Inflammatory Markers and Genes

Epidemiologic Studies on their Roles in Cardiovascular Disease

Isabella Kardys

Acknowledgements

The work presented in this thesis was conducted at the Department of Epidemiology & Biostatistics of Erasmus MC, Rotterdam, the Netherlands.

Most of the studies described in this thesis were performed within the Rotterdam Study, which is supported by Erasmus MC and Erasmus University Rotterdam, the Netherlands Organization for Scientific Research (NWO), the Netherlands Organization for Health Research and Development (ZonMw), the Research Institute for Diseases in the Elderly (RIDE), the Ministry of Education, Culture and Science, the Ministry of Health, Welfare and Sports, the European Commission (DG XII), and the municipality of Rotterdam.

The contributions of the inhabitants, general practitioners and pharmacists of the Ommoord district to the Rotterdam Study are gratefully acknowledged.

The studies on inflammation described in this thesis were supported by grant 948-00-016 from the Research Institute for Diseases in the Elderly (RIDE) of the Netherlands Organization for Health Research and Development (ZonMw). The studies on Lp-PLA2 described in this thesis were supported by an unrestricted grant from GlaxoSmithKline.

Financial support for the publication of this thesis was kindly provided by the Department of Epidemiology & Biostatistics of Erasmus MC, J.E. Jurriaanse Stichting, Astellas Pharma BV, AstraZeneca BV, Sanofi-BMS and Genzyme Nederland.

Cover design: I. Kardys and Optima Grafische Communicatie, Rotterdam
Cover: "Heart on fire", Janne Ahvo
Layout and printing: Optima Grafische Communicatie, Rotterdam

ISBN: 978-90-8559-160-3

Inflammatory Markers and Genes: Epidemiologic Studies on their Roles in Cardiovascular Disease

Epidemiologische studies naar de rol van inflammatoire markers en genen in hart- en vaatziekten

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
Rector Magnificus

Prof.dr. S.W.J. Lamberts

en volgens besluit van het College van Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 19 december 2007 om 15.45 uur

door

Isabella Kardys

geboren te Poznań, Polen

Promotiecommissie

Promotoren:	Prof.dr. J.C.M. Witteman
	Prof.dr. A. Hofman
Overige leden:	Prof.dr. D.J.G.M. Duncker
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Copromotor:	Dr. M.P.M. de Maat

The study described in this thesis was supported by a grant of the Netherlands Heart Foundation (NHF-2002B190).
Financial support by the Netherlands Heart Foundation for the publication of this thesis is gratefully acknowledged.

Voor mijn ouders

Manuscripts based on the studies described in this thesis

Chapter 2.1
Kardys I, De Maat MP, Uitterlinden AG, Hofman A, Witteman JC. C-reactive protein gene haplotypes and risk of coronary heart disease. The Rotterdam Study. *Eur Heart J.* 2006;27(11):1331-7.

Chapter 2.2
Elias-Smale S, Kardys I, Oudkerk M, Hofman A, Witteman JC. C-reactive protein is related to the extent and progression of coronar extra-coronary atherosclerosis; results from the Rotterdam Study. *Atherosclerosis. 2007; in press.*

Chapter 2.3
Kardys I, Klaver CC, Despriet DD, Bergen AA, Uitterlinden AG, Hofman A, Oostra BA, Van Duijn CM, De Jong PT, Witteman JC. A common polymorphism in the complement factor H gene is associated with increased risk of myocardial infarction. The Rotterdam Study. *J Am Coll Cardiol.* 2006;47(8):1568-75.

Chapter 2.4
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Chapter 3.1
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Chapter 4.1

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Chapter 5.1

Kardys I, Deckers JW, Stricker BH, Vletter WB, Hofman A, Witteman JC. Distribution of echocardiographic parameters and their associations with cardiovascular risk factors in the Rotterdam Study. *Submitted.*

Chapter 5.2

Kardys I, Deckers JW, Stricker BH, Vletter WB, Hofman A, Witteman JC. Structural, systolic and diastolic echocardiographic parameters and all-cause mortality: the Rotterdam Study. *Submitted.*

Chapter 5.3

Kardys I, Knetsch AM, Bleumink GS, Deckers JW, Hofman A, Stricker BH, Witteman JC. C-reactive protein and risk of heart failure. The Rotterdam Study. *Am Heart J.* 2006;152(3):514-20.

Chapter 5.4

Van Vark LC, Kardys I, Bleumink GS, Knetsch AM, Deckers JW, Hofman A, Stricker BH, Witteman JC. Lipoprotein-associated phospholipase A2 activity and risk of heart failure. The Rotterdam Study. *Eur Heart J.* 2006;27(19):2346-52.

Chapter 6.1

Kardys I, Vliegenthart R, Oudkerk M, Hofman A, Witteman JC. The female advantage in cardiovascular disease: vascular beds do not contribute equally. *Am J Epidemiol.* 2007;166(4):403-12.

Contents

Chapter 1

Introduction

Established cardiovascular risk factors such as hypertension, hyperlipidemia, diabetes mellitus and smoking do not fully explain the occurrence of cardiovascular disease; although the majority of patients have at least one of these risk factors, a substantial proportion of cases occurs in individuals that have none.[1] As such, further insight is required into the pathophysiology of cardiovascular disease and in factors that may identify individuals at high risk.

One of the most relevant insights in atherosclerosis of the past years is the recognition of the role of inflammation.[2] Research on inflammatory markers, both experimental and epidemiological, has taken flight, and several of these markers have been implicated in cardiovascular disease.[3] This development was accompanied by an expansion of research on genetic variation that may influence inflammatory processes. The field of genetics has rapidly evolved over the last years because of improved technology and methodology in combination with the emergence of large, publicly available genetic databases.[4]

The purpose of this thesis was to expand the knowledge on inflammatory markers and inflammatory genes that may play a part in the pathophysiology of cardiovascular disease. We focused on factors that have drawn increased attention in the recent years, such as C-reactive protein (CRP) and lipoprotein-associated phospholipase A2 (Lp-PLA2), and examined their roles in both atherothrombotic disease and in heart failure. Most studies were conducted within the Rotterdam Study, a population-based cohort study among 7983 men and women aged 55 years and over living in a well-defined suburb of Rotterdam, the Netherlands.[5] During a visit of the participants to the research center, blood was drawn in order to assess inflammatory markers and genetic variation. Several measures of atherosclerosis were assessed at the research center, and furthermore, participants were followed-up for the occurrence of coronary events and heart failure. Specifically, the main research questions we examined were as follows.

With regard to inflammation, atherosclerosis and coronary events:
- Is CRP serum level associated with atherosclerosis and coronary events?
- Is variation in the CRP gene and variation in the complement factor H gene associated with coronary events, and do these genes interact to predict disease?
- Is Lp-PLA2 activity associated with atherosclerosis?

With regard to inflammation and heart failure:
- What is the distribution of echocardiographic parameters in an asymptomatic population, and do these parameters predict mortality?
- Are the inflammatory markers CRP and Lp-PLA2 associated with the occurrence of heart failure?

