker than the association between haplotype H3 and CHD. Moreover, the 4072C allele is also present in haplotypes that are not associated with CHD (H4 and H5), with a combined frequency of 21% versus 15% for H3 (Figure 1). This suggests that the 4072C allele is in linkage disequilibrium with a functional variant instead of representing the functional variant itself.

Plasma angiotensinogen is converted to angiotensin I by renin, when renal perfusion is decreased. ACE converts angiotensin I to angiotensin II, which causes vasoconstriction, release of aldosterone, and a reduction in renal sodium reabsorption.[148] Because plasma angiotensinogen levels in our study population were not available, we were unable to confirm that the polymorphisms and/or haplotypes were associated with such levels. Furthermore, blood pressure levels attained from the medical records were based on single office visits. Instead, we used the diagnosis of hypertension in our analyses, because we could then be certain that more deliberate clinical evaluation had taken place.

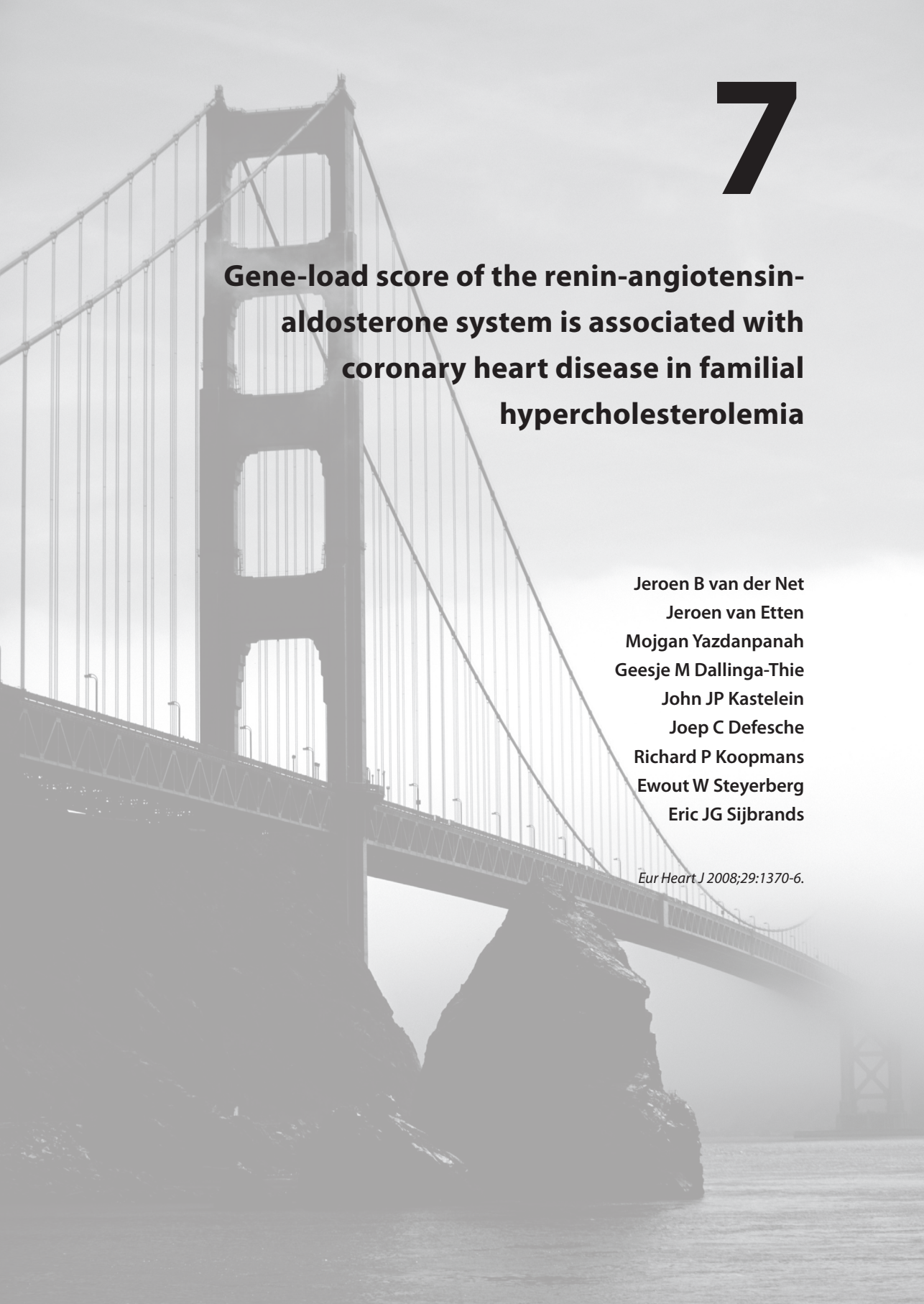
We hypothesized that genetic variation in the angiotensinogen gene would affect CHD risk by influencing blood pressure and/or the risk of hypertension. However, we found no associations of the polymorphisms or haplotypes with hypertension status. This is in line with a previous study, in which the same polymorphisms and haplotypes had no significant relationship with blood pressure in a Caucasian population.[139] In contrast, other studies showed that the 4072C allele was associated with an increase in plasma angiotensinogen levels and increased blood pressure parameters.[133, 137, 146, 147] We found that the association between haplotype H3 and CHD risk was still present after additional adjustment for hypertension status. This suggests that angiotensinogen influences CHD risk through pathways that are not related to blood pressure regulation. The influence of the renin-angiotensin-aldosterone system on the formation of reactive oxygen species supports the existence of such additional effects.[149, 150]

The haplotype analysis presented here was based on a limited number of polymorphisms, whereas Gu et al. performed haplotype analyses based on ten polymorphisms in the angiotensinogen gene.[139] These authors found that the five most frequent haplotypes accounted for 90% of the genetic variation in this gene (based on the ten polymorphisms measured).

These haplotypes were reconstructed with the same four polymorphisms as in the present study. Moreover, apart from the 11535C→A polymorphism, these polymorphisms have been associated with blood pressure or cardiovascular outcomes in earlier studies, supporting the notion that these polymorphisms are located in candidate risk alleles for CHD.[134-138, 143, 151, 152]

Our study has a number of limitations. First, the haplotypes given in this study are estimates and not true haplotypes. However, the posterior probabilities of the estimates in this study were close to one. Nevertheless, we used haplo.stats in order to take the posterior uncertainties into account in the analyses. Second, the findings presented in this study are based on a cohort consisting of patients with heterozygous familial hypercholesterolemia and, therefore, cannot be directly extrapolated to other populations. For this reason, our findings should be validated in other populations. Finally, our study included patients who were referred to lipid clinics and this could lead to selection bias. For instance, 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.[4] Nonetheless, we cannot exclude the possibility that we underestimated the risk.

We showed that genetic variation in the angiotensinogen gene is associated with CHD in patients with FH. Haplotypes of this gene could prove to be helpful in the development of genetic risk profiling, which ultimately will lead to personalized medicine for patients with FH.



7

Gene-load score of the renin-angiotensin-aldosterone system is associated with coronary heart disease in familial hypercholesterolemia

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ABSTRACT

Objectives

Familial hypercholesterolemia (FH) is characterized by premature coronary heart disease (CHD). However, the incidence of CHD varies considerably among FH patients. Genetic variation in the renin-angiotensin-aldosterone system (RAAS) and the adrenalin/noradrenalin system may be of importance in determining the CHD risk in FH, because of their involvement in CHD. We investigated the association between CHD risk and combined genetic variation in the RAAS and adrenalin/noradrenalin system.

Methods

In 2190 FH patients, we genotyped six RAAS polymorphisms and five adrenalin/noradrenalin polymorphisms. For each patient, we calculated two gene-load scores by counting the number of risk genotypes within each pathway.

Results

Four of the six RAAS polymorphisms and none of the polymorphisms in the adrenalin/noradrenalin system were significantly associated with CHD ($p<0.05$). The RAAS gene-load score was significantly associated with CHD ($p_{lineartrend}<0.001$): in patients with a gene-load score of 5 or 6, the CHD risk was 2.3 times as high as in patients with a score of 0 or 1. The gene-load score of the adrenalin/noradrenalin system was not associated with CHD.

Conclusion

Genetic variation in the RAAS contributes gene-dose dependently to CHD risk in patients with FH, whereas genetic variation in the adrenalin/noradrenalin system is not associated with CHD.

INTRODUCTION

Familial hypercholesterolemia (FH) is an autosomal dominant disorder caused by mutations in the low-density lipoprotein (LDL) receptor gene.[1] Clinically, the disease is characterized by severely elevated LDL cholesterol levels, tendon xanthomas, and premature coronary heart disease (CHD).[113] Although FH is a monogenic disorder, there is considerable variation in the onset of cardiovascular disease and all-cause mortality,[4] even among carriers of an identical LDL receptor mutation.[5, 153] Classical risk factors have been shown to influence the burden of FH. In addition, modifier genes may be of importance.[12, 40]

The renin-angiotensin-aldosterone system (RAAS) and adrenalin/noradrenalin system are involved in the development of atherosclerosis and CHD.[154-156] Therefore, the genes of these pathways are important candidate genes for CHD. However, studies conducted so far showed conflicting results concerning the associations between single nucleotide polymorphisms in genes involved in these pathways and CHD.[137, 157] These discrepancies could be explained by the fact that the RAAS and adrenalin/noradrenalin system are regulated by a large number of interacting genes resulting in highly redundant regulation systems. Most likely, genetic variation is only relevant when compensation fails. Therefore, it is appropriate to consider the combined effects of multiple genes when studying the effect of complex pathways on complex phenotypes such as CHD.[158]

We hypothesized that combination of multiple genetic variants of the RAAS and adrenalin/noradrenalin pathways is associated with CHD risk in FH patients. We investigated the combined effect of six RAAS polymorphisms and the combined effect of five adrenalin/noradrenalin polymorphisms in relation to CHD risk in a large cohort of FH patients.

METHODS

Study design, population and data collection

A description of the GIRaFH study population and the definition of FH and CHD are given in Chapter 1.

Genetic analyses

The DNA of 2190 patients was available for the present analysis, because the DNA of the remaining patients was insufficient in amount and/or quality for use in genetic studies. A total of six polymorphisms in genes involved in RAAS and five polymorphisms in genes involved in the adrenalin/noradrenalin system were selected as candidate genes. The 825C→T polymorphism in the G protein β3 subunit gene was involved in both the RAAS and the adrenalin/noradrenalin system. All selected polymorphisms have been associated with CHD, myocardial infarction, blood pressure, and/or hypertension. Seven of the 10 polymorphisms were ana-

Table 1. Primer and probe sequences

Polymorphism		Sequence
CYP11B2	Forward primer	ATCAATTTTGCAATGAACTAAATCTGTGGTATAAA
-344C→T	Reverse primer	AGGGCTGAGAGGAGTAAAATGGAT
	Probe VIC	TCCAAGGCCCCCTCT
	Probe FAM	TCCAAGGCTCCCTCT
	Design strand	Forward
ADRA1A 1039C→T	Forward primer	GCCTTTCAGAATGTCTTGAGAATCC
	Reverse primer	CCAGGGCATGTTTGGAAGACT
	Probe VIC	CTTTCTGCGGAGACAC
	Probe FAM	CTTTCTGCAGAGACAC
	Design strand	Reverse
ADRB1 145A→G	Forward primer	CCGCCCGCCTCGTT
	Reverse primer	CGCTGTCCACTGCTGAGA
	Probe VIC	CAGCGAAAGCCCCGA
	Probe FAM	CAGCGAAGGCCCCGA
	Design strand	Forward

lyzed with a multilocus genotyping assay based on a probe mismatch hybridization method that was published previously:[40] 4072T→C (Met235Thr, rs699) in the angiotensinogen (AGT) gene,[159] insertion/deletion polymorphism (rs1799752) in intron 16 in the angiotensin converting enzyme (ACE) gene,[31, 157] 1166A→C (rs5186) in the angiotensin II type 1 receptor (AGTR1) gene,[131, 160] 614G→T (Gly460Trp, rs4961) in the α-adducin (ADD1) gene,[161] 46G→A (Arg16Gly, rs1042713) and 79C→G (Gln27Glu, rs1042714) polymorphisms in the adrenergic β2 receptor (ADRB2) gene,[162] and 825C→T (rs5443) in the G protein β3 subunit (GNB3) gene.[163] We genotyped the -344C→T polymorphism (rs1799998) in the aldosterone synthase (CYP11B2) gene,[164] 1039C→T (Arg347Cys, rs1048101) in the α-adrenergic receptor 1a (ADRA1A) gene[165] and 145A→G (Ser49Gly, rs1801252) in the β1-adrenergic receptor (ADRB1) gene.[166] The latter genotypes were determined using the fluorescence-based assay-by-design allelic discrimination method using Taqman Universal PCR master mix (Applied Biosystems, Foster City, USA), and a Taqman ABI Prism 7900 Sequence Detection System (Applied Biosystems). Primer and probe sequences of the genotyped polymorphisms are presented in Table 1. Reaction components and amplification parameters were based on the manufacturer's instructions using an annealing temperature of 60° C. Results were scored blinded to CHD status. The mean success rate of the genotyping was 93%.

Gene-load score

The combined effect of genes can be examined by calculating a gene-load score, which measures the number of risk genotypes within a pathway for an individual. In this way, the gene-load score gives a reflection of a individuals' 'genetic burden' and represents the combined effect of genetic variation in multiple genes within a pathway.[158] The effect of the gene-load scores of the two pathways on CHD risk was our primary analysis to test our hypothesis. For each patient, we computed the gene-load scores of the RAAS and adrenalin/noradrenalin

system. The mode of inheritance of each polymorphism was chosen based on literature. The dominant genetic model was chosen for the polymorphisms in the AGTR1, ADD1, ADRA1A, and ADRB2 genes,[131, 161, 162, 165] whereas the recessive model was chosen for the polymorphisms in the AGT, ACE, CYP11B2, and ADRB1 genes.[137, 157, 164, 166] For the polymorphism in the GNB3 gene the literature was inconclusive.[163, 167] Therefore, we chose the recessive mode of inheritance based on the genotypic test (2-df) and Armitage trend test (1-df).

Since we genotyped six polymorphisms in the RAAS, the overall RAAS gene-load score could vary between 0 and 6. The highest RAAS gene-load score category 6 had a low number of patients (n=3) and was therefore combined with category 5. Similarly, category 0 showed a low number (n=14) and was combined with category 1. The same procedure was used for the gene-load score of the adrenalin/noradrenalin system. A reversed coding was applied for the GNB3 polymorphism, because it associated with lower CHD risk: the risk genotypes (CC+CT) were coded as 1 and the TT genotype was coded as 0. Both gene-load scores were analyzed with the lowest gene-load score category as reference category. We assigned the same score to each risk genotype, because it was shown that this yields the same predictive accuracy as a score based on the individual effects of the risk genotypes on CHD.[168] The selected genes were located on different chromosomes. Therefore, the calculation of the score was not complicated by linkage disequilibrium and represents a multilocus effect.

Statistical analyses

Statistical analyses were performed with the SPSS for Windows 12.0.1 statistics program. For differences between groups we used the X^2-test or t-test, and for differences in cumulative CHD risk between groups we used Kaplan-Meier curves and the logrank test. We tested for normality by drawing normal Q-Q plots for the untransformed and log-transformed continuous variables. Plasma triglycerides were used after logarithmic transformation. Hardy-Weinberg equilibrium of the polymorphisms was tested using an exact test.[75] All reported p-values are based on two-sided tests of significance. A p-value of <0.05 was considered statistically significant.

We assessed the association of each polymorphism and gene-load score with CHD by Cox proportional hazards regression. For associations between the gene-load scores and CHD we tested a linear trend by coding these scores as ordinal variables. We used the Cox proportional hazards model, because the age at event was known for the cases and age at data collection was known for the controls, and this model yields more statistical power than logistic regression analysis.[42] Patients without CHD were censored at the date of the last lipid clinic visit or at the date of death attributable to other causes than CHD. The proportional hazards assumption was tested by drawing log minus log plots of the survival function and was met for all Cox proportional hazard models. In the primary model, we adjusted for year of birth, sex and smoking status (categorized as ever/never smoked). In this primary model, we refrained from adjusting for hypertension since blood pressure is a major part of the

pathophysiological pathway studied in this analysis. In secondary models we additionally adjusted for hypertension, to study whether the potential associations were dependent or independent of hypertension. Additional models were constructed to investigate whether potential associations could be explained by possible intermediate covariables, such as diabetes mellitus, BMI, plasma HDL cholesterol and plasma triglycerides. The following variables had missing values (9.1-18.5%): smoking, BMI, plasma HDL cholesterol, and plasma triglycerides. Therefore, we applied the aregImpute function of the R statistical package to impute missing values in the analyses with adjustment for these variables.[49] We calculated the population attributable risks (PARs) of the individual polymorphisms with the use of the formula {P(HR-1) ÷ [P(HR-1)+1]} x 100, in which P is the risk genotype frequency and HR is the hazard ratio. For the calculation of the PAR of the gene-load score we calculated the combined HR for the categories with more than one risk genotype. We then calculated the PAR with category 0+1 as reference category.

The associations of the polymorphisms and gene-load scores in relation to hypertension were studied with logistic regression (univariable as well as multivariable with adjustment for age at last visit to the lipid clinic, sex, and smoking status).

RESULTS

Patient characteristics

The clinical characteristics of the 2400 patients are presented in Table 1 of Chapter 1 and the cumulative risk of CHD at the age of 40, 50, and 60 years in Table 2 of Chapter 1. Over a total of 108925 person years, 618 (28%) patients had at least one CHD event. The mean age of onset of the first CHD event was 48.8 years. The following variables were significantly associated with a higher cumulative CHD risk in the group of 2190 patients: male gender, smoking, plasma total, HDL and LDL cholesterol levels below the median, and plasma triglyceride levels above the median (data not shown). Total genotypes of the RAAS were available for 1756 patients. The 434 remaining patients did not differ with regard to the frequency of CHD and hypertension, although they were more frequently male (54% vs. 48%, p=0.02) and had slightly higher levels of plasma total and LDL cholesterol (9.76 vs. 9.45 mmol/l, p=0.005; and 7.65 vs. 7.28 mmol/l, p=0.001). Total genotypes of the adrenalin/noradrenalin system were available for 1718 patients. The 472 excluded patients did not differ with regard to the frequency of CHD and hypertension (data not shown).

Polymorphisms and CHD

The genotype frequencies of the 10 polymorphisms are shown in Table 2. All polymorphisms were in Hardy-Weinberg equilibrium, except for the ADD1 614G→T polymorphism (p=0.01). The AGT, AGTR1, CYP11B2, and ADD1 polymorphisms were significantly associated with CHD

Table 2. Genotype frequencies

Polymorphism	Genotype	Genotype frequency
RAAS		
AGT 4072T→C (Met235Thr)	TT/TC/CC	37.7/47.2/15.1
ACE I/D intron 16	II/ID/DD	23.6/48.5/27.9
AGTR1 1166A→C	AA/AC/CC	47.4/42.7/9.9
CYP11B2 –344C→T	CC/CT/TT	18.0/49.5/32.4
ADD1 614G1T (Gly460Trp)	GG/GT/TT	62.8/31.7/5.5
GNB3 825C→T	CC/CT/TT	49.9/40.1/10.0
Adrenalin/noradrenalin system		
ADRA1A 1039C1T(Arg347Cys)	CC/CT/TT	19.4/49.3/31.2
ADRB1 145A→G (Ser49Gly)	AA/AG/GG	74.7/23.4/1.8
ADRB2 46G→A (Gly16Arg)	GG/GA/AA	38.9/46.8/14.3
ADRB2 79C→G (Gln27Glu)	CC/CG/GG	32.5/48.0/19.5
GNB3 825C→T	CC/CT/TT	49.9/40.1/10.0

in the primary model (Table 3). Adjustment for hypertension did not change the results, whereas after additional adjustment for diabetes mellitus, BMI, plasma HDL cholesterol and plasma triglycerides, only the AGT, ACE, and ADD1 polymorphisms were associated with CHD (Table 3). None of the polymorphisms in the adrenalin/noradrenalin system was associated with CHD (Table 3).

Table 3. Association between RAAS and adrenalin/noradrenalin polymorphisms and CHD

Polymorphism	Model 1		Model 2		Model 3		PAR
RAAS	HR (95% CI)	p	HR (95% CI)	p	HR (95% CI)	p	%
AGT 4072T→C	1.28 (1.02-1.61)	0.04	1.28 (1.02-1.62)	0.03	1.26 (1.01-1.58)	0.04	3.8
ACE I/D intron 16	1.18 (0.97-1.44)	0.1	1.19 (0.97-1.46)	0.09	1.23 (1.01-1.49)	0.04	6.0
AGTR1 1166A→C	1.21 (1.00-1.45)	0.048	1.22 (1.01-1.46)	0.04	1.12 (0.94-1.34)	0.2	NA
CYP11B2 –344C→T	1.22 (1.01-1.48)	0.04	1.22 (1.00-1.47)	0.047	1.17 (0.97-1.41)	0.09	NA
ADD1 614G→T	1.22 (1.01-1.47)	0.04	1.22 (1.02-1.48)	0.03	1.25 (1.05-1.50)	0.01	8.5
GNB3 825C→T	0.72 (0.52-1.01)	0.06	0.73 (0.52-1.01)	0.06	0.78 (0.57-1.06)	0.1	NA
Adrenalin/noradrenalin	HR (95% CI)	p	HR (95% CI)	p	HR (95% CI)	p	PAR
ADRA1A 1039C→T	0.97 (0.77-1.23)	0.8	0.99 (0.78-1.25)	0.9	0.95 (0.76-1.18)	0.6	NA
ADRB1 145A→G	1.03 (0.51-2.08)	0.9	1.01 (0.50-2.04)	1.0	0.98 (0.48-1.98)	1.0	NA
ADRB2 46G→A	1.17 (0.97-1.41)	0.1	1.17 (0.97-1.42)	0.1	1.16 (0.96-1.39)	0.1	NA
ADRB2 79C→G	1.00 (0.82-1.21)	1.0	1.00 (0.82-1.22)	1.0	1.04 (0.86-1.26)	0.7	NA
GNB3 825C→T	0.72 (0.51-1.01)	0.053	0.73 (0.52-1.01)	0.06	0.78 (0.57-1.06)	0.1	NA

Model 1: Cox proportional hazards model adjusted for sex, year of birth and smoking. Model 2: additional adjustment for hypertension. Model 3: additional adjustment for hypertension, diabetes mellitus, BMI, plasma HDL cholesterol, and plasma triglycerides. HR = hazard ratio, 95% CI = 95% confidence interval. PAR = population attributable risk, based on model 3. NA = not applicable, because polymorphism is not significantly associated with CHD in model 3.

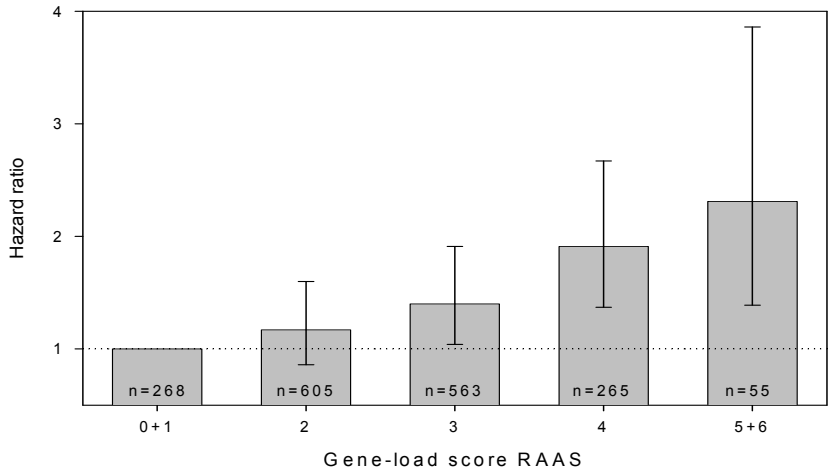


Figure 1. Association between the RAAS gene-load score and CHD. Hazard ratio = hazard ratio for developing coronary heart disease, with lowest gene-load score as reference category, $p_{linear\ trend}<0.001$. Outer categories (0 and 6) were combined with the nearest categories, because of low numbers. Error bars represent 95% confidence intervals.

Gene-load scores and CHD

The RAAS gene-load score was gene-dose dependently associated with CHD ($p_{linear\ trend}<0.001$, Figure 1). CHD risk in patients with a gene-load score of 5 or 6 was 2.3 times as high as in patients with a gene-load score of 0 or 1 (95% CI 1.39-3.86, p=0.001). The association between the RAAS gene-load score and CHD was not influenced by additional adjustment for hypertension, diabetes mellitus, BMI, plasma HDL cholesterol and plasma triglycerides (data not shown). The PAR of the RAAS gene-load score was 26%.

The gene-load score of the adrenalin/noradrenalin system was not significantly associated with CHD ($p_{linear\ trend}=0.06$, Figure 2). Additional adjustment for hypertension, diabetes mellitus, BMI, plasma HDL cholesterol and plasma triglycerides did not change this result ($p_{linear\ trend}=0.3$).

The gene-load scores were not associated with hypertension ($p_{linear\ trend}=0.3$ for RAAS and $p_{linear\ trend}=0.6$ for adrenalin/noradrenalin system, adjusted for age at last visit to the lipid clinic, sex, and smoking).

DISCUSSION

This study demonstrates that variation in RAAS genes is gene-dose dependently associated with CHD in patients with FH. The RAAS gene-load score is a model for multilocus effects within a pathway related to CHD. The presence of at least five risk genotypes in the RAAS resulted in a CHD risk that was more than twice as high as that of one risk genotype or fewer.

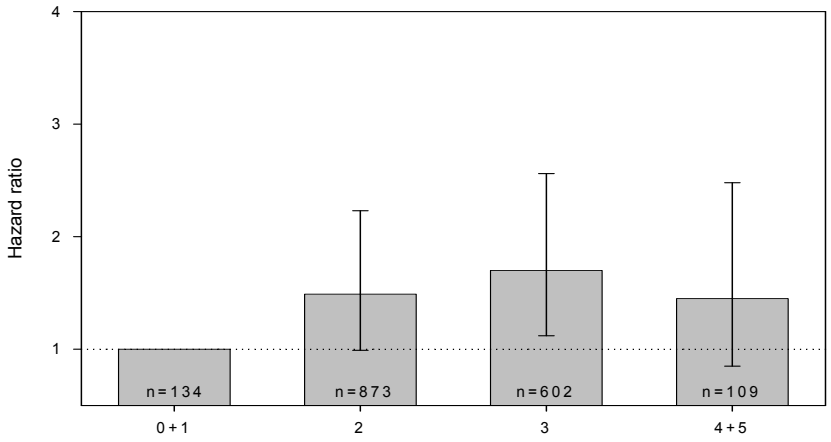


Figure 2. Association between the gene-load score of the adrenalin/noradrenalin system and CHD. Hazard ratio = hazard ratio for developing coronary heart disease, with lowest gene-load score as reference category, $p_{\text{linear trend}}=0.06$. Outer categories (0 and 5) were combined with the nearest categories, because of low numbers. Error bars represent 95% confidence intervals.

This study indicates that despite the fact that the individual polymorphisms revealed small effects, the combinations of polymorphisms may more clearly predict susceptibility to CHD.

Two previous studies investigated the association between genetic variation in the RAAS and CHD in FH populations, and found significant associations for the ACE polymorphism[31] and the AGTR1 polymorphism,[131] which is in line with the present study. These previous studies, however, did not study the combined influence of these polymorphisms on CHD. In contrast, several studies in non-FH populations did investigate interactions between polymorphisms within the RAAS and their combined influence on cardiovascular disease outcomes.[169, 170] To our knowledge, the present study is the first to show that the multilocus effect of six genes involved in the RAAS substantially strengthens the association with CHD. Normally, feedback mechanisms within the RAAS counteract changes in individual components. For instance, a rise in angiotensinogen will be compensated by a decrease in renin, and, consequently, plasma and tissue angiotensin levels are unaltered under such conditions.[171] However, in the presence of multiple risk genotypes, it will become increasingly difficult to normalize the degree of RAAS activity via feedback mechanisms. This most likely explains the current results. Furthermore, the association between the RAAS gene-load score and CHD occurred independently of hypertension. A hypertension-independent association between genetic variation in the RAAS and CHD has already been described in a population-based study,[136] and this finding is therefore not necessarily specific for hypercholesterolemia. Taken together, our observations suggest that the negative consequences of increased RAAS activity predominantly manifest themselves at the tissue level, in agreement with the concept of tissue angiotensin production.[172] Such tissue effects involve multiple mechanisms that are all

associated with atherosclerosis: oxidative stress, inflammation, endothelial dysfunction, and tissue remodeling.

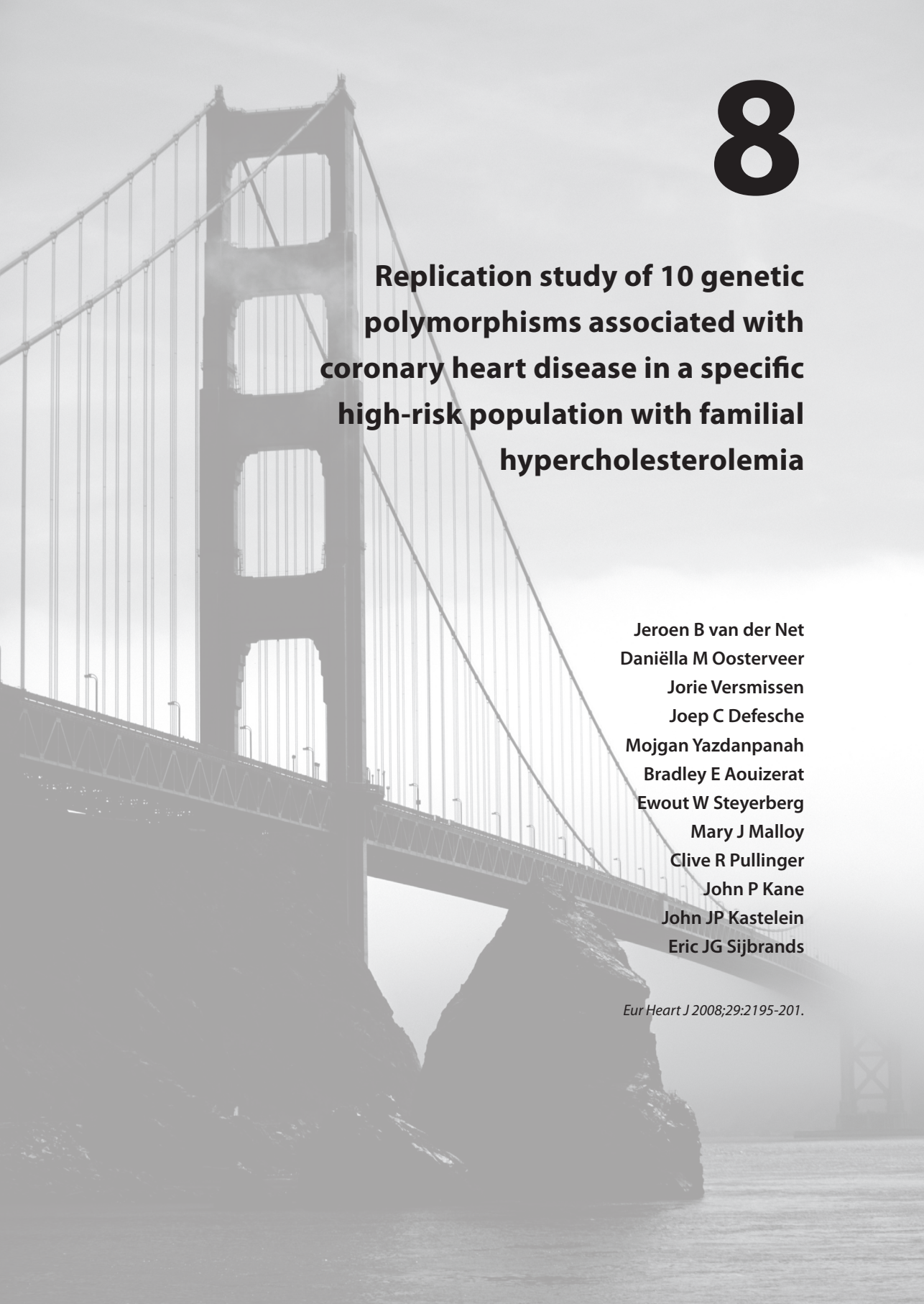
The functional effects of five of the six RAAS polymorphisms have been studied previously. The AGT polymorphism was associated with elevated plasma angiotensinogen levels[137] and CHD,[136] and the ACE polymorphism with elevated serum and tissue ACE levels,[173, 174] and ischemic heart disease.[157] The CYP11B2 polymorphism was found to be associated with plasma renin activity[175] and cardiovascular disease,[176] and the ADD1 polymorphism with greater sensitivity to changes in sodium balance[177] and cardiovascular disease in hypertensive individuals.[161] Furthermore, the GNB3 polymorphism, which leads to a splice variant, was shown to enhance intracellular signaling with a potential to affect vascular reactivity[178] and was associated with risk of CHD.[163] Since G protein-coupled receptors represent the final common pathway in both the RAAS and the adrenalin/noradrenalin system, the GNB3 polymorphism was included in both systems.[179] The AGTR1 polymorphism was shown to be associated with CHD in FH patients and was therefore included in the present study.[131]

The RAAS gene-load score was clearly associated with CHD, but the gene-load score of the adrenalin/noradrenalin system was not associated with CHD. An explanation for this could be that we studied these systems in the setting of severe hypercholesterolemia. Several lines of evidence suggest that there is an interaction between hypercholesterolemia and RAAS in the development of atherosclerosis, in which hypercholesterolemia influences RAAS activity.[180] For instance, it has been shown that LDL cholesterol affects the expression of the angiotensin II type 1 receptor.[181] Although it has been suggested that the plasma noradrenalin concentrations are increased in FH,[182] evidence of an interaction of the adrenalin/noradrenalin system and hypercholesterolemia in the development of atherosclerosis is lacking. We investigated whether there was an interaction between LDL cholesterol and the gene-load scores. We did not find such interactions (data not shown). Notably, higher levels of total and LDL cholesterol were associated with a lower cumulative CHD risk (Table 2 of Chapter 1). An explanation for this paradoxical effect could be that FH patients with total and/or LDL cholesterol levels above the median received cholesterol-lowering therapy at a younger age than patients with levels below the median (42.2 versus 45.0 years, respectively, $p<0.001$, data not shown).