The outline of this thesis is as follows. Part I focuses on inflammation and atherosclerotic disease. Serum levels of C-reactive protein are investigated in relation to coronary events and atherosclerosis, and genetic variation in the C-reactive protein gene and the complement factor H gene is examined in relation to coronary events (chapter 2). The associations of lipoprotein-associated phospholipase A2 activity in plasma with coronary calcification and extracoronary atherosclerosis are described (chapter 3). Hereafter, other emerging genes and markers which are related to inflammation receive attention; genetic variation in the fibrinogen alpha and gamma genes is examined in relation to cardiovascular

outcomes, and the association between plasma level of heat shock protein 27 and cardiovascular disease is investigated in the Women's Health Study (chapter 4). Part II focuses on inflammation and heart failure. Established cardiovascular risk factors are examined in relation to echocardiographic parameters, and the association of echocardiographic parameters with all-cause mortality is described. Associations of C-reactive protein and lipoprotein-associated phospholipase A2 with heart failure are investigated (chapter 5). In Part III, an overview is given of the prevalence of atherosclerosis in the Rotterdam Study in both genders (chapter 6). Finally, in the general discussion (chapter 7), methodological considerations are addressed, the main findings of this thesis are placed in a broader context, and potential clinical implications and directions for future research are discussed.

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Part I

Inflammation, atherosclerosis and coronary events

Chapter 2

C-reactive protein and complement factor H

Chapter 2.1

C-reactive protein level, C-reactive protein gene haplotypes, and coronary heart disease

Abstract

Aims. C- reactive protein is associated with risk of cardiovascular disease. However, whether C-reactive protein is a marker of severity of cardiovascular disease or actually is involved in its pathogenesis remains unknown. We investigated the relation between C-reactive protein haplotypes, representing the comprehensive variation of the C-reactive protein gene, and coronary heart disease.

Methods and results. The Rotterdam Study is a prospective population-based study among men and women aged 55 years and older. C-reactive protein was associated with risk of coronary heart disease, with a multivariate adjusted hazard ratio of 1.9 (95% confidence interval 1.5-2.4) for the highest versus the lowest quartile. Four C-reactive protein haplotypes were present with overall frequencies of 32.8%, 31.7%, 29.5%, and 5.9%. C-reactive protein serum levels were significantly different according to C-reactive protein haplotypes. C-reactive protein haplotypes were not associated with coronary heart disease.

Conclusion. Steady-state C-reactive protein serum level is influenced by C-reactive protein gene haplotypes. Although elevated C-reactive protein level has lately been found to be a consistent and relatively strong risk factor for cardiovascular disease, our study does not support that the common variation in the C-reactive protein gene has a large effect on the occurrence of coronary heart disease.

Introduction

C- reactive protein is associated with cardiovascular disease.[1] However, whether C-reactive protein is merely a marker of severity of cardiovascular disease or actually is involved in its pathogenesis remains unknown. Genetic markers offer a possibility to study this.

Evidence has emerged that C-reactive protein may play a pathogenic role in cardiovascular disease.[2] If this is true, genetic variants associated with high C-reactive protein level may be associated with greater risk of cardiovascular disease. The genes involved in this regulation remain ill-defined. The C-reactive protein gene is likely to play a part, since several studies have demonstrated associations between single nucleotide polymorphisms (SNPs) in the C-reactive protein gene and C-reactive protein level.[3-13] Two recent studies have identified comprehensive sets of common C-reactive protein gene haplotypes and found associations of these haplotypes with C-reactive protein level.[14,15] One of these studies has also examined the association between these haplotypes and myocardial infarction or ischemic stroke in a nested case-control study within the Physicians' Health Study cohort,[15] but the association between C-reactive protein variants and baseline C-reactive protein did not correlate with the effects of those variants on clinical cardiovascular events in this study.

SeattleSNPs (part of the National Heart Lung and Blood Institute's Programs for Genomic Applications) reports that four C-reactive protein gene haplotypes are present in populations of European descent. These haplotypes represent all common variation across the C-reactive protein gene in these populations. To further clarify the role of C-reactive protein in coronary heart disease, we set out to investigate the relation between these four C-reactive protein gene haplotypes, C-reactive protein serum level and coronary heart disease prospectively in all participants of the large, population-based Rotterdam Study.

Participants and Methods

Study population and baseline data collection

The present study is part of the Rotterdam Study, a population-based cohort study aimed at assessing the occurrence of and risk factors for chronic diseases in the elderly. Objectives and methods of the Rotterdam Study have been described in detail elsewhere.[16] The Rotterdam Study cohort includes 7983 men and women aged 55 years and over (78% of the eligible population), living in a well-defined suburb of the city of Rotterdam, The Netherlands. The medical ethics committee of Erasmus Medical Center, Rotterdam, approved the study. Participants gave written informed consent and permission to retrieve information from treating physicians. This study complies with the Declaration of Helsinki.

Baseline data were collected from 1990 until 1993, as described previously.[17] A trained interviewer visited all participants at home and collected information on current health status, medical history, drug use, and smoking, using a computerized questionnaire. Additionally, in 7129 participants, established cardiovascular risk factors were measured at the research center.

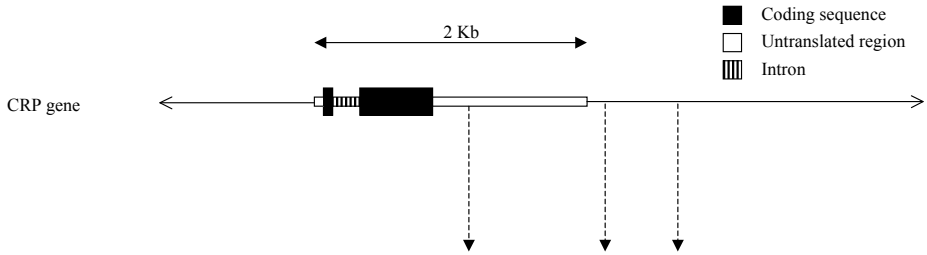
Measurement of C-reactive protein

At baseline, non-fasting blood was collected. All tubes were stored on ice before and after blood sampling. High-sensitivity C-reactive protein was determined in serum, which was stored at -20°C until performance of the C-reactive protein measurements in 2003-2004. C-reactive protein was measured using Rate Near Infrared Particle Immunoassay (Immage® Immunochemistry System, Beckman Coulter, USA). This system measures concentrations from 0.2 to 1440 mg/l, with a within-run precision < 5.0%, a total precision < 7.5% and a reliability coefficient of 0.995.

Genotyping

The Seattle SNPs Program for Genomic Applications has identified 31 SNPs in the C-reactive protein gene and has established that, based on SNPs with overall frequencies above 5%, four common C-reactive protein gene haplotypes are present in 23 unrelated individuals of European descent from the CEPH pedigrees (http://pga.gs.washington.edu/data/crp, "visual haplotype" option). These four haplotypes can be identified by "haplotype tagging" SNPs. By genotyping three haplotype tagging SNPs we were able to infer all four haplotypes and consequently to describe the common variation across the C-reactive protein gene (figure 1). These three tagging SNPs were chosen partly based on their presence in existing literature and on their proximity to the C-reactive protein gene. Other SNPs were also eligible, since a range of SNP trios across the C-reactive protein gene captures the four most common haplotypes among European participants.

Figure 1. The C-reactive protein gene, C-reactive protein gene polymorphisms determined in this study and common C-reactive protein gene haplotypes.