The strength of our study is determined by the availability of a large, well-phenotyped study population consisting of FH patients, who have a severely elevated risk of CHD. However, the effect of any single gene on the susceptibility to a complex disease is likely to be modest and the studied pathways are highly redundant systems. Therefore, we investigated the association between the combined effect of genetic variants and CHD in our population with its corresponding high power to detect potential small but accumulating effects of the genetic variants. Nevertheless, our findings need to be validated in independent FH populations. In the present study, we did not adjust for multiple testing, but it is important to note that even a Bonferroni correction would not have changed the association between the gene-load scores and CHD.

Our study has a number of limitations. First, it was a retrospective cohort study which primary source of data was from medical records. Nonetheless, the primary endpoint CHD was well defined by using guidelines, which were developed before the data collection of this population.[15] Second, our findings may solely apply to people with FH. Third, the ADD1 polymorphism was not in Hardy-Weinberg equilibrium. This deviation is probably not based on mixed ethnic groups, because over 99% of our FH population is Caucasian and we excluded apparent relatives. Because the polymorphism was in Hardy-Weinberg equilibrium in the FH patients with CHD, no apparent selection has taken place on the basis of CHD status. Most likely, therefore, this deviation from Hardy-Weinberg equilibrium was found by chance. It seems unlikely that genotyping errors are the cause; less than 0.5% of discordant results were found in a re-analysis of several polymorphisms in the original genotyping assay.[40] Finally, the association between the RAAS gene-load score and CHD suggests an additive effect of the underlying risk genotypes. However, we cannot rule out interactions between the risk genotypes. We analyzed fully unlinked loci on separate chromosomes, but the size of our population is too small to investigate all potentially functional interactions between the polymorphisms.

In conclusion, we have shown that genetic variation in the RAAS contributes to CHD risk in patients with FH in a gene-dose dependent way. Pathway specific gene-load scores, like that of RAAS, may offer opportunities for the development of improved individual CHD risk assessments in patients with FH.



8

Replication study of 10 genetic polymorphisms associated with coronary heart disease in a specific high-risk population with familial hypercholesterolemia

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ABSTRACT

Objectives

Recent large genetic association studies have revealed associations between genetic polymorphisms and myocardial infarction (MI) and coronary heart disease (CHD). We performed a replication study of 10 polymorphisms and CHD in a population with familial hypercholesterolemia (FH), individuals at extreme risk of CHD.

Methods

We genotyped 10 polymorphisms in 2145 FH patients. We studied the association between these polymorphisms and CHD in Cox proportional hazards models.

Results

We confirmed the associations between four polymorphisms and CHD; the rs1151640 polymorphism in the olfactory receptor family 13 subfamily G member 1 (OR13G1) gene (HR 1.14, 95% CI 1.01-1.28, p=0.03), the rs11881940 polymorphism in the heterogeneous nuclear ribonucleoprotein U-like 1 (HNRPUL1) gene (HR 1.27, 95% CI 1.07-1.51, p=0.007), the rs3746731 polymorphism in the complement component 1 q subcomponent receptor 1 (CD93) gene (HR 1.26, 95% CI 1.06-1.49, p=0.01), and the rs10757274 polymorphism near the cyclin-dependent kinase N2A and N2B (CDKN2A and CDKN2B) genes (HR 1.39, 95% CI 1.15-1.69, p<0.001).

Conclusion

We confirmed previously found associations between four polymorphisms and CHD, but refuted associations for six other polymorphisms in our large FH population. These findings stress the importance of replication before genetic information can be implemented in prediction of CHD.

INTRODUCTION

Coronary heart disease (CHD), and especially myocardial infarction (MI), is one of the most common causes of morbidity and mortality, and has a strong genetic component.[183] The complexity of CHD and MI is illustrated by the many cell types that are involved in the atherosclerotic plaque and by the multiple processes that determine CHD risk, such as inflammation and thrombosis. Given this complexity, it is not clear which genes harbor the variation responsible for the genetic component of CHD. Recently, we conducted three large association studies to identify novel genetic variants associated with MI and early-onset MI.[184-186] A total of eight polymorphisms were found to be associated with MI or early-onset MI in three independent populations. Collaborators in these studies also found another polymorphism which showed an association with MI in two independent populations.[187] More recently, two genome-wide association (GWA) studies found an additional association between polymorphisms nearby the cyclin-dependent kinase N2A and N2B (CDKN2A/B) genes and CHD. These latter polymorphisms were consistently associated with MI and CHD in independent populations.[188, 189]

Replication of genetic associations in independent populations is essential to reduce the number of false-positive results and to further define the role of these variants in the susceptibility to complex disease. We therefore performed a replication study in a specific population of patients with familial hypercholesterolemia (FH), who have an extremely high risk of CHD and MI, to test whether these previous findings can be generalized to these high-risk patients. FH is an autosomal dominant disorder caused by mutations in the low-density lipoprotein (LDL) receptor gene and results in severely premature CHD.[74, 113, 190, 191] The incidence and age of onset of CHD varies considerably among individuals with FH.[4, 5, 153] Classical risk factors explain this variability to only a minor degree.[14] Probably, a substantial part of the variation in the incidence of CHD in this disorder is due to genetic factors outside the LDL receptor gene.[12, 40]

The aim of this study was to replicate the associations between CHD and the eight polymorphisms discovered by our association studies, the polymorphism found by our collaborators, and the polymorphism near CDKN2A/B genes which showed the strongest association with CHD in previous GWA studies, in a specific population with extreme CHD risk.

METHODS

Study population

A description of the GIRaFH study population and the definition of FH and CHD are given in Chapter 1.

Genetic analyses

The DNA of 2145 patients was available for the present analysis. We selected 10 polymorphisms of which we expected to have enough statistical power (>80%) based on effect sizes and genotype frequencies in literature.[184-189] These a priori power calculations were based on a person-years approach as applied in the present study. The following polymorphisms were investigated: rs12510359 in the palladin (PALLD) gene,[185] rs619203 in the v-ros UR2 sarcoma virus oncogene homolog 1 (ROS1) gene,[185] rs1376251 in the taste receptor type 2 member 50 (TAS2R50) gene,[185] rs1151640 in the olfactory receptor family 13 subfamily G member 1 (OR13G1) gene,[185] rs4804611 in the zinc finger protein 627 (ZNF627) gene,[185] rs1010 in the vesicle-associated membrane protein 8 (VAMP8) gene,[186] rs11881940 in the heterogeneous nuclear ribonucleoprotein U-like 1 (HNRPUL1) gene,[186] rs3746731 in the complement component 1 q subcomponent receptor 1 (C1QR1 or CD93) gene,[184] rs11666735 in the Fc fragment of IgA receptor (FCAR) gene,[187] and rs10757274 approximately 100 kb upstream of the cyclin-dependent kinase N2A and N2B (CDKN2A and CDKN2B) genes.[189] All genotypes were determined using fluorescence-based TaqMan allelic discrimination assays and analyzed on an ABI Prism 7900 Sequence Detection System (Applied Biosystems). The rs619203 polymorphism in the ROS1 gene was not in Hardy-Weinberg equilibrium (p=0.01 in the whole group, and p=0.01 in the patients without CHD). To ensure that this was not due to technical reasons, we genotyped the rs529038 polymorphism, which was in almost complete linkage disequilibrium with the rs619203 polymorphism in our original study with only four discordant calls.[185] In our population these polymorphisms were concordant in >99%. The further analyses were therefore conducted with the rs619203 polymorphism. Primer and probe sequences are presented in Table 1. Reaction components and amplification parameters were based on the manufacturer's instructions using an annealing temperature of 60° C. Results were scored blinded to CHD status. The genotyping of all polymorphisms had success rates between 92% and 94%. A total of 204 random duplicate samples showed highly concordant results (>99%).

Statistical analyses

For differences in cumulative CHD risk between groups we used Kaplan-Meier curves and the log-rank test. We tested for normality by drawing normal Q-Q plots for the untransformed and log-transformed continuous variables. Plasma triglycerides were tested after logarithmic transformation. Hardy-Weinberg equilibrium of the polymorphisms was tested with an exact test.[75]

Since there is little literature about the studied polymorphisms, we chose the mode of inheritance on the basis of the genotypic test (2-df). This resulted in the use of a dominant genetic model for the PALLD, TAS2R50, and FCAR polymorphisms, the recessive genetic model for the ROS1, VAMP8, and CD93 polymorphisms and the polymorphism near the

Table 1. Primer and probe sequences

Polymorphism		Sequence
rs12510359	Gene	PALLD
	Forward primer	GTTCAATATCCCAAGCCCAGAAAGA
	Reverse primer	GCTGAGTCAAAGCAGCTGAATTTG
	Probe VIC	CCTTTAAGGATGAACTCA
	Probe FAM	CTTTAAGGACGAACTCA
rs619203	Gene	ROS1
	Assay number	C__11315168_10
	Amino acid change	C2229S
rs529038	Gene	ROS1
	Assay number	C__11315171_20
	Amino acid change	N2213D
rs1376251	Gene	TAS2R50
	Assay number	C__12107274_10
	Amino acid change	Y203C
rs1151640	Gene	OR13G1
	Assay number	C___1449414_10
	Amino acid change	I132V
rs4804611	Gene	ZNF627
	Assay number	C__25992024_20
rs1010	Gene	VAMP8
	Assay number	C___2091644_20
rs11881940	Gene	HNRPUL1
	Forward primer	GGATTACAGGCGTGAGCCA
	Reverse primer	GGGAGAAGGTGAAGAAAACTGGAAT
	Probe VIC	CCTTCTGTCTATGTCTTT
	Probe FAM	CCTTCTGTCTTTGTCTTT
rs3746731	Gene	CD93
	Assay number	C___1212713_10
	Amino acid change	P541S
rs11666735	Gene	FCAR
	Assay number	C___7841642_10
	Amino acid change	D113N
rs10757274	Gene	Near CDKN2A/B
	Assay number	C__26505812_10

CDKN2A/B genes. The additive model was chosen for the OR13G1, ZNF627, and HNRPUL1 polymorphisms.

To determine the association between the polymorphisms and CHD we used Cox proportional hazards models.[99] Patients without CHD were censored at the date of the last lipid clinic visit or at the date of death attributable to causes other than CHD. The proportional hazards assumption was tested by drawing log minus log plots of the survival function and was met for all Cox proportional hazard models. In the primary model, we adjusted for year of birth, sex, and smoking. For smoking we implemented a linearly decreasing risk effect for the 6 years after cessation.[51] A secondary model was constructed to investigate whether potential associations could be explained by possible intermediary variables, such as hypertension,

diabetes mellitus, BMI, plasma HDL cholesterol, and plasma triglycerides. Postmenopausal women are at increased risk of developing CHD compared to premenopausal women.[192] Unfortunately, we do not have information about the age of menopause in our cohort. Alternatively, we studied the presence of an age effect among women by additionally adjusting the Cox proportional hazards models for age tertiles,[93] which were defined by cut-off values of 42.7 and 56.6 years. This adjustment did not change the results (data not shown).

The following covariables had missing values: smoking (9.4%), hypertension (1.0%), BMI (14.0%), plasma HDL cholesterol (18.6%), and plasma triglycerides (15.9%). Therefore, we applied the multiple imputation method of the aregImpute function of the R statistical package to impute these missing values.[49] Imputation methods substitute the missing values with plausible values on the basis of the relationship between the variable with missing values and the available information. With multiple imputation, 10 completed data sets were created, and subsequently 10 analyses were performed by treating each completed data set as a real complete data set. Finally, the results from these analyses were combined to obtain the effect estimates, while properly taking into account the uncertainty in the imputed values. It has been shown that imputation is beneficial for handling missing data in epidemiologic methods.[193]

Since testing multiple polymorphisms could have led to false-positive associations due to multiple testing, we estimated the false-discovery rate (FDR) and considered an FDR <5% acceptable.[194] An exact description of the calculation of the FDR has been published previously.[185]

We further investigated the associations between the polymorphisms and cardiovascular risk factors (age, sex, smoking, hypertension, diabetes mellitus, BMI, plasma LDL and HDL cholesterol, and plasma triglycerides), by using the χ^2-test, t-test, and ANOVA.

All data are provided as mean ± standard deviation, unless stated otherwise, and all reported p-values are based on two-sided tests of significance. A p-value of <0.05 was considered statistically significant. All statistical analyses were performed with the SPSS for Windows 12.0.1 statistics program and the R statistical package.[49]

RESULTS

Patient characteristics

Table 1 of Chapter 1 shows the the clinical characteristics of the 2400 patients, whereas the cumulative lifetime risks of CHD till the age of 40, 50, and 60 years are presented in Table 2 of Chapter 1. In the group of 2145 patients, 607 (28%) patients had at least one CHD event during a total of 106772 person years. The mean age of onset of the first CHD event was 48.8 ± 10.7 years. The following variables were associated with a higher cumulative CHD risk in the

Table 2. Frequency distributions of polymorphisms

Gene	Polymorphism	Genotype	Frequency
PALLD	rs12510359	AA/AG/GG	12.0/44.5/43.5
ROS1	rs619203	GG/GC/CC	57.3/35.4/7.3
TAS2R50	rs1376251	TT/TC/CC	10.1/43.9/45.9
OR13G1	rs1151640	AA/AG/GG	19.8/48.7/31.4
ZNF627	rs4804611	GG/GA/AA	6.7/40.0/53.3
VAMP8	rs1010	AA/AG/GG	32.0/48.3/19.7
HNRPUL1	rs11881940	TT/TA/AA	2.3/26.0/71.7
CD93	rs3746731	CC/CT/TT	19.5/49.5/31.1
FCAR	rs11666735	GG/GA/AA	86.5/12.8/0.8
Near CDKN2A/B	rs10757274	AA/AG/GG	28.1/49.8/22.0

group of 2145 patients: sex, smoking, plasma total, HDL and LDL cholesterol levels below the median, and plasma triglyceride levels above the median (data not shown).

Polymorphisms and CHD

Table 2 shows the genotype frequencies of the 10 polymorphisms. All polymorphisms were in Hardy-Weinberg equilibrium, except for the rs619203 polymorphism in the ROS1 gene (p=0.01). The associations between the polymorphisms and CHD are presented in Table 3. Carrriers of one G-allele of the OR13G1 polymorphism had a 14% higher risk of CHD, whereas carriers of two G-alleles had a 30% higher risk of CHD, compared to carriers of two A-alleles of this polymorphism (p=0.03, primary model, Table 3). Carriers of one A-allele of the HNRPUL1 polymorphism had a 27% higher risk of CHD, whereas carriers of two A-alleles had a 61%

Table 3. Association between polymorphisms and CHD

Gene	Polymorphism	Genetic mode	Primary model		Secondary model		
			HR (95% CI)	p-value	HR (95% CI)	p-value	FDR**
PALLD	rs12510359	Dominant	1.09 (0.85-1.41)	0.5	1.03 (0.80-1.34)	0.8	0.80
ROS1	rs619203	Recessive	0.88 (0.63-1.23)	0.5	0.84 (0.60-1.18)	0.3	0.38
TAS2R50	rs1376251	Dominant	1.31 (0.97-1.78)	0.08	1.25 (0.93-1.70)	0.1	0.20
OR13G1	rs1151640	Additive	1.14* (1.01-1.28)	0.03	1.15* (1.02-1.30)	0.02	0.04
ZNF627	rs4804611	Additive	0.98* (0.87-1.12)	0.8	0.97* (0.85-1.11)	0.7	0.78
VAMP8	rs1010	Recessive	0.87 (0.70-1.08)	0.2	0.87 (0.70-1.08)	0.2	0.29
HNRPUL1	rs11881940	Additive	1.27* (1.07-1.51)	0.007	1.28* (1.15-2.32)	0.006	0.03
CD93	rs3746731	Recessive	1.26 (1.06-1.49)	0.01	1.24 (1.05-1.48)	0.01	0.03
FCAR	rs11666735	Dominant	1.16 (0.91-1.46)	0.2	1.16 (0.92-1.47)	0.2	0.29
Near CDKN2A/B	rs10757274	Recessive	1.39 (1.15-1.69)	<0.001	1.39 (1.15-1.69)	<0.001	0.01

95% CI = 95% confidence interval, CHD = coronary heart disease, HR = hazard ratio. Primary model adjusted for sex, year of birth and smoking. Secondary model additionally adjusted for hypertension, diabetes mellitus, BMI, plasma HDL cholesterol, and plasma triglycerides. *Hazard ratio per risk allele. **FDR = false-discovery rate.

higher risk of CHD, compared to carriers of two T-alleles of that polymorphism (p=0.007, primary model, Table 3). Patients homozygous for the T-allele of the CD93 polymorphism had a 26% increased risk of CHD compared to patients with at least one C-allele of that polymorphism (p=0.01, primary model, Table 3). Patients homozygous for the G-allele of the polymorphism near the CDKN2A/B genes had a 39% higher risk of CHD than patients with at least one A-allele of that polymorphism (p<0.001, primary model, Table 3). The other polymorphisms were not significantly associated with CHD (Table 3). Additional adjustment for hypertension, diabetes mellitus, BMI, plasma HDL cholesterol, and plasma triglycerides yielded similar results (Table 3).

Polymorphisms and cardiovascular risk factors

The TAS2R50 polymorphism was associated with a slightly increased BMI (25.2 ± 3.6 kg/m^2 for the TC+CC genotypes vs. 24.6 ± 3.2 kg/m^2 for the TT genotype, p=0.04). The ZNF627 polymorphism showed an association with increased total cholesterol levels (9.0 ± 1.7/9.5 ± 1.9/9.6 ± 2.0 mmol/l for the GG/GA/AA genotypes, respectively, p for trend=0.01). The VAMP8 was associated with an increased BMI (25.6 ± 3.8 kg/m^2 for the GG genotype vs. 25.0 ± 3.5 kg/m^2 for the AA+AG genotypes, p=0.01). The CD93 polymorphism was associated with the presence of hypertension (11.0 % for the TT genotype vs. 7.8 % for the CC+CT genotype genotypes, p=0.02). Finally, the polymorphism near CDKN2A/B was associated with the presence of diabetes mellitus (6.6 % for the GG genotype vs. 4.1 % for the AA+AG genotypes, p=0.02).

DISCUSSION

We confirmed associations between four polymorphisms and CHD in this study of FH patients. These four polymorphisms were among a set of 10 that were recently found associated with MI or CHD in genome-wide or gene-centric association studies. The replicated polymorphisms are in the OR13G1 gene,[185] the HNRPUL1 gene,[186] the CD93 gene,[184] and near the CDKN2A/B genes.[189]

The rs10757274 polymorphism that is located approximately 100 kb upstream of the CDKN2A/B genes was discovered by a large GWA study, and the association with CHD was confirmed in four Caucasian populations.[189] The locus on chromosome 9p21 in which this polymorphism is located was also associated with MI and CHD in two other independent GWA studies,[188, 195] and a recent prospective meta-analysis gave further evidence of the involvement of this locus in CHD.[196] The CDKN2A/B genes are tumor-suppressor genes involved in the regulation of cell proliferation, cell aging, and apoptosis,[197] which are all important in atherogenesis.[198] This locus might therefore play a role in cell cycle checkpoints, which are important in repair of DNA that has been damaged by for example oxidative stress in athero-

sclerotic plaques. Future studies are required to elucidate the exact underlying mechanism by which this polymorphism or locus affects CHD risk.

The three other polymorphisms are located in genes that are relatively unknown in the field of cardiovascular disease and atherosclerosis. HNRPUL1 encodes a heterogeneous nuclear ribonucleoprotein and plays a role in RNA transport, processing, and transcriptional regulation. Furthermore, it has been speculated that this gene is involved in cell cycle regulation,[199, 200] which might constitute a link with the proposed functionality of the polymorphism near the CDKN2A/B genes. In our original study, the HNRPUL1 polymorphism was associated with early-onset MI,[186] which might be the reason why we were able to replicate this polymorphism, as FH is an important cause of severely premature CHD. It has been suggested that the CD93 gene is involved in intercellular adhesion, and leukocyte extravasation.[201] These are two important processes in the development of atherosclerosis[198] and could be the pathophysiological mechanisms underlying the association between variation within the CD93 gene and CHD. The mechanism through which the OR13G1 polymorphism influences CHD is unknown but might be related to dietary choices.

We did not find associations for the polymorphisms in the PALLD, ROS1, TAS2R50, ZNF627, VAMP8, and FCAR genes in our FH population. We could not find support for the hypothesis that hypercholesterolemia explains why these polymorhisms were not significant, whereas the four other polymorphisms were. The simplest explanation is that these associations were false-positive findings in the earlier studies, or false-negative findings in the present study. Lack of power is a well-known problem for small effects. Our a priori power calculations based on the effect sizes and genotype frequencies of the original studies showed sufficient statistical power for all polymorphisms (>80%). However, mostly we found smaller effect sizes for the polymorphisms than in the original studies, which in is line with a study by Ioannidis *et al*.[202] If these lower effect sizes are true for FH populations, we might have had insufficient statistical power for the detection of these associations.

In contrast to the present study, one population-based replication study showed a significant association between the ROS1 polymorphism and MI, whereas the PALLD, TAS2R50, OR13G1, and ZNF627 polymorphisms were not associated with MI.[203] Yet another study found that none of these polymorphisms was significantly associated with MI in a case-control design.[204] The reason for these discrepancies could be found in the genetic heterogeneity or differences in functionality of this polymorphism across different populations. This could also be the reason for the fact that we did not find an association between CHD and the other non-significant polymorphisms in this study.

Two topics regarding the statistical analysis merit discussion. First, association studies of multiple polymorphisms could lead to false-positive findings due to multiple testing. We addressed this multiple-testing issue by calculating the FDR for all polymorphisms.[185, 194] All four significant variants met the FDR criterion of 5%, indicating that the expected proportion of false-positives among all significant tests is below 5%. A Bonferroni correction would have

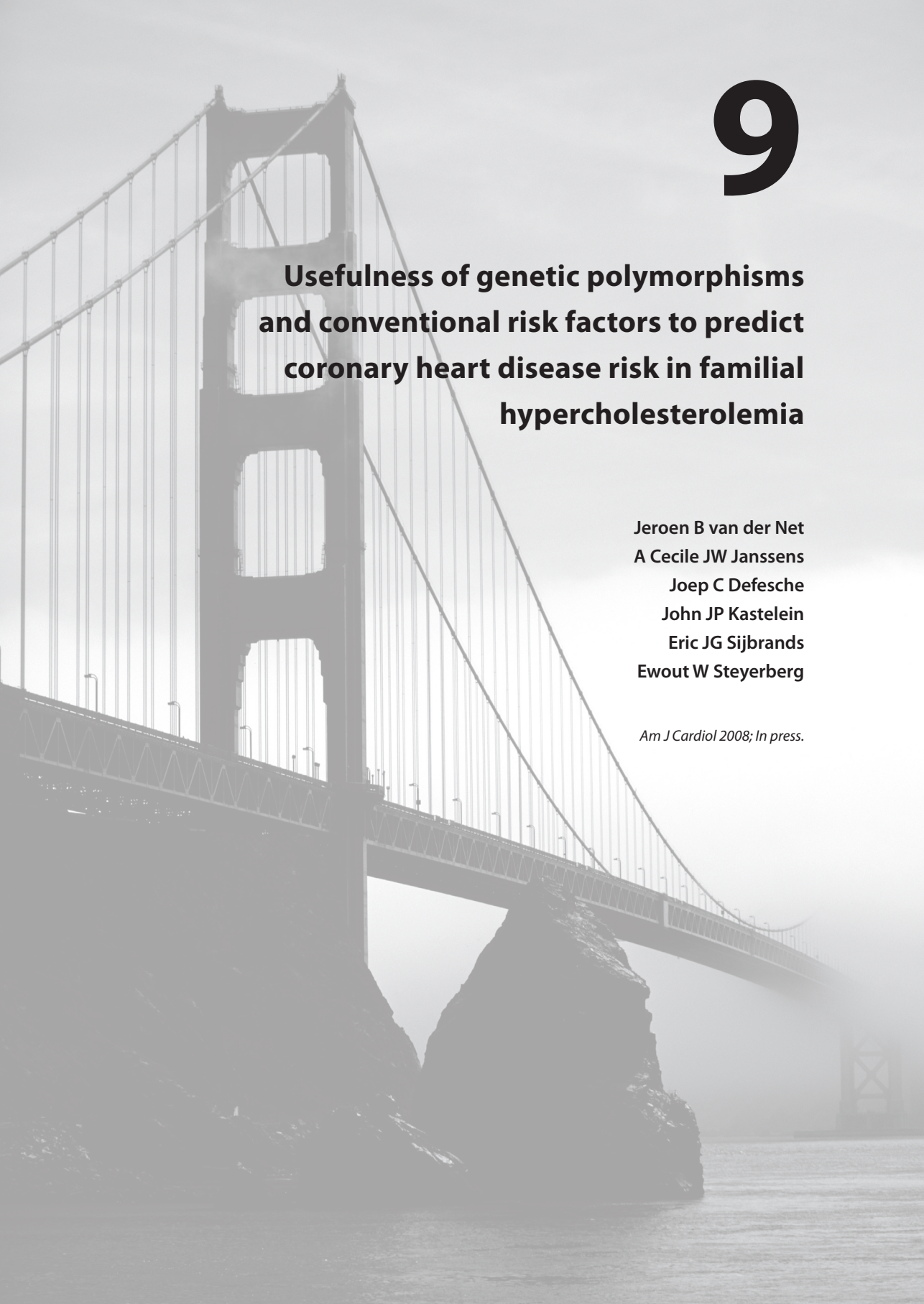
been strongly over-punitive in case of low false-positive proportions.[20] Second, women who are menopausal are at increased risk of developing CHD.[192] Information on age of menopause was not available in our study, but we estimated that approximately half of the women had passed menopause at the end of follow-up. Among women, we adjusted for age tertiles in order to take this possible confounder into account, but this did not change our results (data not shown). Age did not confound our findings, but we are aware that our findings in women may only apply to populations with a similar distribution of age and menopause.

In the present population, higher levels of total and LDL cholesterol were associated with a lower cumulative CHD risk (Table 2 of Chapter 1). An explanation for this paradoxical effect could be that FH patients with total and/or LDL cholesterol levels above the median received cholesterol-lowering therapy at a younger age than patients with levels below the median (42.2 versus 45.0 years, respectively, $p<0.001$, data not shown).

In conclusion, we have confirmed the previously found associations between four polymorphisms and CHD in a large population of patients with FH. Further studies should elucidate the pathophysiological mechanisms underlying these associations. Genetic association studies will lead to further identification of potential modifier genes for CHD in FH patients or other high-risk populations. If replicated, these genetic risk factors can be incorporated into better tools for CHD risk prediction.

PART IV

Prediction of coronary heart disease risk by genetic polymorphisms



9

Usefulness of genetic polymorphisms and conventional risk factors to predict coronary heart disease risk in familial hypercholesterolemia

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ABSTRACT

Objectives

Familial hypercholesterolemia (FH) is an autosomal dominant disorder with an associated high risk of coronary heart disease (CHD). The considerable variation in the age of onset of CHD among FH patients is believed to arise from conventional risk factors as well as from genetic variation other than in the low-density-lipoprotein gene. The degree to which currently known genetic variants can improve the prediction of CHD risk beyond conventional risk factors in this disorder was investigated.

Methods

Fourteen genetic variants recently identified for association with CHD in a Dutch FH population were considered. Prediction models were constructed using Cox proportional hazards models, and predictive value was assessed using a concordance statistic (c statistic).

Results

A total of 1337 patients with FH were completely genotyped for all genetic variants. Hazard ratios of the genetic variants ranged from 0.61 to 0.74 and from 1.24 to 2.33. The c statistic of the CHD prediction model based on genetic variants was 0.59, denoting little discrimination. The model based on conventional risk factors had a c statistic of 0.75, denoting moderate discrimination. Adding genetic test results to this model increased the c statistic to 0.76.

Conclusion

The contribution of 14 genetic variants to the prediction of CHD risk in patients with FH was limited. To improve genome-based prediction of CHD, larger numbers of genetic variants need to be identified that either on their own or in gene-gene interaction have substantial effects on CHD risk.

INTRODUCTION

During the last decades, a number of genetic susceptibility variants for coronary heart disease (CHD) risk have been identified in familial hypercholesterolemia (FH) populations of modest size.[23, 26-29, 31, 36-38, 131] These associations could not be replicated in subsequent studies of independent populations, possibly because of lack of statistical power.[25, 30, 131] We have conducted several candidate gene association studies in our large Dutch FH population (n=2400) and found 14 genetic variants that showed significant associations with CHD risk, with hazard ratios ranging from 0.65-0.69 and from 1.14-2.44.[40, 205-208] Because effect sizes of genetic susceptibility variants for CHD are small, single variants are not useful for CHD prediction. Genetic variants may provide better predictive information when they are considered in combination, either on their own or when added to conventional risk factors.[209] We therefore aimed to study the extent to which the combination of the 14 genetic variants identified to date can predict CHD risk, and distinguish FH patients who will develop CHD from those who will not.

METHODS

Study design, population and data collection

A description of the GIRaFH study population and the definition of FH and CHD are given in Chapter 1.

Genetic analyses

In the present analysis we considered the 14 genetic variants previously associated with CHD in our population (Table 1)[40, 205-208]; 13 polymorphisms and a 4-marker haplotype.[119] Genotyping was performed in several rounds. First, polymorphisms in the α-adducin (ADD1), angiotensinogen (AGT), angiotensin II type 1 receptor (AGTR1), apolipoprotein A4 (APOA4), apolipoprotein C3 (APOC3), prothrombin (FII), and paraoxonase-2 (PON2) genes were identified using a multilocus genotyping assay based on a probe mismatch hybridization method.[40] In this analysis, 1958 patients were completely genotyped for all seven polymorphisms.[40] In three subsequent genotyping rounds, DNA was available of 2145 of the 2400 patients, because DNA of the remaining patients was insufficient in amount and/or quality. Of this group, 1587 patients were completely genotyped for the six polymorphisms and the 4-marker haplotype.[40, 205-208] Hence, 1337 patients were completely genotyped for all 14 genetic variants. Patients who were completely genotyped had slightly higher mean plasma total and low-density lipoprotein (LDL) cholesterol levels than patients who were not completely genotyped (9.66 vs. 9.43 mmol/l, p=0.01, and 7.53 vs. 7.26 mmol/l, p=0.002, respectively). There were no differences in the presence of CHD or other conventional factors between

Table 1. Genetic variants considered in the present analysis

Gene (abbreviated)	Gene (full name)	Rs number	Nucleotide change
ADD1	α-adducin	rs4961 (Gly460Trp)	G→T
AGT	Angiotensinogen	rs699 (Met235Thr)	T→C
AGTR1	Angiotensin II type 1 receptor	rs5186	A→C
ALOX5AP	5-lipoxygenase activating protein	HapB*	
APOA4	Apolipoprotein A4	rs675 (Thr347Ser)	A→G
APOC3	Apolipoprotein C3	rs4520	C→T
CD93	Complement component 1 q subcomponent receptor 1	rs3746731 (Pro541Ser)	C→T
Near CDKN2A/B	Cyclin-dependent kinase N2A and N2B	rs10757274	A→G
CFH	Complement factor H	rs1061170 (Tyr402His)	T→C
CYP11B2	Aldosterone synthase	rs1799998	C→T
FII	Prothrombin	rs1799963	G→A
HNRPUL1	Heterogeneous nuclear ribonucleoprotein U-like 1	rs11881940	T→A
OR13G1	Olfactory receptor family 13 subfamily G member 1	rs1151640 (Ile132Val)	A→G
PON2	Paraoxonase-2	rs7493 (Ser311Cys)	G→C

*Haplotype based on four polymorphisms: SG13S377 (rs17216473), SG13S114 (rs10507391), SG13S41

patients with and without complete genotype information. For the latter three genotyping rounds, genotypes were determined using fluorescence-based TaqMan allelic discrimination assays and analyzed on an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, California). Primer and probe sequences were previously published.[205-207] The primer and probe sequences of the polymorphism in the complement factor H (CFH) gene were: forward primer CTTTATTTATTTATCATTGTTATGGTCCTTAGGAAAATGTTATTT, reverse primer GGCAGGCAACGTCTATAGATTTACC, VIC probe TTTCTTCCATGATTTTG, and FAM probe TTCTTCCATAATTTTG. Reaction components and amplification parameters were based on the manufacturer's instructions. Results were scored blinded to CHD status. Genotyping of polymorphisms had success rates of 92% to 96%. A total of 192 duplicate samples showed highly concordant results (>99%).