Haplotypes in population of European descent (n=6007)

Haplotype	1184	2042	2911	frequency
1	C	T[a]	C	32.8%
2	T[a]	C	C	31.7%
3	C	C	C	29.5%
4	C	C	G[a]	5.9%
Others				< 0.001%

[a] Tagging SNP for that haplotype

All participants were genotyped for the 1184 C>T, 2042 C>T and 2911 C>G SNPs of the C-reactive protein gene. The polymorphisms are described in relation to the start of the coding sequence of exon 1 using the Human May 2004 (hg 17) assembly (http://genome.ucsc.edu). These polymorphisms have also been described at http://www.ncbi.nlm.nih.gov/SNP under identification numbers rs1130864 (1184 C>T), rs1205 (2042C>T) and rs3093068 (2911 C>G).

DNA was extracted according to standard procedures. DNA was solubilized in double-distilled water and stored at -20°C until used for DNA amplification. Genotypes were determined in 2 ng genomic DNA with the Taqman allelic discrimination assay (Applied Biosystems, Foster City, CA, USA). Primer and probe sequences were optimized by using the SNP assay-by-design service of Applied Biosystems (http://store.appliedbiosystems.com). Reactions were performed with the Taqman Prism 7900HT 384 wells format. Haplotype alleles present in the population were inferred by means of the haplo.em function of the program Haplo Stats (http://cran.r-project.org/src/contrib/Descriptions/haplo.stats.html), which computes maximum likelihood estimates of haplotype probabilities.[18,19] Haplotype reconstruction resulted in seven haplotypes, but the fifth, sixth and seventh haplotypes were present in <0.001% of the alleles and were therefore not used in the analyses. Haplotype alleles were coded as haplotype numbers 1 through 4 in order of decreasing frequency in the population: coding from 1184 C>T, 2042C>T and 2911 C>G, haplotype 1= C-T-C, 2= T-C-C, 3= C-C-C and 4= C-C-G (figure 1).

Follow-up procedure

Follow-up started at the baseline examination and for the present study lasted until January 1st, 2002. Information on fatal and non-fatal cardiovascular endpoints was obtained from general practitioners (GPs) and letters and discharge reports from medical specialists.[17] Two research physicians independently coded all reported events according to the International Classification of Diseases, 10th edition (ICD-10).[20] In case of disagreement, consensus was reached. A medical expert in cardiovascular disease, whose judgment was considered final, reviewed all events.

We defined incident coronary heart disease as myocardial infarction, coronary artery bypass grafting (CABG), percutaneous transluminal coronary angioplasty (PTCA) and cardiac death. In identifying myocardial infarctions, all available information, which included ECG, cardiac enzyme levels, and the clinical judgment of the treating specialist, was used. We defined cardiac death as death from myocardial infarction or other ischemic heart disease (ICD-10: I20-I25), sudden cardiac death (I46), sudden death undefined (R96), or death from heart failure (I50).

Population for analysis

C-reactive protein serum levels were available for 6658 participants. C-reactive protein measurements were lacking for participants who did not visit the research center (854) and for participants of whom no blood was available due to logistic reasons (471). After excluding participants with coronary heart disease at baseline (870), defined as a history of myocardial infarction, PTCA or CABG, 5788 participants were left for the analysis of the association between C-reactive protein serum levels and coronary events.

DNA was available for 6571 participants. Genotyping of all 3 polymorphisms was successful in 6007 participants. For 5584 of these participants, C-reactive protein serum levels were available. After

excluding participants with coronary heart disease at baseline, 5231 participants were left for the analysis of the association between C-reactive protein haplotypes and coronary events.

Data analysis

Linear regression was used to investigate the association between C-reactive protein serum levels and established cardiovascular risk factors. After log-transformation of C-reactive protein, the residuals were normally distributed with a constant variance.

Subsequently, participants with coronary heart disease at baseline were excluded, and Cox proportional hazards analysis was used to determine the relative risks of coronary heart disease and myocardial infarction associated with increasing C-reactive protein quartiles (cut-points 0.9, 1.8 and 3.5 mg/l). The proportional hazards assumption was tested by drawing log minus log plots of the survival function. We adjusted for age and sex (model 1), and subsequently for age, sex, body mass index, systolic blood pressure, diastolic blood pressure, total cholesterol, HDL-cholesterol, smoking, and diabetes mellitus (model 2).

Hardy-Weinberg equilibrium of the three C-reactive protein gene polymorphisms was tested using a Chi square test. Differences in serum C-reactive protein levels (log transformed) and established cardiovascular risk factors for the three polymorphisms were examined by using analysis of covariance, adjusting for age and sex, categorizing the participants by their genotypes. We used the Bonferroni correction to account for multiple testing (three genotypes). All the above analyses were performed by using SPSS 11.0 for Windows.

To test the associations of C-reactive protein gene haplotypes with cardiovascular risk factors, we used the program Haplo Stats (http://cran.r-project.org/src/contrib/Descriptions/haplo.stats.html).[18,19,21] The probability for each haplotype pair in each individual was assigned and then an individual's phenotype was directly modeled as a function of each inferred haplotype pair, weighed by their estimated probability, to account for haplotype ambiguity. The haplo.score function of Haplo Stats was used to test the associations. Details on the background and theory of score statistics can be found in Schaid et al.[21] We adjusted for age and sex and we computed global simulation P-values and simulation P-values for each haplotype. The number of simulations was set as 1000.

Since haplo.score does not provide the magnitude of the effect of each haplotype, the association between C-reactive protein gene haplotypes and C-reactive protein serum level, coronary heart disease and myocardial infarction was investigated by using the haplo.glm function of Haplo Stats.[19] This approach is based on a generalized linear model, and computes the regression of a trait on haplotypes and other covariates. For the analysis regarding the disease outcomes, the haplotype that was found to be associated with the lowest serum C-reactive protein levels served as the reference category. First, we adjusted for age and sex, and second, we additionally adjusted for body mass index, systolic blood pressure, diastolic blood pressure, total cholesterol, HDL-cholesterol, smoking, and diabetes mellitus. Haplo.em, haplo.score and haplo.glm were all implemented in the Haplo Stats software using the R language.

Values for cardiovascular covariates were missing in less than 4% of participants. These missing values were handled by single imputation using the expectation-maximization algorithm in SPSS 11.0. All tests were two-sided.

Results

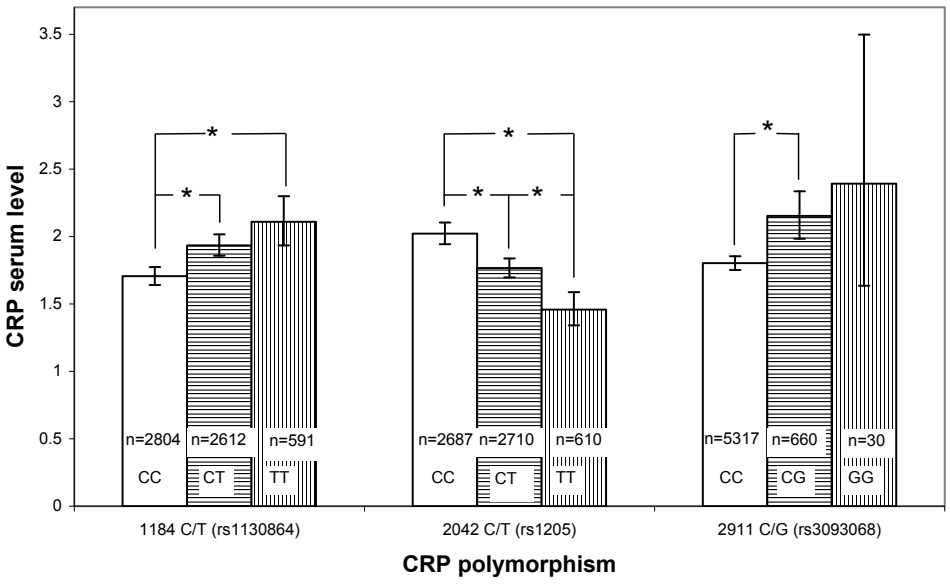
Table 1 shows baseline characteristics of the total cohort and their associations with C-reactive protein serum level. All studied characteristics, except for total cholesterol, were significantly associated with C-reactive protein serum level.