Statistical analyses

Genotype assay failure was assessed by testing Hardy-Weinberg equilibrium using an exact test.[75] All genetic variants were in Hardy-Weinberg equilibrium.

For the descriptive analyses, we investigated the association between cardiovascular risk factors and CHD by calculating the cumulative risk of CHD at the age of 40, 50, and 60 years using Kaplan-Meier curves and tested the associations using log-rank tests.

Associations between individual genetic variants and CHD risk were tested using a Cox proportional hazards model.[99] Follow-up was supposed to start at birth and patients without CHD were censored at the date of the last visit to the lipid clinic or at the date of death attributable to causes other than CHD. The proportional hazards assumption was tested by drawing log minus log plots of the survival function and was met for all models. Genetic variants were entered as categorical variables with 2 df. We adjusted for sex, year of birth, and smoking in the primary analyses, and additionally for conventional risk factors (hypertension, diabetes mellitus, BMI, plasma high-density lipoprotein (HDL) and LDL cholesterol, and plasma triglycerides) in secondary analyses. Because of skewed distribution, plasma triglycerides were analyzed after logarithmic transformation. For smoking, we implemented a linearly decreasing risk effect for the six years after cessation.[51] We adjusted for year of birth to adjust for a possible cohort effect.

We focused on three CHD prediction models. The first model was based on genetic variants only; the second on conventional risk factors (sex, smoking, BMI, hypertension, diabetes mellitus, plasma HDL and LDL cholesterol, and plasma triglycerides), and the third was based on both genetic variants and conventional risk factors. The association between a risk-allele score and the predicted CHD risks at the age of 60 years derived from the first model was investigated and visualized in a scatterplot. The risk-allele score was calculated by counting the number of risk alleles. The risk allele was defined as the allele asscociated with increased CHD risk. The discriminative accuracy of the three prediction models was evaluated using a concordance statistic (c statistic).[210] The c statistic is the equivalent of the area under the receiver-operating characteristic curve (AUC) for binary outcomes.[211]

The internal validity of the prediction models was assessed using bootstrapping techniques. A total of 100 random bootstrap samples were drawn with replacement from the group of 1337 patients. The discriminative accuracy of the 100 prediction models as fit on these bootstrap samples was determined for each bootstrap sample and for the original group (n=1337). This comparison gives an impression of how "overoptimistic" the model is, i.e., how much the performance of the model would deteriorate when applied to a new group of similar patients.[212]

Variables with missing values were hypertension (1%), smoking (10%), BMI (14%), plasma HDL cholesterol (19%), plasma LDL cholesterol (20%), and plasma triglycerides (16%). We applied a multiple imputation method (aregImpute function of the R statistical package; version 2.5.1; www.r-project.org) to impute these missing values in our Cox proportional

hazards models,[49] because imputation decreases bias in the hazard ratios that may occur when patients with incomplete information are excluded from the analysis.[193] In a secondary analysis we used the full data set (n=2145) and multiple imputation to impute both missing values for conventional risk factors and missing genotype data. This analysis gave discriminative accuracies for the three prediction models virtually identical to the analysis without imputation of missing genotype data (results not shown).

Statistical analyses were performed with SPSS (version 14.0; SPSS Inc., Chicago Illinois) and the R statistical package.[49] All reported p-values are based on 2-sided tests of significance without correction for multiple testing. A p-value of <0.05 was considered statistically significant.

RESULTS

Clinical characteristics of study population

Table 1 of Chapter 1 shows the the clinical characteristics of the 2400 patients, whereas the cumulative lifetime risks of CHD till the age of 40, 50, and 60 years are presented in Table 2 of Chapter 1. In the group of 1337 patients, 387 patients had at least one CHD event during a total of 66904 person years. Mean age of onset of the first CHD event was 49 ± 11 (SD) years. Sex, smoking, plasma total, HDL and LDL cholesterol levels below the median, and plasma triglyceride levels above the median were significantly associated with a higher cumulative CHD risk in the group of 1337 patients (data not shown).

Individual effects of genetic variants on CHD risk

A total of eight genetic variants showed significant association with CHD risk (Table 2). Adjustment for hypertension, diabetes mellitus, BMI, plasma HDL and LDL cholesterol and plasma triglycerides did not change the results (data not shown).

Risk allele score

The Figure shows the positive correlation between the risk-allele score and predicted cumulative CHD risks at the age of 60 years for patients with and without CHD. Mean predicted cumulative CHD risks at the age of 60 years were 44% for patients who eventually developed CHD and 41% for those who did not, suggesting that the risk-allele score has limited value in discriminating between patients with FH who will and will not develop CHD.

Discriminative accuracy

The prediction model on the basis of genetic information only (model 1) yielded a c statistic of 0.62 (95% CI 0.60 – 0.65). For the risk-allele score, the c statistic was slightly lower (0.59 (95% CI 0.55 – 0.62)). Prediction of CHD risk based on conventional risk factors (sex, smoking,

Table 2. Genotypic association between genetic variants and coronary heart disease (n=1337)

Gene	Genotype	Risk allele for allele score	Frequency	Hazard ratio (95% CI)	p-value
ADD1	GG	T	62%	1.0	
rs4961	GT		33%	1.24 (1.00-1.53)	0.05
	TT		5.4%	0.94 (0.59-1.49)	0.79
AGT	TT	C	38%	1.0	
rs699	TC		47%	1.15 (0.92-1.45)	0.23
	CC		15%	1.37 (1.03-1.83)	0.03
AGTR1	AA	C	47%	1.0	
rs5186	AC		43%	1.11 (0.89-1.38)	0.34
	CC		10%	1.36 (0.97-1.90)	0.07
ALOX5AP	B-/B-	B+	88%	1.0	
HapB	B-/B+		12%	1.48 (1.10-1.98)	0.01
	B+/B+		0.5%	0.76 (0.11-5.44)	0.79
APOA4	AA	T	68%	1.0	
rs675	AT		27%	1.00 (0.79-1.25)	0.97
	TT		4.9%	1.23 (0.80-1.88)	0.35
APOC3	CC	C	56%	1.0	
rs4520	CT		36%	1.02 (0.83-1.27)	0.84
	TT		8.2%	0.61 (0.40-0.94)	0.02
CD93	CC	T	18%	1.0	
rs3746731	CT		51%	0.94 (0.71-1.25)	0.69
	TT		31%	1.29 (0.96-1.74)	0.09
CDKN2A/B	AA	G	28%	1.0	
rs10757274	AG		50%	0.74 (0.58-0.94)	0.01
	GG		23%	1.21 (0.92-1.59)	0.16
CFH	TT	T	41%	1.0	
rs1061170	TC		47%	0.99 (0.80-1.22)	0.90
	CC		12%	0.64 (0.44-0.93)	0.02
CYP11B2	CC	T	18%	1.0	
rs1799998	CT		51%	1.02 (0.78-1.35)	0.87
	TT		32%	1.15 (0.86-1.55)	0.35
FII	GG	A	98%	1.0	
rs1799963	GA		1.9%	2.33 (1.27-4.27)	0.01
HNRPUL1	TT	A	2.4%	1.0	
rs11881940	TA		25%	1.47 (0.64-3.39)	0.36
	AA		72%	1.66 (0.74-3.74)	0.22
OR13G1	AA	G	20%	1.0	
rs1151640	AG		49%	1.58 (1.17-2.11)	0.002
	GG		32%	1.59 (1.16-2.17)	0.004
PON2	GG	G	59%	1.0	
rs7493	GC		35%	0.95 (0.77-1.17)	0.63
	CC		5.5%	0.66 (0.39-1.11)	0.12

95% CI = 95% confidence interval, CHD = coronary heart disease. Models adjusted for sex, year of birth and smoking (time-dependent).

BMI, hypertension, diabetes mellitus, plasma HDL and LDL cholesterol, and plasma triglycerides, model 2) had a c statistic of 0.75 (95% CI 0.73 – 0.78). Finally, the prediction model based on both genetic variants and conventional risk factors (model 3) resulted in a c statistic of

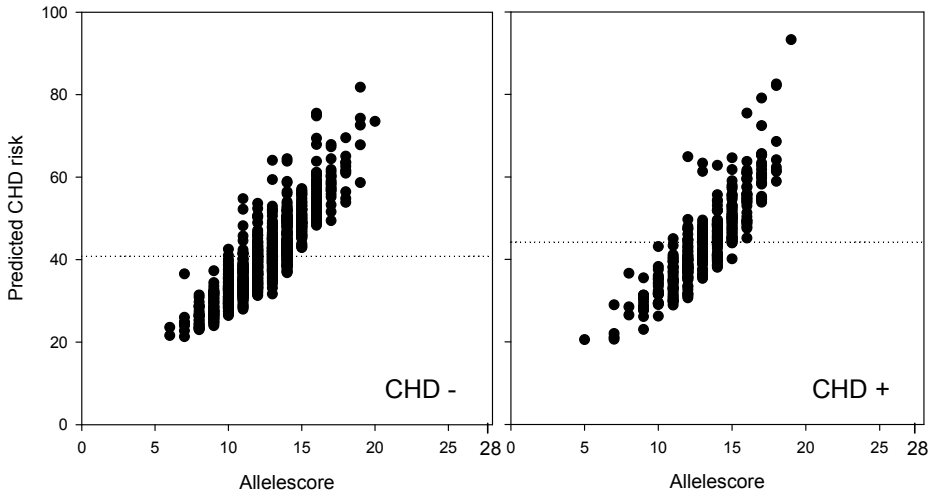


Figure: Predicted cumulative coronary heart disease risk at the age of 60 years as a function of the risk allele score. Left panel represents familial hypercholesterolemia patients without coronary heart disease, right panel represents familial hypercholesterolemia patients with coronary heart disease. The horizontal lines represent the mean predicted coronary heart disease risks at 60 years of age.

0.78 (95% CI 0.76 – 0.80). Validation of the models by means of bootstrapping reduced the c statistics to 0.58 (risk-allele score), 0.59 (model 1), 0.75 (model 2), and 0.76 (model 3).

DISCUSSION

The present study showed that information on 14 currently known genetic variants associated with CHD risk yielded little discrimination for the prediction of CHD in patients with FH (c statistic 0.59). The c statistic of a prediction model already containing the conventional CHD risk factors increased from 0.75 to 0.76 when the 14 genetic variants were added to this prediction model. The clinical relevance of such a small increase in discriminative accuracy is questionable.

A number of methodological issues merit discussion. First, six of the 14 genetic variants were not significantly associated with CHD in the present study, whereas the effect sizes were similar to those of our previous studies in the same FH population.[40, 205, 207] This was likely caused by lack of statistical power, because the present analyses were performed in a smaller data set considering only patients who were completely genotyped for all genetic variants. Because effect sizes were similar to the original studies, this might not have had substantial effect on the reported c statistics. Second, our assessment of the predictive accuracy of genetic markers may still be too optimistic, because the 14 genetic variants were the most predictive of a larger set of 87 variants identified in the same population. Although such selection is an important source of overoptimism, this is not corrected by the bootstrap

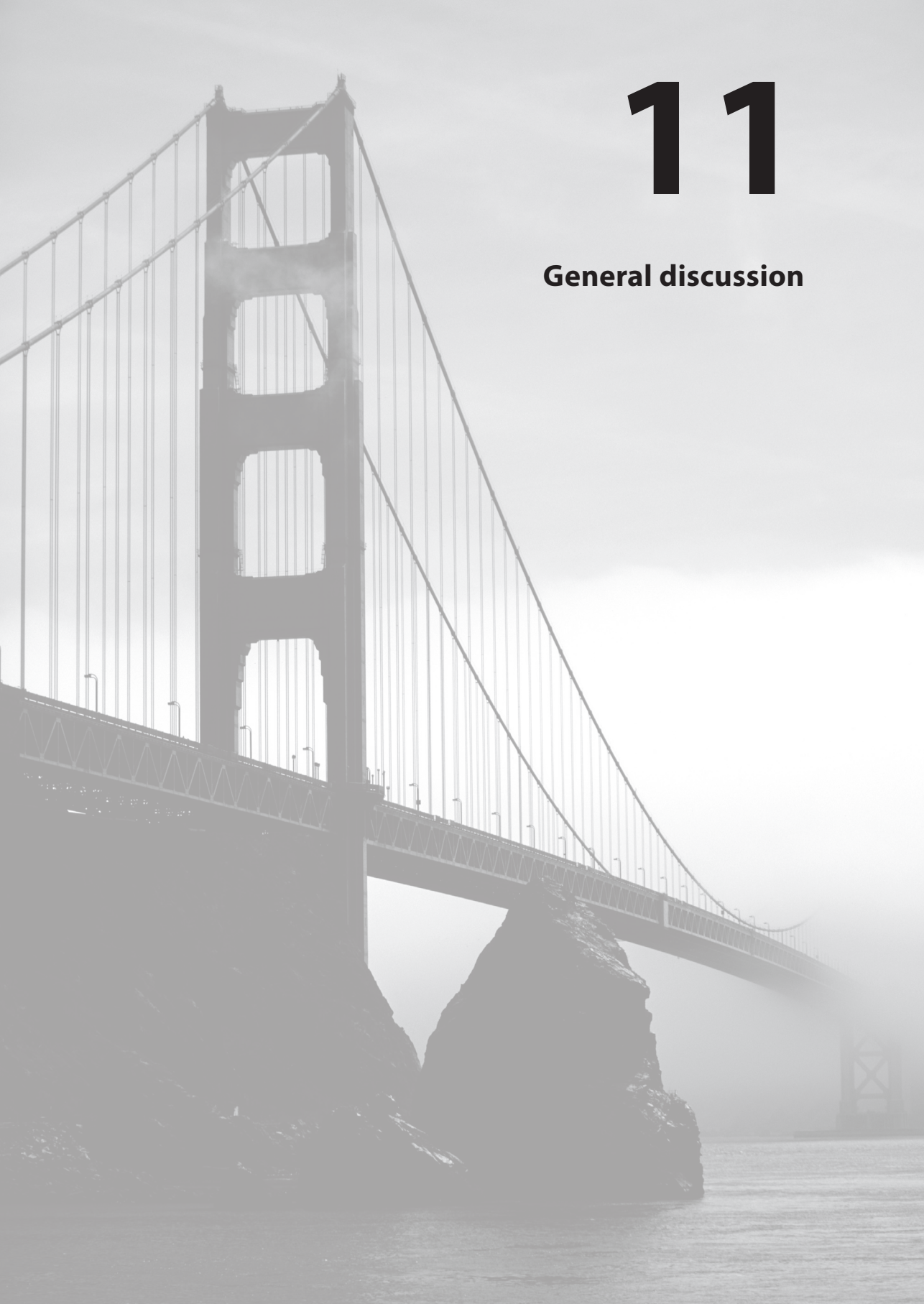
validation procedure.[213] Third, six of the seven genetic variants significantly associated with CHD or cardiovascular disease in other FH populations [26-28, 31, 37, 38] were not significant in our FH population.[40] We noted that the other study populations were substantially smaller than our FH population, with a mean of approximately 100 cases,[26-28, 31, 37, 38] and it could be that their results were false positives. Additional replication studies are needed to confirm or refute their association with CHD risk in patients with FH. Finally, higher levels of total and LDL cholesterol were associated with a lower cumulative CHD risk (Table 2 of Chapter 1). This paradoxical effect could be explained in part because patients with FH with total and/or LDL cholesterol levels higher than the median received cholesterol-lowering therapy at a younger age than patients with levels lower than the median (42 vs 45 years, respectively; p=0.04; data not shown).

Our finding of a modest discriminative accuracy of genetic risk factors outside the LDL receptor gene over and above that of conventional risk factors agrees with empirical studies in non-FH populations focusing on CHD and other complex diseases. These studies also showed limited added value to date.[214-217] For example, a cardiovascular risk score based on conventional CHD risk factors yielded an AUC of 0.76 in Caucasians in a previous study of CHD risk.[216] The AUC improved to 0.77 by adding 11 genetic risk factors in the prediction model. This improvement, although statistically significant, is unlikely to be clinically relevant.

To date, no CHD risk prediction tool is available for FH populations. Determining an accurate estimate of the CHD risk of a patient with FH is very important to identify patients with FH with a severely increased CHD risk who will benefit most from targeted therapeutic interventions such as aggressive lipid lowering therapy.[218] The present study showed that considerable discriminative accuracy can be obtained with information on sex, smoking behavior, BMI, presence of hypertension and diabetes mellitus, plasma HDL and LDL cholesterol, and plasma triglycerides (c statistic 0.75). This discriminative accuracy was similar to that of CHD risk predictors commonly used in clinical practice for general populations. For instance, the Prospective Cardiovascular Munster (PROCAM) and Framingham risk scores,[219, 220] which are also based on conventional risk factors for CHD, resulted in c statistics between 0.75 and 0.82 in the original studies.[220, 221] Nevertheless, before the prediction model in this study should be considered a prediction tool in clinical practice, its discriminative accuracy must be externally validated in independent FH populations.