The mean follow-up time was 8.1 years (standard deviation 3.0 years). Among participants without history of coronary heart disease, 584 (8.8%) participants experienced incident coronary heart disease during follow-up, including 224 myocardial infarctions. Hazard ratios for coronary heart disease and myocardial infarction increased significantly across quartiles of C-reactive protein (table 2). When we repeated the analysis without excluding participants with coronary heart disease at baseline, the results did not change materially.

Genotype distributions for the three haplotype tagging SNPs were in Hardy-Weinberg equilibrium. Both using the Seattle SNPs website and the HapMap website (http://www.hapmap.org), the SNPs were found to lie in one linkage disequilibrium block. The 1184 T allele was present in 31.6% of 12014 chromosomes, the 2042 T allele in 32.7% and the 2911 G allele in 6.0%. Figure 2 shows differences in serum C-reactive protein levels according to the genotype of the three C-reactive protein polymorphisms. For all three polymorphisms we observed an allele dose effect. No associations were present between genotypes and established cardiovascular risk factors (data not shown). Genotypes were not associated with coronary heart disease and myocardial infarction (data not shown).

Haplotype alleles were present in the following frequencies: haplotype 1 (C-T-C) in 32.8%; haplotype 2 (T-C-C) in 31.7%; haplotype 3 (C-C-C) in 29.5%, haplotype 4 (C-C-G) in 5.9%; and remaining haplotypes (T-C-G, T-T-C and C-T-G) in less than 0.001%.

Figure 2. Geometric means and 95% confidence intervals of serum levels of C-reactive protein (mg/l) for three C-reactive protein gene polymorphisms.



* P-value $<10^{-3}$.

Table 1. Baseline characteristics of the population and age- and sex-adjusted regression coefficients for cardiovascular risk factors, describing the increase in log C-reactive protein per unit increase in each risk factor.

Variable	Total (n=6658)	Regression coefficient (95% confidence interval)	P- value
Age (years)*	69.6±9.2	0.020 (0.017, 0.022)	10^{-45}
Women#	3970 (60%)	-0.137 (-0.188, -0.086)	10^{-7}
Body mass index (kg/m^2)	26.3±3.7	0.065 (0.058, 0.071)	10^{-79}
Systolic blood pressure (mm Hg)	139±22	0.005 (0.004, 0.006)	10^{-16}
Diastolic blood pressure (mm Hg)	74±12	0.003 (0.002, 0.006)	10^{-4}
Total cholesterol (mmol/l)	6.6±1.2	0.006 (-0.015, 0.027)	0.6
HDL-cholesterol (mmol/l)	1.3±0.4	-0.470 (-0.540, -0.399)	10^{-38}
Diabetes mellitus	704 (11%)	0.321 (0.239, 0.402)	10^{-14}
Smokers			
- Never	2289 (35%)		
- Current (vs never)	1479 (23%)	0.420 (0.347, 0.493)	10^{-29}
- Former (vs never)	2708 (42%)	0.100 (0.035, 0.164)	10^{-3}
History of myocardial infarction	783 (13%)	0.255 (0.176, 0.333)	10^{-10}

Categorical variables are expressed as count (percentage). Valid percentages may vary for some counts because of missings in the variables. Values of continuous variables are expressed as mean ± standard deviation.

* Adjusted for sex. # Adjusted for age

Table 2. Hazard ratios for coronary heart disease and myocardial infarction for quartiles of C-reactive protein in participants without history of coronary heart disease at baseline.

	Events/ participants	Hazard ratio (95% confidence interval)	
		Model 1	Model 2
Coronary heart disease			
Quartile 1 (<=0.9)	92/ 1450	1.0 (reference)	1.0 (reference)
Quartile 2 (>0.9-1.8)	133/ 1450	1.4 (1.1-1.8)	1.3 (1.0-1.7)
Quartile 3 (>1.8-3.5)	158/ 1446	1.7 (1.3-2.1)	1.5 (1.1-1.9)
Quartile 4 (>3.5)	201/ 1442	2.2 (1.7-2.8)	1.9 (1.5-2.4)
P for trend		<0.01	<0.01
Myocardial infarction			
Quartile 1 (<=0.9)	32/ 1450	1.0 (reference)	1.0 (reference)
Quartile 2 (>0.9-1.8)	62/ 1450	2.0 (1.3-3.0)	1.8 (1.1-2.7)
Quartile 3 (>1.8-3.5)	67/ 1446	2.2 (1.4-3.3)	1.8 (1.2-2.8)
Quartile 4 (>3.5)	63/ 1442	2.1 (1.4-3.2)	1.7 (1.1-2.7)
P for trend		0.07	0.20

Model 1: adjusted for age and sex.

Model 2: adjusted for age, sex, body mass index, systolic blood pressure, diastolic blood pressure, total cholesterol, HDL-cholesterol, smoking, and diabetes mellitus.

Table 3. Association of C-reactive protein haplotypes with C-reactive protein serum level.

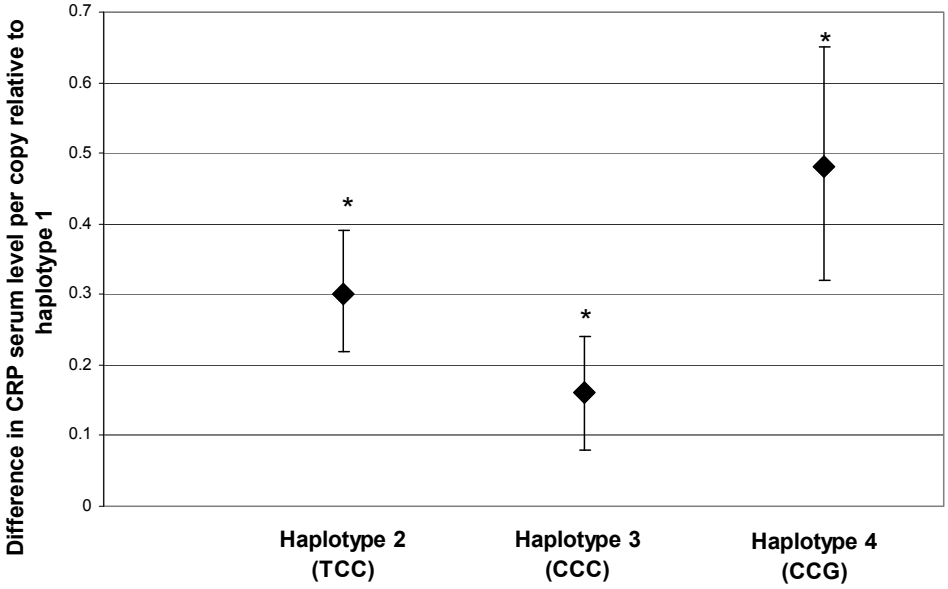
Variable	Coefficient	Standard error	t	P value
Intercept	-0.83	0.11	-7.56	10^{-14}
Age	0.02	0.002	12.51	$< 10^{-16}$
Female sex	-0.15	0.03	-5.45	10^{-7}
Haplotype 2 (T-C-C)	0.18	0.02	7.50	10^{-13}
Haplotype 3 (C-C-C)	0.10	0.02	3.99	10^{-4}
Haplotype 4 (C-C-G)	0.28	0.04	6.39	10^{-10}

t statistics and P values were calculated from the coefficients and standard errors within the best-fit multivariable model by the haplo.glm function of the Haplo Stats R package. Regression coefficients for each haplotype reflect the difference in mean ln(C-reactive protein) per copy relative to haplotype 1, the most frequent haplotype.

Table 4. Age- and sex-adjusted odds ratios for coronary heart disease and myocardial infarction for C-reactive protein haplotypes in participants without history of coronary heart disease.

	Odds ratio (95% confidence interval)	
	Coronary heart disease	Myocardial infarction
Haplotype 1 (C-T-C)	1.00 (reference)	1.00 (reference)
Haplotype 2 (T-C-C)	0.93 (0.79-1.10)	0.98 (0.76-1.25)
Haplotype 3 (C-C-C)	1.00 (0.85-1.17)	1.16 (0.91-1.48)
Haplotype 4 (C-C-G)	0.84 (0.63-1.13)	1.02 (0.67-1.56)

Figure 3. Relative effects of C-reactive protein gene haplotypes on C-reactive protein serum levels (mg/l).



* P-value $< 10^{-3}$. Regression coefficients were estimated with haplo.glm for each haplotype, adjusted for age and sex. Coefficients reflect difference in mean ln(C-reactive protein) per copy relative to haplotype 1, the most frequent haplotype. Values were transformed back to normal scale. Haplotypes 2, 3 and 4 were all significantly higher than haplotype 1.

Haplotype 4 was associated with lower BMI (p=0.03), haplotype 1 with higher systolic blood pressure (p=0.04) and haplotype 3 with higher percentage of prevalent myocardial infarction (p=0.04). No other associations with cardiovascular risk factors were present (data not shown). All haplotypes provided significantly higher C-reactive protein levels than haplotype 1 (table 3). The effect of haplotype on C-reactive protein level, calculated from the regression coefficients, is displayed in figure 3. Additional adjustment for body mass index, systolic blood pressure, diastolic blood pressure, total cholesterol, HDL-cholesterol, smoking, and diabetes mellitus did not materially change the results.

In table 4, age-and sex-adjusted odds ratios for coronary heart disease and for myocardial infarction are displayed for different C-reactive protein haplotypes. Since haplotype 1 was associated with the lowest serum C-reactive protein levels, it served as the reference. For both outcomes, the odds ratios for all haplotypes were around one. Additional adjustment for body mass index, systolic blood pressure, diastolic blood pressure, total cholesterol, HDL-cholesterol, smoking, and diabetes mellitus did not materially change the point estimates, and neither did repeating the analysis without excluding participants with coronary heart disease at baseline.

Discussion

In this population-based study, elevated C-reactive protein serum level was a strong and independent marker of increased risk of coronary heart disease and myocardial infarction in participants without a history of coronary heart disease. C-reactive protein haplotypes were associated with C-reactive protein serum levels. However, C-reactive protein haplotypes were not associated with coronary heart disease and myocardial infarction.

The approach we used in this study has also been termed "Mendelian randomization". This approach has been used recently in studies of C-reactive protein and hypertension and metabolic syndrome.[8,22] It deals with residual confounding, as alleles of the C-reactive protein gene that influence C-reactive protein level are transmitted from parent to offspring at random, and factors that could confound associations of C-reactive protein level with cardiovascular disease should be evenly distributed in those who do, and those who do not, have alleles that cause high C-reactive protein level. Furthermore, it deals with reverse causation, because genotype is determined before onset of disease.[23]

The present study uses haplotypes describing the total common variation of the C-reactive protein gene. So far, two other studies have used this approach. First, Carlson et al.[14] defined all common genetic variation across the C-reactive protein gene region by resequencing the region in a multiethnic variation discovery panel (24 African Americans and 23 European Americans), and selected SNPs for genotyping in a larger panel (CARDIA study), in which associations between common haplotypes and C-reactive protein levels were investigated. Carlson et al. used a population that was partly of European descent and partly of African descent, and used all haplotypes that occur in these populations. We used a population of European descent; therefore, the studies are not strictly comparable. In approximation, Carlson et al.'s haplotypes 4, 5 and 7 concur with our haplotypes 3, 2 and 4, respectively, and Carlson et al.'s haplotype 1 and 2 taken together concur with our haplotype 1. Remaining haplotypes in the Carlson et al. study were only present in African Americans. Carlson et al. found

that their haplotypes 5 and 7 were associated with high C-reactive protein levels, haplotype 1 and 2 with the lowest levels, and haplotype 4 with intermediate levels. These results are in agreement with ours. Furthermore, Carlson et al. did a promoter transcriptional analysis of the C-reactive protein gene, which suggested that the C-reactive protein haplotype-phenotype associations are at least partially attributable to functional changes at promoter sites rs3093062 (SNP 1421 in their paper) and rs3091244 (SNP 1440 in their paper).

Secondly, Miller et al. resequenced 192 individuals to ascertain a comprehensive set of common variants in the C-reactive protein gene, studied their association with C-reactive protein level in (subsets of) three cohorts, and also studied their association with myocardial infarction or ischemic stroke in a nested case-control study within the Physicians' Health Study cohort.[15] Interestingly, after resequencing this large number of individuals, Miller et al. found a haplotype pattern similar to the pattern of SeattleSNPs. Miller et al's haplotypes 1, 2 and 5 concur with haplotypes 3, 2 and 4 in our study, respectively. There were only two minor differences: Miller et al's haplotypes 3 and 4 together constitute our haplotype 1, and we did not determine Miller et al's haplotype 6, but the mean frequency of this haplotype was only 2.1%.[15] Miller et al's haplotypes 2 and 5 were associated with higher C-reactive protein levels and haplotypes 3 and 4 with lower C-reactive protein levels; these results are again in agreement with ours. Also, Miller et al. found that the minor allele of SNP rs2794521 was associated with reduced risk of atherothrombotic events. However, this SNP was associated with higher C-reactive protein level, so the association between the C-reactive protein variant and baseline C-reactive protein did not correlate with the effect of this variant on clinical cardiovascular events. We did not determine this SNP in our study. Our study has the advantage that we had data available on all cases and non-cases in a large, population-based cohort.