PART V

GENERAL DISCUSSION



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General discussion

Heterozygous familial hypercholesterolemia (FH) is an inherited disorder of lipid metabolism caused by mutations in the low-density lipoprotein (LDL) receptor gene. Patients with FH have high plasma levels of LDL cholesterol, which leads to accelerated development of atherosclerosis and consequently to coronary heart disease (CHD) at a relatively young age.[2] Although a large proportion of FH patients develops CHD before the age of 60 years (premature CHD),[9] there is considerable variation in the risk of CHD among FH patients.[4, 5] This suggests that the etiology of CHD in FH is complex, and that the risk of CHD is most likely modified by conventional CHD risk factors and genetic risk factors for CHD outside the LDL receptor gene.[13]

The genetics of CHD involves a large number of genetic susceptibility variants. Although numerous studies have investigated the association between genetic variants and CHD in general populations, only a small number of genetic variants have been evidently associated with CHD in meta-analyses,[225] indicating that a large part of the genetic basis of CHD is still unknown. It has been argued that FH can be considered an exemplary model to study genetic risk factors for CHD, because of the high CHD risk.[29] Common but modest genetic effects may become more evident in this high-risk population.[73] However, it is also possible that the genetic basis of CHD in FH is different from that in general populations, because FH patients develop CHD at a much younger age than individuals in other populations. Premature CHD in FH populations and CHD in general populations may not be the same phenotype and may therefore have their own distinct genetic basis. In FH populations, the number of genetic association studies that have been performed is still small (see Table 3 of Chapter 1), which makes it impossible to perform meta-analyses.

Basically, the primary aim of genetic association studies of CHD is to identify genetic variants that play a role in the etiology of CHD. The identification of such genetic variants serves 2 purposes. First, the genetic risk factors for CHD may be useful to predict CHD risk of an individual. This could ultimately lead to a more personalized approach of medical care, in which therapeutic decisions are based on an individual's risk of CHD. The second purpose is to develop pharmacological therapies based on the novel basic disease mechanisms. Although the genome cannot be changed yet with gene therapies, the consequence of genetic variants at the mRNA or protein levels, or further downstream, can potentially be specifically targeted with new pharmacological compounds.

The aim of the studies described in this thesis was to identify genetic variants that play a role in the etiology of CHD in FH, and to investigate the extent to which these genetic risk factors could distinguish FH patients who will develop CHD from those who will not. In this final chapter, the findings of this thesis are summarized and discussed. First, the methodological issues concerning the design and statistical analysis of the GIRaFH cohort (Genetic Identification of Risk factors in FH) and of genetic association studies in general are briefly reviewed. Then, the results of the genetic association studies are considered in the context of

ongoing research. Subsequently, the potential use of genetic variants in CHD risk prediction is discussed. Finally, directions for future research are addressed.

METHODOLOGICAL ISSUES

Design of the GIRaFH cohort

The major strength of the genetic association studies described in this thesis is determined by the availability of a large, well-phenotyped cohort of FH patients (GIRaFH). The details of this cohort are presented in Chapter 1. Here, we discuss a number of methodological issues regarding the design of this cohort.

First, we included patients who were referred to lipid clinics and this could have led to selection bias. Patients with the most harmful genetic profiles might have died before referral, although extreme premature deaths were not observed in mortality studies.[4, 12] Nonetheless, we cannot exclude that we underestimated the effect sizes of the genetic variants studied in this thesis.

Second, all patients in the GIRaFH cohort were clinically diagnosed as having FH based on internationally established criteria.[14] Only 52% of these patients had a demonstrated LDL receptor mutation.[240] This raises the question if this cohort fully consists of patients with FH, or that other causes of hypercholesterolemia were included, such as familial combined hyperlipidemia.[240] However, even if this is true, it probably will not have major consequences for clinical practice, because CHD risk is increased to about the same degree in patients with and without demonstrable mutations.[241] Therefore, both groups of patients receive the same treatment to lower their CHD risk. Furthermore, eventual prediction models as proposed in Chapter 9 will be based on and used for all patients at lipid clinics with a clinical FH diagnosis, and thus not only the patients with a known LDL receptor mutation. Therefore, we did not exclude these patients from our research.

Statistical analysis

Several topics concerning the statistical analyses of our genetic association studies merit further discussion.

First, the GIRaFH cohort has a relatively short period of follow-up. Genetic association studies without follow-up or with short follow-up are generally analyzed with logistic regression models. However, since genotype status does not change over time and hence also represents genotype status at birth, age at event can be considered as follow-up time. If the age at event is known, genetic association studies could be analyzed with Cox proportional hazards models, even in the absence of prospectively studying follow-up. As we have demonstrated in Chapter 2, the Cox proportional hazards model is the preferred regression model in cross-sectional genetic association studies, because it yields more statistical power than the lo-

gistic regression model. An explanation for the higher statistical power of Cox proportional hazards models is that these models take the time until events occur into account, thereby changing the unit of analysis from persons to person-years. Logistic regression models do not use the time until events occur, but give 'early' and 'late' events the same weight in the analysis.[41, 44] Young individuals who have had no event (yet) are classified as 'no event', while some would have experienced the event at an older age. This is a form of misclassification in terms of outcome. The superiority of the Cox proportional hazards model over the logistic regression model in analyzing longitudinal data has been mathematically proven for models which consider one dichotomous covariate[42] and models with multiple covariates.[43] Based on the results described in Chapter 2, all genetic association studies described in this thesis were performed with the Cox proportional hazards model.

Second, throughout the genetic association studies described in this thesis we considered a primary statistical model in which we adjusted for year of birth, sex and smoking. These factors are most likely not influenced by the genetic variants, but could be possible confounders in the association between genetic variants and CHD if they are statistically associated with both the independent variable (genetic variant) and the dependent variable (CHD). We adjusted for year of birth to adjust for a possible cohort effect. This choice was based on a family-tree mortality study, in which the excess mortality from untreated FH varied largely over time.[4] Genetic variants might express their effects on CHD risk through intermediate traits, such as hypertension, diabetes mellitus, body mass index (BMI), and plasma lipid levels. To investigate whether the potential associations could be explained by these intermediate traits we constructed secondary Cox proportional hazards models, in which we additionally adjusted for these factors.

Third, a number of intermediate traits in our genetic association studies had missing values. Missing values lead to a more limited set of patients with complete data compared to the ideal situation of complete original data. Standard statistical software for regression analysis excludes patients with any missing value from the analysis.[242] This approach discards information from patients who have information on some but not all variables, and is therefore statistically inefficient. Hence, we applied a multiple imputation method that substitutes the missing values with plausible values on the basis of the relationship between the missing data and the available information. It has been shown that imputation reduces the bias in the hazard ratios that may occur when patients without complete information are excluded from the analysis.[193]

GENETIC ASSOCIATION STUDIES

The rationale for choosing the candidate genes described in this thesis was based on their involvement in pathophysiological processes underlying CHD, such as inflammation (Chap-

ter 5), or their relation to conventional CHD risk factors, such as hypertension, dyslipidemia, and BMI (Chapters 3, 4, 6 and 7). In Chapter 8, we describe a replication study of 10 genetic variants that had been identified in large genome-wide association (GWA) studies or gene-centric association studies. The pathophysiology underlying these associations is largely unknown.

ABCG8 gene

The adenosine triphosphate binding cassette G8 (ABCG8) gene is involved in plant sterol metabolism. The ABCG8 protein limits the intestinal absorption of dietary sterols by effluxing sterols from enterocytes into the intestinal lumen and promotes the excretion of sterols from the liver into the bile. Previous studies have demonstrated associations between the H-allele of the D19H polymorphism and lower plasma plant sterol levels,[67, 68] whereas we found a modestly *increased* CHD risk associated with the H-allele. Based on previous studies that showed associations between elevated levels of plasma plant sterol levels with an increased CHD risk,[58, 60, 61] we a priori expected that the H-allele would be associated with a *decreased* CHD risk. Nevertheless, our finding is in line with a recent study in which elevated plasma plant sterol levels or a high plant sterol content of the diet were associated with a reduced cardiovascular risk.[66, 76] Moreover, adding plant sterols to margarine and other foods is currently used to optimize cardiovascular risk profile by decreasing plasma cholesterol concentrations.[77] Interestingly, carriers of the risk genotypes of both the D19H and T400K polymorphism had a more evidently increased CHD risk, which gives further support of the hypothesis that plant sterol metabolism plays a role in CHD in FH. Unfortunately, data on plasma plant sterols were not available in the GIRaFH, so we were unable to confirm that this association was in fact due to differences in plasma plant sterol levels.

Glucocorticoid receptor (GR) gene

Glucocorticoids play an important role in the pathophysiology of atherosclerosis by their contribution to the development of hypertension, dyslipidemia, impaired glucose tolerance, and central adiposity. The effects of glucocorticoids are mediated primarily by binding to the intracellular GR. The sensitivity to glucocorticoids varies considerably among individuals. Four functional variants in the GR gene, the N363S, *Bcl*I, ER22/23EK, and 9β polymorphisms, have been associated with the sensitivity to glucocorticoids, CHD and CHD-related traits, such as BMI, blood pressure, and plasma lipid levels.[84, 88, 90-92, 94-96, 102, 243-245] We found that the haplotype containing the *Bcl*I polymorphism was associated with CHD in men. The increased susceptibility to CHD in male carriers of the *Bcl*I haplotype is in line with previous studies in middle-aged individuals in whom the *Bcl*I (A→G) polymorphism was associated with an unfavorable cardiovascular risk profile.[91, 92, 102, 244] Furthermore, heterozygous and homozygous G allele carriers showed an increased responsiveness to glucocorticoids.[88] Studies restricted to middle-aged, non-obese individuals showed associations with increased abdominal visceral

obesity.[91, 92, 102, 244] We conclude that men with FH and the *Bcl*I haplotype of the GR gene had a more severely increased CHD risk. Interestingly, this effect was independent of known conventional risk factors, suggesting that the increased risk for CHD might be due to until now unknown risk factors for CHD.

ALOX5AP gene

The 5-lipoxygenase-activating protein (FLAP), encoded by the arachidonate 5-lipoxygenase-activating protein (ALOX5AP) gene, is an important mediator of the activity of 5-lipoxygenase, a key enzyme in the biosynthesis of leukotrienes.[114] Leukotrienes are proinflammatory signaling molecules that are produced by inflammatory cells.[115] Because it has been suggested that the arterial wall of hypercholesterolemic patients is continuously subjected to an inflammatory challenge,[116, 117] the ALOX5AP gene could be an important candidate gene for CHD in FH patients. This is further supported by the accumulating evidence that atherosclerosis, the main cause of CHD, is a chronic inflammatory disorder.[118] We found a significant association between HapB, a haplotype defined by four single nucleotide polymorphisms, and CHD in our FH population. This is in line with the study by Helgadottir *et al*., in which the association between HapB and myocardial infarction (MI) was first described.[119] However, subsequent studies showed conflicting results regarding the association of HapB with MI in Caucasian populations: no association was found with MI in the prospective Physician's Health Study[123] and a German case-control study,[124] whereas a significant association was found with CHD in an Italian cohort[121] and with MI in another German case-control study.[122]

Interestingly, we observed a modifying effect of plasma LDL cholesterol on this association. FH patients with a plasma LDL cholesterol level above the median had a particularly increased CHD risk, whereas patients with a plasma LDL cholesterol level below the median had a non-significantly increased CHD risk. These findings are in line with an interaction between hypercholesterolemia and inflammation on the risk of MI and CHD as described in previous studies, suggesting that the consequences of the inflammatory state are particularly deleterious in FH patients whose LDL cholesterol levels are among the highest of the population.[129] An explanation for this finding could be that patients with severely raised LDL cholesterol levels have more oxidized LDL, which has been shown to increase the 5-lipoxygenase activity by increasing the expression of the ALOX5AP gene.[130] Taken together, these findings emphasize the importance of inflammation in the pathogenesis of CHD, particularly in FH patients.

Genes involved in the renin-angiotensin-aldosterone system (RAAS)

Because the RAAS is not only involved in the development of hypertension, but also contributes independently to the development of atherosclerosis and CHD,[156] RAAS genes are important candidate modifier genes for CHD in FH. The RAAS involves the following enzymatic reactions: plasma angiotensinogen (AGT) is converted to angiotensin I by renin, when renal perfusion is decreased. Angiotensin converting enzyme (ACE) converts angiotensin I to

angiotensin II, which causes vasoconstriction, release of aldosterone, and a reduction in renal sodium reabsorption.[148]

We first studied the association between CHD risk and haplotypes of the AGT gene (Chapter 6). Haplotype H3, containing the minor allele of the 4072T→C (Met235Thr) polymorphism and the wild type alleles of three other polymorphisms, was associated with an increased CHD risk. The 4072T→C polymorphism itself was also associated with an increased CHD risk, which is in line with previous studies.[134-138] This polymorphism has been associated with elevated AGT levels and an increased risk of hypertension in white subjects.[133, 137, 146, 147] Because plasma AGT levels in our study population were not available, we were unable to confirm this.

Studies conducted so far showed conflicting results concerning the associations between single nucleotide polymorphisms in RAAS genes and CHD.[137, 157] These discrepancies could be explained by the fact that the RAAS is regulated by multiple interacting genes resulting in a redundant regulation system. Therefore, it is appropriate to consider the combined effects of multiple genes when studying the effect of this pathway on complex phenotypes such as CHD.[158] Hence, we investigated the combined effect of six RAAS polymorphisms on CHD risk by calculating a gene-load score, which reflects the number of risk genotypes within a pathway for each individual.[158] In Chapter 7, we demonstrated the gene-dose dependent association between variation in RAAS genes and CHD in patients with FH. The presence of at least five risk genotypes in the RAAS resulted in a CHD risk that was more than twice as high as that of one risk genotype or fewer. This indicates that despite the fact that the individual polymorphisms revealed small effects, the combination of polymorphisms may more clearly predict susceptibility to CHD.

We hypothesized that genetic variation in RAAS genes would affect CHD risk by influencing blood pressure and/or the risk of hypertension. However, we found no associations of the polymorphisms, haplotypes or gene-load score with hypertension. Moreover, the RAAS gene-load score and AGT haplotype H3 remained significantly associated with CHD after additional adjustment for hypertension. This suggests that the RAAS influences CHD risk through pathways that are not related to blood pressure regulation. Our observations therefore suggest that the negative consequences of increased RAAS activity predominantly manifest themselves at the tissue level.[172] Such tissue effects involve multiple mechanisms that are all associated with atherosclerosis: oxidative stress, inflammation, endothelial dysfunction, and tissue remodeling.

Replication study of 10 novel polymorphisms

Recently, GWA and gene-centric association studies were conducted to identify novel genetic variants associated with MI, early-onset MI, and CHD.[184-187] Gene-centric studies only consider polymorphisms that could influence gene function, e.g. by affecting the amino acid sequence of the encoded protein or by their location in transcription-factor binding sites.[185]

A total of 10 polymorphisms were found to be associated with MI, early-onset MI and CHD in multiple independent populations.[184-189, 195]

Replication of genetic associations in independent populations is essential to reduce the number of false-positive results and to further define the role of these variants in the susceptibility to complex disease. We therefore performed a replication study by investigating the association between these 10 polymorphisms and CHD in our FH population (Chapter 8). We confirmed associations between four polymorphisms and CHD. The replicated polymorphisms are in the olfactory receptor family 13 subfamily G member 1 (OR13G1) gene,[185] the heterogeneous nuclear ribonucleoprotein U-like 1 (HNRPUL1) gene,[186] the complement component 1 q subcomponent receptor 1 (C1QR1 or CD93) gene,[184] and the CDKN2A/B locus.[189] The pathophysiology underlying these associations is largely unknown, and these associations might therefore open avenues to novel CHD-related pathways. For instance, both the CDKN2A/B and HNRPUL1 genes are involved in cell cycle regulation.[197, 199, 200] These loci might play a role in cell cycle checkpoints which are important in repair of DNA that has been damaged by for example oxidative stress in atherosclerotic plaques. Future studies are required to elucidate the exact underlying mechanism by which these polymorphisms or loci affect CHD risk.