Remaining studies on C-reactive protein gene haplotypes and risk of cardiovascular events have mostly been conducted in smaller numbers of high-risk patients.[24-26] The haplotypes used in these studies have been constructed without consideration of the patterns of variation across the locus as a whole. Remaining studies that have examined the association of C-reactive protein gene haplotypes with C-reactive protein levels[6,7,12,26,27] are not comparable to our study, because of different polymorphisms used to reconstruct the haplotypes and different populations used in terms of health status, ethnicity or age. The results of these studies are diverse, some finding associations with C-reactive protein levels, and some not. Interesting to note is the finding of Szalai et al.,[27] that haplotypes reconstructed from the -409G/A (rs3093032) and –390C/T/A (rs3091244) C-reactive protein gene promoter polymorphisms affect transcription factor binding, alter transcriptional activity, and associate with differences in baseline serum C-reactive protein level. According to SeattleSNPs, the latter polymorphism is present in all participants with haplotypes 2 and 4 in our study, and therefore it may result in functional differences between the haplotypes in our study, leading to different serum C-reactive protein levels. Several, mostly smaller, studies have demonstrated associations between various C-reactive protein SNPs and C-reactive protein levels.[3-13] These studies were different in design and were conducted in various populations, and are therefore not similar to ours.

In this study, we found an independent association between serum C-reactive protein levels and coronary heart disease. Since our earlier report on the role of C-reactive protein in prediction of myocardial infarction in the Rotterdam Study, which was then investigated by means of a nested case-control study,[28] data from 4 more years of follow-up have become available and C-reactive protein has

been determined in the total cohort. This may in part explain the difference with the previous results that showed a lack of association after multivariable adjustment.

An issue that warrants consideration in this study is that C-reactive protein measurements were lacking for 854 participants who did not visit the research center at baseline. These participants generally had a higher age and worse general health. However, the association that we found between C-reactive protein serum level and coronary heart disease is in line with the results from previous studies,[1] and this suggests that although we cannot entirely exclude the presence of selection bias, it is not likely that it has substantially influenced the results.

Although serum C-reactive protein levels were found to influence risk of coronary heart disease and C-reactive protein gene haplotypes were found to influence steady state serum C-reactive protein levels, no association could be demonstrated between C-reactive protein haplotypes and coronary heart disease. Power calculation for the present study shows that, with a power of 80% and an alpha of 0.05, in reference to haplotype 1, (the most common haplotype, frequency 32.8%), we were able to demonstrate relative risks for coronary heart disease of at least 1.21 (for haplotype 2, frequency 31.7%).[29] Therefore, either there indeed is no association between C-reactive protein gene haplotypes and coronary heart disease, or, otherwise, the relative risk is of relatively small magnitude. Application of the instrumental variables approach[22] to our data is in compliance with the latter; the expected relative risks of coronary heart disease for haplotypes 2, 3 and 4, as estimated from the association between C-reactive protein serum level with coronary heart disease and the association between haplotypes and C-reactive protein serum level, were 1.03 (95% CI 0.90-1.20), 1.02 (95% CI 0.88-1.18) and 1.05 (95% CI 0.82-1.36), respectively, as compared to haplotype 1. We were not able to demonstrate estimates of such small magnitude in the present study. Therefore, the door may still be open for a pathophysiological role of C-reactive protein in the development of cardiovascular disease.

Another explanation of the absence of an association in the present study is that baseline C-reactive protein levels are not solely determined by the variation in the C-reactive protein gene, but also by its interaction with several transcription factors induced by regulatory cytokines such as IL-6 and IL-1β,[30] which may have a larger influence on serum C-reactive protein levels. Furthermore, high C-reactive protein levels may exert their harmful effects in the acute phase of a coronary event, with high peak C-reactive protein levels leading to enhanced infarct size and more complications such as arrhythmias.[31] Since high C-reactive protein responders may not necessarily have high baseline serum C-reactive protein levels, this aspect needs to be studied by means of a study design different from ours.

In conclusion, this study confirms that steady-state C-reactive protein serum level is predictive of coronary heart disease. Furthermore, it demonstrates that steady-state C-reactive protein serum level is influenced by C-reactive protein haplotypes. Although elevated C-reactive protein level has lately been found to be a consistent and relatively strong risk factor for cardiovascular disease, our study does not support that the common variation in the C-reactive protein gene has a large effect on the occurrence of coronary heart disease.

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Chapter 2.2

C-reactive protein level and measures of coronary and extracoronary atherosclerosis

Abstract

Aims. Although prospective studies have unequivocally shown that C-reactive protein (CRP) is an independent predictor of future cardiovascular events, studies on the association between CRP and atherosclerosis have provided inconsistent results. We investigated the association of CRP with extent and progression of atherosclerosis in multiple vessel beds in a large, population-based cohort study.
Methods. In the Rotterdam Study, standardized measurements of coronary and extra-coronary atherosclerosis were performed in 1962 persons and 6582 persons, respectively. Progression of extra-coronary atherosclerosis during a mean follow-up period of 6.4 years was assessed in 3757 persons.
Results. Independent and graded associations were found of CRP with the number of carotid plaques and carotid plaque progression (OR 1.72; 95% C.I. 1.14-2.59) for severe progression in participants with CRP > 3 mg/dl versus participants with CRP< 1 mg/dl). Similarly, CRP showed an independent and graded association with ankle-arm index (AAI) and worsening AAI over the years (OR 1.99; 95% C.I. 1.37-2.88) for severe progression in participants with CRP > 3 mg/dl versus participants with CRP< 1 mg/dl). Although CRP was independently related to the highest level of carotid intima-media thickness (IMT), the association with change in IMT was not significant. Furthermore, there was an independent, graded relation between CRP and aortic calcification, but no independent association was observed with progression of aortic calcification, nor with the amount of coronary calcification.
Conclusion. In this population-based study, independent and graded associations were present of CRP with extent and progression of carotid plaques and AAI, while associations with carotid IMT and aortic and coronary calcification were less pronounced.

Introduction

Multiple prospective studies have unequivocally shown that C-reactive protein (CRP) is an independent predictor of future cardiovascular events including myocardial infarction, stroke and peripheral vascular disease.[1-3] Studies published on the association between CRP and established measures of atherosclerosis, however, have provided inconsistent results. In some studies the association was independently present in the whole study population,[2,4-11] in women only[12] or in men only.[13-16] Other studies found a positive association that was lost after adjustment for cardiovascular risk factors.[13,17-19] Several studies did not find any relation between CRP and atherosclerosis.[20,21]

Studies performed thus far have several limitations. Firstly, most studies are cross-sectional rather than longitudinal while inflammation is thought to play an important role in the progression of atherosclerosis.[22] Secondly, some studies measured artery calcification which is considered to represent stable atherosclerosis, or increased intima-media thickness (IMT) which is thought to reflect early atherosclerosis,[23] stages in which CRP may play a less important role.[22,24] Thirdly, most studies did not look at CRP in relation to graded levels of atherosclerosis. Finally, many studies had selected or small study populations and most studies examined one vessel bed only.

The Rotterdam Study is a large, prospective, population-based cohort study with standardized measurements of atherosclerosis. We studied the association of CRP with extent and progression of atherosclerosis in multiple vessel beds. We were able to substantially increase the number of subjects available for analysis compared to earlier work on this topic in a limited random sample of the Rotterdam Study,[6] thus increasing precision leading to stronger conclusions.

Methods

Study population

The Rotterdam Study is a prospective, population-based cohort study including 7983 men and women ≥55 years of age (response rate 78%). A detailed description of the rationale and design of the Rotterdam Study has been given elsewhere.[25]

Between visit 1 (1990 to 1993) and 3 (1997 to 1999), 1859 (23.3%) participants died and 135 (1.7%) were lost to follow-up or not invited due to logistic reasons. Furthermore, 1193 (14.9%) persons did not participate in the third visit. At the third visit, 2,063 subjects (response rate 61%) underwent an electron beam CT scan (EBT).[26] The Medical Ethics Committee of the Erasmus Medical Center Rotterdam approved the study, and all participants gave informed consent.