Causes of failure to replicate genetic variants

The initial enthusiasm about genetic association studies is partially tempered because published positive associations could often not be replicated in subsequent studies. We will address this issue by using Chapter 8 as an example. The 10 polymorphisms investigated in the replication study were originally found in gene-centric association studies or GWA studies that applied a multistage design.[184-186, 188, 189] In the multistage design the first stages are conducted to identify polymorphisms putatively associated with CHD, whereas in the following stages the hypotheses are tested that these polymorphisms are in fact associated with CHD. In this way, the number of false-positive associations is restricted. Hence, we expected that the 10 polymorphisms were mainly true-positives in the context of general populations. Yet, we only replicated four of the 10 polymorphisms in our FH population. This can be explained in three different ways.

First, the six polymorphisms that were not replicated are probably false-negative findings in our study, because of insufficient statistical power to detect associations (type II error). Our a priori power calculations based on the effect sizes and genotype frequencies of the original studies showed sufficient statistical power for all polymorphisms (>80%). However, mostly we found smaller effect sizes for the polymorphisms than in the original studies, which is in line with a study by Ioannidis *et al*.[202] If these lower effect sizes are true for FH populations, we had insufficient statistical power to reach statistical significance for these associations.

Second, the contrasting findings could have been caused by true variability in association of different phenotypes among different populations.[246] If the influence of a genetic variant

on CHD risk is only manifest on a certain genetic or environmental background, then differences in genetic and environmental factors could account for irreproducibility. True heterogeneity between the populations may also exist if there is variation in linkage disequilibrium between the polymorphism being studied and the true causal variant among populations, which could lead to variation in the strength of an association between populations. Moreover, as indicated earlier in this chapter, FH is an autosomal disorder that may cause CHD at a much younger age than in other populations. Premature CHD in FH populations and CHD in general populations may not be the same phenotype and may have different etiology.

Third, the six non-replicated polymorphisms could have been false-positives in the original studies due to multiple testing (type I error). Yet, the authors of the gene-centric association studies and GWA studies applied a multistage design to restrict the number of false-positives,[184-186, 188, 189] which makes this explanation less likely. Other methods that are often used to deal with multiple testing are the Bonferroni correction and the calculation of the false-discovery rate (FDR). In the Bonferroni correction the nominal p-value is multiplied by the number of hypotheses tested.[246] This correction is too conservative, because many true positive associations between genetic variants and phenotypes may not become significant. The FDR gives an impression of the expected proportion of false-positives among all significant tests, and was applied in Chapter 8.[194] Nevertheless, there is no clear consensus which statistical method is most effective in distinguishing true-positives from false-positives in genetic association studies. We can only speculate on which of these explanations is true. Future replication studies in both FH and general populations will help to clarify this issue.

PREDICTION OF CHD BASED ON GENETIC VARIANTS

The unraveling of the genetic basis of CHD will not only lead to better understanding of the etiology of CHD, it is also expected that genetic risk factors can eventually be used to predict CHD risk in individuals.[73, 214, 225, 226] Because the effect sizes of genetic susceptibility variants for CHD are generally small (as shown in Chapter 3-8), single genetic variants are not useful for CHD prediction. Genetic variants need to be considered in combination, for instance in a genetic profile, i.e. the simultaneous testing of multiple genetic variants. One way in which genetic profiles are currently investigated is by calculating gene-load scores, such as the RAAS gene-load score (Chapter 7). Such a gene-load score approach may stress the importance of a specific pathway in the development of CHD and might be helpful in identifying subgroups of FH patients with a very high CHD risk. However, the question remains if these scores, or genetic profiles in general, will be able to accurately predict the CHD risk of an individual and if they subsequently will be used as a screening tool to distinguish FH patients who will and who will not develop CHD.[209] Moreover, a genetic profile needs to provide additional predictive information above that given by conventional CHD risk factors.

Discriminative accuracy is a first requirement for a valuable predictive test, and can be expressed as the area under the receiver operating characteristic curve (AUC) or the concordance statistic ('c'). The c statistic is the equivalent of the AUC for binary outcomes.[211] The discriminative accuracy that is required in preventive or clinical care depends on the goal of testing, the burden of disease, the availability of (preventive) treatment and the adverse effects of false-positive and false-negative test results. For instance, the identification of individuals at increased CHD risk will result in prescription of medication that partly reduces this CHD risk and is generally well tolerated by the patient. Here, a small proportion of false-positives and/or false-negatives is acceptable. Therefore, an AUC between 0.70 and 0.80 would in this example indicate moderate performance, whereas an AUC above 0.80 would denote good performance. In contrast, the AUCs needed for decisions about invasive and irreversible interventions or for presymptomatic diagnosis are much higher. The discriminative accuracy for CHD prediction of the RAAS gene-load score was 0.55, indicating a low predictive value. Clearly, more genetic variants are needed to accurately predict CHD in FH.

Prediction of CHD in FH

We investigated the extent to which genetic variants identified so far in our FH population can distinguish between FH patients who will and who will not develop CHD (Chapter 9). We considered 14 genetic variants that were significantly associated with CHD in the GIRaFH cohort, of which eight were described in this thesis (Chapters 5, 7, and 8). The other six were previously identified for association with CHD in the GIRaFH cohort.[40, 208] Prediction models were constructed using Cox proportional hazards models, and the predictive value was assessed by the c statistic. The c statistic of the CHD prediction model based on the genetic variants was 0.62, denoting little discrimination. The model based on the conventional risk factors had a c statistic of 0.75. Adding the genetic test results to this model increased the c statistic to 0.76. In conclusion, the contribution of 14 genetic variants to the prediction of CHD risk in FH patients is limited. The additional value beyond conventional risk factors for CHD is particularly low. Larger numbers of genetic variants need to be identified and validated, preferably in etiological pathways for which intermediate outcomes are not included in the prediction model.

Prediction of CHD in general populations

Previous studies in general populations that investigated the discriminative accuracy for prediction of CHD generally found small discriminative accuracies of genetic profiles (AUCs in the order of 0.60).[214, 216] Whereas these previous studies only considered genetic profiles consisting of a limited number of genetic variants, a much larger number of genetic variants with small effects is likely involved in CHD.[209] Inclusion of more genetic variants in genetic profiles may further improve their discriminative accuracy. The expectations are high,[73, 214, 225, 226] but the question remains whether genetic profiling will indeed become useful for the prediction of CHD.

A recent study investigated the combined influence on CHD risk of a genetic profile consisting of 10 genetic variants that were all significantly associated with CHD in published meta-analyses.[225] In a simulation study the authors determined the expected frequency of individuals with a different number of risk genotypes, and estimated their combined effect on CHD risk. Although this study showed that individuals with a high number of risk genotypes had a very high CHD risk compared to individuals with a low number of risk genotypes, the authors did not investigate whether this genetic profile discriminates between individuals who will and will not develop CHD.

In our simulation study presented in Chapter 10, we showed that the discriminative accuracy (measured by the AUC) that could be obtained with such a set of genetic variants is 0.59. We studied two scenarios that could improve the discriminative accuracy of these 10 genetic variants. First, we estimated the expected AUC when two, five and ten times as many genetic variants with the same distribution of odds ratios (ORs) and genotype frequencies would be known, i.e. genetic profiles consisting of 20, 50 and 100 genetic variants in total. The AUCs increased to 0.63, 0.69, and 0.76, respectively. Second, we investigated conditions in which genetic profiling yields a similar discriminative accuracy as conventional CHD risk prediction scores. For different genotype frequencies, we investigated the magnitude of the ORs of 1 to 100 additional genetic variants needed to obtain an AUC of 0.75 in a panel already consisting of the 10 genetic variants described in the first scenario. An AUC of 0.75 could be obtained when 10 additional genetic variants would be identified that have an OR of at least 1.8, depending on the genotype frequency. For 20, 50, or 100 additional genetic variants the minimal ORs were 1.5, 1.3, and 1.2, of course also depending on the genotype frequency.

Taken together, the degree to which genetic profiling is able to predict CHD risk will not easily be better than the prediction by conventional CHD risk factors. In order to obtain AUCs similar to those of the conventional CHD risk prediction tools, a considerable number of additional common genetic variants with preferably strong effects needs to be identified. This may prove difficult, because the genetic variants with the strongest effects may already have been discovered, meaning that even a higher number of genetic variants with small effect sizes is needed. With the latest developments in GWA studies, it is expected that the knowledge about genetic risk factors for CHD will expand.[189, 195] The question remains whether the effect sizes and genotype frequencies of these additional genetic variants will be high enough to substantially influence the discriminative accuracy for CHD risk prediction. The conclusions from this simulation study are probably also true for FH populations. Although the effect sizes of polymorphisms may differ between general populations and FH populations, they are likely similar on average.

Advantages and limitations of prediction based on genetic variants

A potential advantage of genetic profiling above conventional CHD risk factors in the prediction of CHD risk is that most of the conventional risk factors, such as blood pressure and

plasma lipid levels, are subject to measurement errors and may show substantial variation over the entire lifespan. In contrast, genetic risk factors can be accurately measured and do not change between intrauterine life and death. Therefore, genetic profiles could theoretically predict CHD risk before conventional risk factors become apparent, although it is important to keep in mind that the expression of genes may in fact change during life. This increases the opportunities for prevention of CHD as damaging effects of long-term exposure to conventional risk factors can be prevented. Individuals could for instance start with lipid-lowering medication earlier in life, or be screened for CHD when they belong to a group with a particularly increased CHD risk.

The possible contribution of genetic risk factors to the prediction of CHD risk, however, must be considered carefully. The discriminative accuracy of the prediction model based on the conventional risk factors is often quite high (c statistic 0.76 in GIRaFH cohort), which makes it more difficult for the genetic variants to have substantial additional predictive value. Moreover, most candidate gene association studies to date have focused on genetic variants that are related to conventional risk factors for CHD, which often can be considered intermediate traits in the relationship between the genetic variant and CHD, such as blood pressure or plasma lipids. Although such a genetic variant may be strongly and consistently associated with CHD risk, there may be little or no predictive value of this genetic variant in addition to that of the intermediate trait,[247] as demonstrated in Chapter 9. Therefore, to further improve the predictive value of genetic profiles in addition to that of the conventional risk factors, strong genetic risk factors that are not involved in pathophysiological pathways of known conventional risk factors are needed (Figure). Unfortunately, this cannot be expected for many of the currently known genetic susceptibility variants for CHD.

Prediction models based on genetic risk factors for CHD need to be developed for specific populations or for specific ethnicities. Because of the genetic heterogeneity between different populations, it is unlikely that a genetic prediction model can be applied worldwide or even for a specific race. Similarly, the AUCs of the Framingham and PROCAM risk scores[219] were originally reported to be between 0.75 and 0.82,[220, 221] but much lower when applied in other populations (AUCs ranging between 0.61 and 0.68).[235, 236]

Recently, the value of the AUC as a measure of the discriminative accuracy has been questioned.[237] Critics claim that researchers should not solely rely on the change in AUC to evaluate the additional value of risk factors for the prediction of complex diseases, but propose to determine the extent of reclassification in clinically important risk categories.[237] Despite the fact that individuals might be reclassified into different risk categories with consequent changes in treatment decisions, the question remains if this reclassification is accurate and correct, since the discriminative accuracy often does not change much.

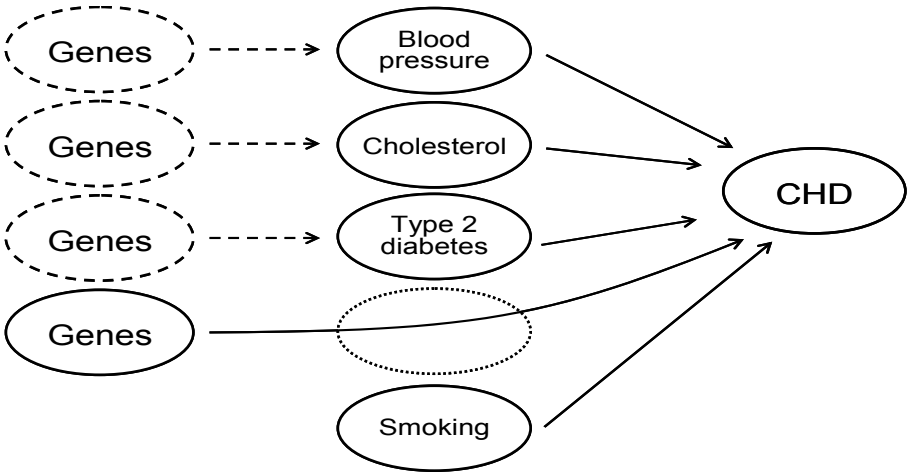

Figure. Schematic presentation of pathways involved in CHD. All interactions between the risk factors have been omitted. The dotted circle indicates unmeasured or unknown intermediate factors in other pathways. Obtained from Janssens AC, van Duijn CM. Genome-based prediction of common diseases: advances and prospects. *Hum Mol Genet* 2008;17:R166-73.

DIRECTIONS FOR FUTURE RESEARCH

Identification of novel genetic susceptibility variants in GWA studies

Since the recent availability of panels of single nucleotide polymorphisms (SNPs) that tag the whole genome, it is now feasible to conduct GWA studies to identify novel genetic susceptibility variants for CHD without relying on any prior hypothesis. The rationale of this approach is that, if unknown susceptibility variants are present somewhere in the genome, they may be detected through their linkage disequilibrium with tagging SNPs.[248] Therefore, if a strong association is detected between a SNP and CHD, the real disease-associated variant should be found by scanning nearby genes for variants that plausibly satisfy the requirement for having an effect on CHD. Because of the recent progress in GWA studies and with the lowering of genotyping costs, it is expected that novel genetic susceptibility variants for CHD in FH will be identified, which will elucidate novel pathophysiological processes underlying CHD.[188, 189, 195] Genotyping FH individuals at the extremes of the phenotype distribution can increase statistical power of a GWA. For example, the genotypes of FH patients with severe premature onset of CHD symptoms can be compared with older FH patients who did not experience CHD during life. This study design will improve the power to detect variants with relatively large effects.

The newly identified genetic susceptibility variants for CHD will gain evidence by replication of the associations with the same phenotype and the same direction of the effect in independent populations. Ultimately, meta-analyses should be performed to assess the overall

evidence for a role of the genetic variants in CHD. This may prove difficult for FH populations, because of the low number of large study populations focusing on FH patients.

Collaboration between centers

Lack of statistical power is a drawback for the identification of genetic variants with relatively small effects, and for analyzing gene-gene and gene-environment interactions. One of the solutions is collaboration between centers in order to obtain larger populations for genetic association analyses. This is especially important for FH populations, since these study populations are generally small.

Development of prediction models

The novel genetic susceptibility variants for CHD may be added to prediction models to further improve individual CHD prediction for FH patients, although we have shown in Chapter 10 that this will not be easy to accomplish. Genetic variants with strong effects have the largest predictive value. Therefore, it may be worthwile to consider gene-gene or gene-environment interactions in prediction models, because the phenotypic expression of a genetic variant may depend on the genetic background or on conventional risk factors (Chapter 5). Before the prediction model can be applied in clinical practice, it should first be internally validated by means of bootstrapping techniques (Chapter 9) and subsequently externally validated in independent FH populations to assess its overall performance.

Elucidating the pathophysiological mechanism underlying genetic associations

The pathophysiological mechanisms underlying the associations between the genetic variants presented in Chapter 8 are largely unknown, and it is expected that more novel genetic susceptibility variants for CHD will be identified in future GWA studies. Additional studies should be conducted to reveal the pathophysiological mechanisms through which the newly identified genetic variants influence CHD risk.

CONCLUSION

This thesis describes studies on several genetic susceptibility variants for CHD in FH. Identification of such genetic variants leads to a better understanding of the pathophysiology underlying atherosclerosis and CHD in FH patients. Yet, the currently known genetic susceptibility variants are not able to accurately predict CHD risk of an individual FH patient. Therefore, it is not reasonable to test genetic variants in routine clinical practice to improve individual CHD risk prediction in FH patients. The question remains if this will be possible in future, because a large number of common genetic susceptibility variants for CHD with preferably strong effects needs to be identified in upcoming candidate gene association studies and GWA studies.

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Summary

Familial hypercholesterolemia (FH) is an inherited disorder of lipid metabolism, caused by mutations in the low-density lipoprotein (LDL) receptor gene. It is characterized by high plasma LDL cholesterol levels, tendon xanthomas, and premature coronary heart disease (CHD). Despite the fact that a large proportion of FH patients develops CHD before the age of 60 years, there is considerable variation in the risk of CHD among FH patients, which is influenced by conventional and genetic risk factors for CHD. The aim of this thesis was to identify genetic risk factors for CHD in patients with FH, and to investigate whether these genetic risk factors could be helpful in distinguishing FH patients who will develop CHD from those who will not.

The thesis starts with a description of FH and CHD, together with their etiology and epidemiology (**Chapter 1**). This chapter also describes the GIRaFH cohort, which consists of 2400 unrelated FH patients, aged 18 years and older, who where identified at Dutch lipid clinics between 1989 and 2002. This study population was used for all genetic association studies described in this thesis. Finally, the chapter provides an overview of the studies that have been performed on conventional and genetic risk factors for CHD in the GIRaFH cohort and other FH populations.