Measurement of CRP

At visits 1 and 3, C-reactive protein was measured in serum using a nephelometric method (Immage; Beckman Coulter). This technique has a within-run precision <5.0%, a total precision <7.5%, and a reliability coefficient of 0.995. The serum was kept frozen at –20°C (1990-1993) or –80°C (1997-1999).

Measures of extent of atherosclerosis

Ultrasonography of both carotid arteries was performed with a 7.5-MHz linear-array transducer and a duplex scanner (ATL UltraMark IV, Advanced Technology Laboratories). The common carotid artery, carotid bifurcation, and internal carotid artery were examined on both the left and right sides for the presence of a plaque. Per location a point was added to a plaque score when this location showed the presence of atherosclerotic plaque. Thus, a total plaque score between 0 and 6 was obtained for each participant.[27] The categories of 0-6 plaques comprised 40.4%, 15.2%, 18.3%, 8.9%, 9.9%, 3.7%, 3.6% of the study population, respectively.

Common carotid IMT was determined as the average of near- and far-wall measurements, and the average of left and right common carotid IMT was computed.[28] For the analyses, IMT was categorized into deciles (cut-off values (mm): 0.62, 0.66, 0.70, 0.74, 0.78, 0.81, 0.86, 0.91, 1.00).

Aortic calcification was diagnosed by radiographic detection of calcified deposits in the abdominal aorta on a lateral abdominal film. Calcification of the posterior abdominal aortic wall was scored according to the length of the involved area along the lumbar spine (L1 to L4) with scores of 0 to 5 corresponding to 0, ≤1, 1.1 to 2.4, 2.5 to 4.9, ≥5.0 to 9.9 and ≥10 cm, respectively. The categories of 0-5 plaques comprised 33.1%, 9.4%, 26.9%, 17.7%, 10.6%, 2.2% of the study population, respectively.

The ratio of the systolic blood pressure at the ankle (8-MHz continuous-wave Doppler probe (Huntleigh 500D, Huntleigh Technology)) to the systolic blood pressure at the arm (random-zero sphygmomanometer) was computed to obtain the ankle-arm index (AAI).[4] Because arterial rigidity prevents arterial compression leading to spuriously high values of the AAI, an AAI >1.50 was considered invalid.[29] For the analyses, we used the leg with the lowest value of AAI and categorized AAI into deciles (cut-off values: 1.29, 1.23, 1.18, 1.13, 1.09, 1.05, 0.99, 0.91, 0.76).

Coronary calcifications in the epicardial coronary arteries were detected on EBT scans. Imaging was performed with a C-150 Imatron scanner (GE-Imatron) as described previously.[26] Calcium scores were calculated according to Agatston's method[30] and divided into 5 categories: 0-10, >10-100, >100-500, >500-1000, >1000. The median duration between visit 3 and EBT scanning was 50 days.

Measures of progression of atherosclerosis

Progression of atherosclerosis was computed for each of the 4 extra-coronary measures by subtracting the extent of atherosclerosis at visit 1 from the extent at visit 3. We categorized this new variable into no, mild, moderate and severe progression. To ensure comparable categories between the different measures of atherosclerotic progression (i.e. progression of aortic calcification, carotid plaques, IMT and AAI), we chose cut-off points for each variable that resulted in more or less the same number of subjects in that category (i.e. no, mild, moderate or severe progression) for all progression variables. We chose to categorize the progression variables instead of analyzing the data in a continuous way because we expected a non-linear relation with CRP, assuming the strongest relation with severe progression.[22,23]

For progression of aortic calcification, baseline and follow-up X-ray films were examined in pairs. We defined no, mild, moderate, and severe progression of aortic calcification as a progression 0, ≤1, 1.1 to 2.4, and ≥2.5 cm of aortic calcification along the lumbar spine. None of the participants showed a decrease in the extent of aortic calcification. We defined no, mild, moderate, and severe progression of carotid plaques as an increase of 0, 1, 2, or ≥3 plaque locations, respectively. Participants with a de-

crease in plaque score were added to the group with no progression. We based the categories of no, mild, moderate and severe progression of the continuous variables IMT and AAI (leg with largest decrease) on the 30th, 60th, and 90th percentile of the sample distribution. The mean interval between extra-coronary measurements at visits 1 and 3 was 6.4±0.4 years.

Assessment of covariates

At visits 1 and 3, covariates were ascertained using standard procedures as described previously.[26,28] Diabetes mellitus was considered to be present when fasting blood glucose exceeded 7.0 mmol/L, non-fasting glucose exceeded 11.0 mmol/L and/or anti-diabetic medication was used. History of cardiovascular disease included history of myocardial infarction, stroke or presence of peripheral artery disease according to the Rose criteria.[31]

Populations for analyses

The following participants were included: (A) 6582 persons in whom CRP and at least one measure of extra-coronary atherosclerosis was assessed at visit 1. Within this group, measurements of carotid plaques, IMT, aortic atherosclerosis, and AAI were available for 5267, 4385, 5429 and 5959 participants, respectively. (B) 3757 participants in whom information on CRP at visit 1 and at least one measure of atherosclerosis at visit 1 and visit 3 were available. Within this group, information on progression of carotid plaques, IMT, aortic calcification and AAI was available for 2661, 2301, 2565 and 3298 participants, respectively. (C) 1962 participants of visit 3 in whom both CRP and a coronary calcification score were obtained.

Statistical analyses

We performed analyses of variance to compute geometric means of CRP for categories of atherosclerosis and used a Student *t* test to compare categories against the reference group (lowest amount of atherosclerosis). Linear regression analysis was used as a test for trend. Because the distribution of CRP was highly skewed, log CRP was used for analysis of variance and linear regression analysis. Outliers (values > 3*SD of the population distribution of log CRP; study population A: n=25, B: n=4, C: n=6) were excluded.

Using multinomial logistic regression analysis, we examined the association of CRP measured at visit 1 with mild, moderate, and severe progression of atherosclerosis. CRP was divided in categories of <1 mg/L, 1 to 3 mg/L and > 3 mg/L.[32] These categories comprised 34%, 42% and 24% of the study population, respectively. CRP<1 mg/L served as the reference category. Participants who already had the maximum number of carotid plaques at visit 1 were excluded because of lacking ability of progression (n=52).

In all analyses, we used two models. In model 1, analyses were adjusted for age and sex. In model 2, we additionally adjusted for smoking status and number of pack-years, systolic and diastolic blood pressure, antihypertensive medication, total cholesterol, high-density lipoprotein (HDL) cholesterol, cholesterol-lowering medication, body-mass-index (BMI), hormone replacement therapy (for women), cardiovascular history and, in case of analyses of progression, duration of follow-up and baseline level of atherosclerosis. To test whether the relation between CRP and extent or progression of atherosclerosis was different for men and women, we added an interaction term to the regression model

(atherosclerosis measure x gender for the linear regression, CRP x gender for the multinomial regression). If the interaction term was statistically significant ($P<0.05$), we conducted analyses for men and women separately.