Chapter 2 reports on a comparison of the statistical power of Cox proportional hazards models and logistic regression models in cross-sectional genetic association analyses. Because genetic variation is present at birth, under certain conditions both strategies can be used to study the association between genetic variation and common diseases. We found that Cox proportional hazards models have more statistical power, especially in the range of effect estimates that are expected for genetic associations in common diseases.

The contribution to CHD risk of variation in the adenosine triphosphate binding cassette G8 (ABCG8) gene is described in **Chapter 3**. This candidate gene was investigated because it is involved in plasma plant sterol levels. We found a marginally significant association between the H-allele of the D19H polymorphism and an increased CHD risk, whereas the T400K polymorphism was not associated with CHD risk. We conclude that genetic variation in the ABCG8 gene is not a major contributor for differences in CHD risk among patients with FH.

Glucocorticoids play a role in the development of hypertension, dyslipidemia, impaired glucose tolerance, and central adiposity. Four polymorphisms in the glucocorticoid receptor (GR) gene have been reported to alter glucocorticoid sensitivity and have been associated with cardiovascular risk factors. In **Chapter 4** we report on the relationship between CHD and haplotypes of the GR gene, which were defined by these four polymorphisms. One common haplotype in the GR gene was associated with CHD risk in men, but none of the haplotypes was associated with CHD in women. We therefore conclude that variation in the GR gene only modestly influences CHD risk in men with FH.

The arachidonate 5-lipoxygenase-activing protein (ALOX5AP) gene is required for the synthesis of leukotrienes, a protein family involved in inflammatory responses. Recently, a haplotype of this gene was associated with myocardial infarction in an Icelandic and British population. Since FH is characterized by chronic inflammation of the arterial wall, and subsequent premature CHD, the ALOX5AP gene could be an important candidate gene for CHD in FH. In **Chapter 5** we report that HapB, a haplotype defined by four polymorphisms, is associated with CHD in FH, and particularly in patients with the highest plasma LDL cholesterol levels. These findings support the important role of inflammation in the pathogenesis of CHD in FH.

Chapters 6 and 7 describe the relation between genetic variation in the renin-angiotensin-aldosterone system (RAAS) and CHD risk in FH. Because the RAAS is involved in the development of atherosclerosis and CHD, RAAS genes are important candidate genes for CHD. We first studied the association between CHD risk and haplotypes of the angiotensinogen gene (**Chapter 6**). These haplotypes were based on four polymorphisms. Haplotype H3, defined by the minor allele of the 4072T→C (Met235Thr) polymorphism, was associated with an increased CHD risk.

Because the RAAS is regulated by a large number of interacting genes resulting in a highly redundant regulation system, it is appropriate to consider the combined effects of multiple genes when studying the effect of complex pathways on complex phenotypes such as CHD. Hence, we investigated the combined effect of six RAAS polymorphisms in relation to CHD risk in **Chapter 7**. The presence of at least five risk genotypes in the RAAS results in a CHD risk that is more than twice as high as that of one risk genotype or fewer. This suggests that despite the fact that the individual polymorphisms revealed small effects, the combination of polymorphisms may more clearly predict susceptibility to CHD.

Recent candidate gene and genome-wide association studies (GWAs) have revealed associations between novel genetic polymorphisms and CHD. **Chapter 8** presents a replication study of 10 novel polymorphisms and CHD. We confirmed previously found associations between four polymorphisms and CHD, but refuted associations for the six other polymorphisms. The replicated polymorphisms are located in the olfactory receptor family 13 subfamily G member 1 (OR13G1) gene, the heterogeneous nuclear ribonucleoprotein U-like 1 (HNRPUL1) gene, and the complement component 1 q subcomponent receptor 1 (C1QR1 or CD93) gene, and near the cyclin-dependent kinase N2A and N2B (CDKN2A and CDKN2B) genes. Future studies are required to elucidate the exact underlying mechanism by which these polymorphisms or loci affect CHD risk.

In **Chapter 9,** we address the extent to which genetic variants identified so far in our FH population can predict CHD in FH patients. We considered 14 genetic variants that were significantly associated with CHD in the GIRaFH cohort, of which eight were described in this thesis (**Chapters 5, 7, and 8**). We concluded that the contribution of these genetic variants

to the prediction of CHD risk in FH patients is limited. The additional value over conventional risk factors for CHD is particularly low.

Chapter 10 describes a simulation study in which we investigated to what degree CHD risk in general populations can be predicted by testing multiple genetic variants (genetic profiling). We showed that the predictive value of 10 established genetic susceptibility variants for CHD was limited. The predictive ability with genetic susceptibility variants could theoretically become similar to that of conventional CHD risk prediction models if a considerable number of additional common genetic variants were identified with strong effects, either on their own or in interaction with other variants or environmental factors.

Chapter 11 gives a general discussion in which the findings of the thesis are discussed. First, the methodological issues relevant to the studies described in this thesis are reviewed. Then, the results of the genetic association studies are considered in the context of ongoing research, and the potential use of genetic variants in CHD risk prediction is discussed. Subsequently, directions for future research are addressed. Finally, we conclude that the identification of genetic susceptibility variants for CHD leads to a better understanding of the pathophysiology underlying CHD in FH patients, which theoretically could lead to novel pharmacological therapies. Yet, the currently known genetic susceptibility variants are not able to accurately predict CHD risk of an individual FH patient. The question remains if this will be possible in future. A large number of common genetic susceptibility variants for CHD with strong effects needs to be identified in upcoming candidate gene association studies and GWAs to enable prediction in individual patients.

Samenvatting

Familiaire hypercholesterolemie (FH) is een erfelijke vorm van verhoogd cholesterol, veroorzaakt door mutaties in het *low-density lipoprotein* (LDL) receptor gen. Het wordt gekarakteriseerd door hoge waarden van LDL cholesterol ('slecht cholesterol') in het bloed, vetophopingen op pezen (peesxanthomen) en verkalking van de kransslagaderen (coronaire hartziekten). Ondanks het feit dat een groot deel van de FH patiënten coronaire hartziekten (CHZ) ontwikkelt voor de leeftijd van 60 jaar, is er een grote variatie in de kans op CHZ tussen FH patiënten. Deze variatie wordt beïnvloed door conventionele en genetische risicofactoren voor CHZ. Het doel van dit promotieonderzoek was tweeledig. Ten eerste om genetische risicofactoren voor CHZ te ontdekken bij patiënten met FH en ten tweede om te onderzoeken in hoeverre deze genetische risicofactoren in staat zijn om FH patiënten die CHZ zullen ontwikkelen te onderscheiden van patiënten die geen CHZ zullen ontwikkelen.

Het proefschrift start met de definitie, de ontstaanswijze en de epidemiologie van FH en CHZ (**Hoofdstuk 1**). In dit hoofdstuk wordt ook het GIRaFH cohort beschreven. Het GIRaFH cohort (Genetische Identificatie van Risicofactoren bij FH) bestaat uit 2400 niet-verwante FH patiënten, met een leeftijd van 18 jaar en ouder, die werden behandeld op Nederlandse lipidenpoliklinieken tussen 1989 and 2002. Deze studiepopulatie werd gebruikt voor alle genetische associatiestudies die in dit proefschrift worden beschreven. Ten slotte geeft dit hoofdstuk een uiteenzetting van de studies die gedaan zijn naar conventionele en genetische risicofactoren voor CHZ in het GIRaFH cohort en andere FH populaties.

In **Hoofdstuk 2** wordt de statistische kracht van 'Cox proportional hazards' modellen en logistische regressiemodellen in genetische associatiestudies vergeleken. De statistische kracht is de kans dat een bestaand verband ook daadwerkelijk gevonden wordt door een statistisch model. Omdat genetische variatie al bij de geboorte aanwezig is, kunnen onder bepaalde voorwaarden beide strategieën gebruikt worden om de associatie tussen genetische variatie en veelvoorkomende ziekten te onderzoeken. Wij tonen in dit hoofdstuk aan dat Cox proportional hazards modellen de geprefereerde statistische methode is ten opzichte van logistische regressiemodellen bij het onderzoeken van associaties tussen genetische varianten en veelvoorkomende ziekten, aangezien de verwachte grootte van het effect relatief klein is.

De bijdrage van variatie in het 'adenosine triphosphate binding cassette G8 (ABCG8)' gen aan het risico op CHZ wordt beschreven in **Hoofdstuk 3**. Dit gen werd onderzocht omdat het betrokken is bij de concentratie van plantensterol in het bloed. Wij vonden een marginaal significante associatie tussen het H-allel van het D19H polymorfisme en een verhoogd CHZ risico, terwijl het T400K polymorfisme niet geassocieerd was met CHZ risico. Wij concluderen dat genetische variatie in het ABCG8 gen geen belangrijke bijdrage levert aan het verschil in CHZ risico tussen FH patiënten.

Glucocorticoïden spelen een rol in de ontwikkeling van hoge bloeddruk, hoog cholesterol, verminderde glucose tolerantie en centrale vetzucht. Vier polymorfismen in het glucocorticoïd receptor (GR) gen zijn geassocieerd met de gevoeligheid voor glucocorticoïden en met risicofactoren voor hart- en vaatziekten. In **Hoofdstuk 4** beschrijven wij de relatie tussen CHZ en haplotypen van het GR gen, welke gebaseerd zijn op deze vier polymorfismen. Een veelvoorkomende haplotype in het GR gen was geassocieerd met CHZ risico bij mannen, maar geen van de haplotypes was geassocieerd met CHZ bij vrouwen. Wij concluderen daarom dat variatie in het GR gen slechts in beperkte mate het risico op CHZ beïnvloedt bij FH patiënten.

Het arachidonaat 5-lipoxygenase-activerende proteïne (ALOX5AP) gen is noodzakelijk voor de aanmaak van leukotriënen, een soort eiwitten die betrokken zijn bij onstekingsreacties. Een recent onderzoek in een IJslandse en Britse populatie toonde aan dat een haplotype van dit gen geassocieerd was met hartinfarcten. Omdat FH gekenmerkt wordt door chronische ontsteking van de arteriële vaatwand resulterend in premature CHZ, kan het ALOX5AP gen een belangrijk kandidaat gen zijn voor CHZ bij FH patiënten. In **Hoofdstuk 5** laten we zien dat HapB, een haplotype gebaseerd op vier polymorfismen, geassocieerd is met CHZ bij FH, en voornamelijk bij patiënten met de hoogste LDL cholesterol waarden. Deze bevindingen ondersteunen de belangrijke rol van ontsteking in het ontstaan van CHZ bij FH.

Hoofdstukken 6 en 7 beschrijven de relatie tussen genetische variatie in het renine-angiotensine-aldosteron systeem (RAAS) en CHZ risico bij FH patiënten. Omdat het RAAS betrokken is bij de ontwikkeling van aderverkalking en CHZ, zijn RAAS genen belangrijke kandidaat genen voor CHZ. We onderzochten eerst de associatie tussen CHZ risico en haplotypes van het angiotensinogeen gen (**Hoofdstuk 6**). Deze haplotypes waren gebaseerd op vier polymorfismen. Haplotype H3, gedefinieerd door het C-allel van het 4072T→C (Met235-Thr) polymorfisme, was geassocieerd met een verhoogd CHZ risico.

Het RAAS wordt gereguleerd door een groot aantal genen, wat resulteert in een complex regulatiesysteem. Daarom moet rekening worden gehouden met de gecombineerde effecten van meerdere genen wanneer het effect van complexe regulatiesystemen op complexe ziekten, zoals CHZ, worden bestudeerd. In **Hoofdstuk 7** onderzochten wij het gecombineerde effect van zes RAAS polymorfismen op het CHZ risico. FH patiënten met minstens vijf genetische risicovarianten van het RAAS hadden een CHZ risico dat meer dan verdubbeld was ten opzichte van het CHZ risico van FH patiënten met één genetische risicovariant of minder. Dit suggereert dat ondanks het feit dat de individuele polymorfismen slechts kleine effecten hebben, de combinatie van polymorfismen wel waarde heeft voor de voorspelling van het CHZ risico.

Recente genetische associatiestudies en genoomwijde associatiestudies hebben associaties gevonden tussen nieuwe genetische polymorfismen en CHZ. In **Hoofdstuk 8** wordt een replicatiestudie gepresenteerd van 10 nieuwe polymorfismen and CHD. Eerder beschreven associaties tussen vier polymorfismen en CHZ werden in deze replicatiestudie bevestigd, terwijl zes andere associaties niet werden bevestigd. De gerepliceerde polymorfismen liggen

in het 'olfactory receptor family 13 subfamily G member 1 (OR13G1)' gen, het 'heterogeneous nuclear ribonucleoprotein U-like 1 (HNRPUL1)' gen en het 'complement component 1 q subcomponent receptor 1 (C1QR1 or CD93)' gen, en vlakbij de 'cyclin-dependent kinase N2A and N2B (CDKN2A and CDKN2B)' genen. Toekomstige studies zijn nodig om de exacte mechanismen die ten grondslag liggen aan deze associaties op te helderen.

In **Hoofdstuk 9** staan we stil bij de mate waarin de tot nu toe in onze FH populatie gevonden genetische risicovarianten het CHZ risico van FH patiënten kunnen voorspellen. Voor deze analyse gebruikten wij 14 genetische varianten die significant geassocieerd waren met CHZ in het GIRaFH cohort, waarvan er acht beschreven staan in dit proefschrift (**Hoofdstuk 5, 7, en 8**). Wij concludeerden dat de bijdrage van deze genetische varianten aan de voorspelling van het CHZ risico beperkt is. De extra waarde ten opzichte van conventionele risicofactoren voor CHZ is met name laag.

Hoofdstuk 10 beschrijft een simulatiestudie waarin we onderzocht hebben in welke mate CHZ risico in de algemene bevolking voorspeld zou kunnen worden door meerdere genetische varianten (genetisch profiel). We toonden aan dat de waarde van 10 bewezen genetische risicovarianten voor de voorspelling van CHZ beperkt was. De voorspellende waarde op basis van genetische risicovarianten zou theoretisch vergelijkbaar kunnen worden met dat van gebruikelijke CHZ risicovoorspellers als in de toekomst een aanzienlijk aantal additionele en veelvoorkomende genetische varianten met sterke effecten geïdentificeerd zullen worden.

In **Hoofdstuk 11** worden de belangrijkste bevindingen van dit proefschrift toegelicht. Ten eerste worden methodologische kwesties besproken die relevant zijn voor de studies beschreven in dit proefschrift. Daarna worden de resultaten van de genetische associatiestudies besproken en in verband gebracht met de huidige literatuur over dit onderwerp. Verder wordt er stilgestaan bij de potentiële waarde van genetische varianten voor de voorspelling van CHZ risico en de mogelijkheden voor toekomstig onderzoek. Tenslotte concluderen we dat het ontdekken van genetische risicovarianten voor CHZ leidt tot een beter begrip van de ziekteprocessen die ten grondslag liggen aan CHZ bij patiënten met FH, wat theoretisch zou kunnen leiden tot nieuwe medicamenteuze behandelingen. Echter, de nu bekende genetische risicovarianten zijn niet in staat om nauwkeurig het CHZ risico van een patiënt met FH te voorspellen. De vraag blijft of dit wel mogelijk zal zijn in de toekomst. Een groot aantal veelvoorkomende genetische risicovarianten voor CHZ met sterke effecten moeten worden ontdekt in aankomende genetische associatiestudies en genoomwijde associatiestudies om voorspelling van CHZ risico mogelijk te maken voor de individuele patiënt.

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Curriculum Vitae

Jeroen van der Net was born on March 7th, 1979 in Rotterdam. After graduating from secondary school in 1997 (Emmaus College, Rotterdam), he spent two years studying Economics at the Erasmus University in Rotterdam. In 1999 he started studying Medicine at the Erasmus University in Rotterdam. During his medical study, he followed a Master of Science program in Clinical Epidemiology at the Netherlands Institute for Health Sciences (NIHES). As part of this program, he attended a summer course at the Harvard School of Public Health, Boston, USA (July - August 2003). He obtained both master degrees in Medicine and Clinical Epidemiology in the summer of 2003. After obtaining his qualification as a medical doctor in August 2005 cum laude, he started the research described in this thesis at the departments of Internal Medicine and Public Health of the Erasmus MC, University Medical Center Rotterdam. As part of this research project, he worked at the Cardiovascular Research Institute of the University of California San Francisco, USA (September - November 2007) under supervision of prof.dr. J.P. Kane, for which he received a grant from the Netherlands Heart Foundation. In September 2008, he started his specialty training in Internal Medicine (supervisors: prof.dr. J.L.C.M. van Saase, Erasmus MC, University Medical Center Rotterdam, and drs. A.P. Rietveld, Sint Franciscus Gasthuis in Rotterdam).