Missing data on covariates were imputed by single imputation using the Expectation Maximization (EM) algorithm. Values for cardiovascular risk factors were missing mainly due to logistic reasons (e.g. absence of sonographers). Analyses were performed with SPSS 11.0 for Windows (SPSS Inc.)

Results

Table 1 displays the baseline characteristics of the 3 study populations. Baseline characteristics of the populations with measurements of extra-coronary extent and progression of atherosclerosis were assessed at visit 1 (1990-1993) while characteristics of the population with coronary calcification measurement were assessed at visit 3 (1997-1999).

Geometric mean CRP levels (mg/L) for categories of measures of coronary and extra-coronary atherosclerosis are presented in Figure 1. After adjusting for age and sex (model 1), a strong and graded increase in CRP level was observed with increasing number of carotid plaques, whereas CRP levels only rose across the highest 3 deciles of carotid IMT, corresponding to an IMT above 0.86 mm. A graded increase of CRP levels was also seen across incremental categories of aortic calcification. Furthermore, CRP levels gradually rose with decreasing AAI, but this was most pronounced in the highest 2 deciles, corresponding to an AAI below 0.91. With increasing level of coronary artery calcification, a gradual rise in CRP was observed, but this was only modest compared to the extra-coronary measures. After multivariable adjustment (model 2), the association between CRP level and extent of atherosclerosis was attenuated in the extra-coronary measures but remained statistically significant for the highest levels of atherosclerosis. No independent association was found between CRP and coronary artery calcification. Tests for trend were all significant except for coronary calcification after multivariable adjustment (Fig.1).

Since the interaction term of gender x atherosclerosis level was statistically significant for carotid IMT and AAI, we analyzed the relation of CRP with IMT and AAI for men and women separately. For both measures, the association between CRP and extent of atherosclerosis showed a similar pattern compared to the overall results on IMT and AAI, but was stronger in men than in women (Fig. 2). The tests for trend were all significant except for IMT of women after multivariable adjustment: (beta's ; 95% C.I.) for model 2 were (0,03 ; 0,01 to 0,05) for men and (0,01 ; -0,01 to 0,03) for women and for AAI (0,04 ; 0.03 to 0,06) for men and (0,02 ; 0,01 to 0,03) for women (Fig.2).

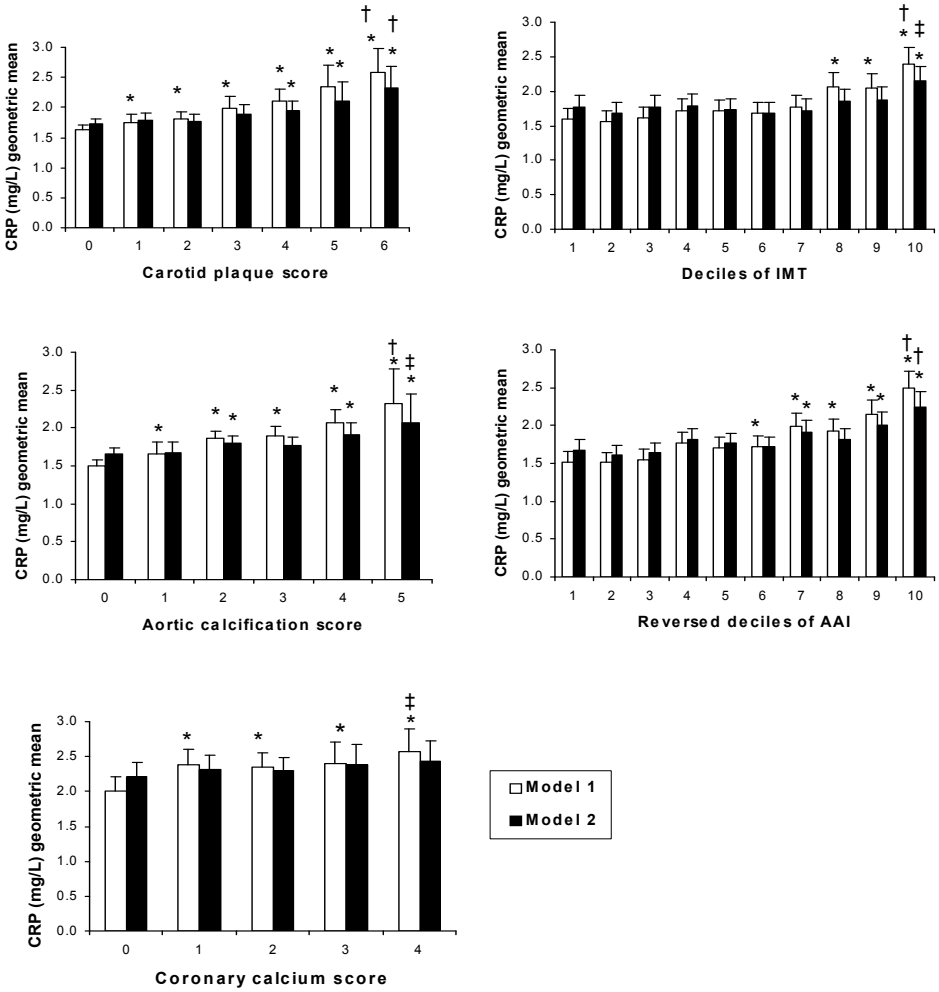
Table 2 shows the odds ratios for risk of mild, moderate and severe progression of atherosclerosis in comparison to no progression for categories of CRP level. CRP levels >3 mg/L were independently related to severe carotid plaque progression and severe decrease in AAI over time. The association with progression of aortic calcification was weaker and there was no clear relation between CRP and change in carotid IMT. Risk estimates did not change when baseline level of atherosclerosis was not included in the model.

Table 1. Baseline characteristics of the study populations

	Visit 1		Visit 3
Variable	**Extra-coronary atherosclerosis (n=6582)**	**Progression of atherosclerosis (n=3757)**	**Coronary atherosclerosis (n=1962)**
Age, y	69.5±9.2	65.7±6.9	71.3±5.7
Male, %	40.6	42.5	46.6
Body-mass-index, kg/m^2	26.3±3.7	26.2±3.5	27.0±3.9
Systolic blood pressure, mmHg	139±22	136±21	143±21
Diastolic blood pressure, mmHg	74±12	74±11	76±11
Total cholesterol, mmol/L	6.6±1.2	6.7±1.2	5.7±1.0
HDL cholesterol, mmol/L	1.3±0.4	1.4±0.4	1.3±0.4
Smokers, %			
- Current	22.9	21.5	16.0
- Past	42.0	45.2	54.4
Pack-years smokers	22.5 (9.0, 40.0)	21.5 (9.0, 37.5)	18.5 (8.0, 32.2)
Diabetes mellitus, %	11.0	7.2	12.1
Cardiovascular history*, %	16.1	11.2	17.0
Hypertension medication, %	17.8	16.2	28.7
Cholesterol-lowering medication, %	3.3	4.2	19.2
Hormone-replacement therapy, %	15.6	18.8	20.7
Carotid plaque score > 0, %	59.6	42.4	-
Intima-media-thickness, mm	0.80±0.16	0.76±0.14	-
Aortic plaque score > 0, %	66.9	69.2	-
Ankle-arm index	1.05±0.23	1.11±0.18	-
Coronary calcification score > 0, %	-	-	77.0
CRP, mg/L	1.87 (0.91, 3.64)	1.62 (0.79, 3.06)	2.44 (1.26, 4.45)

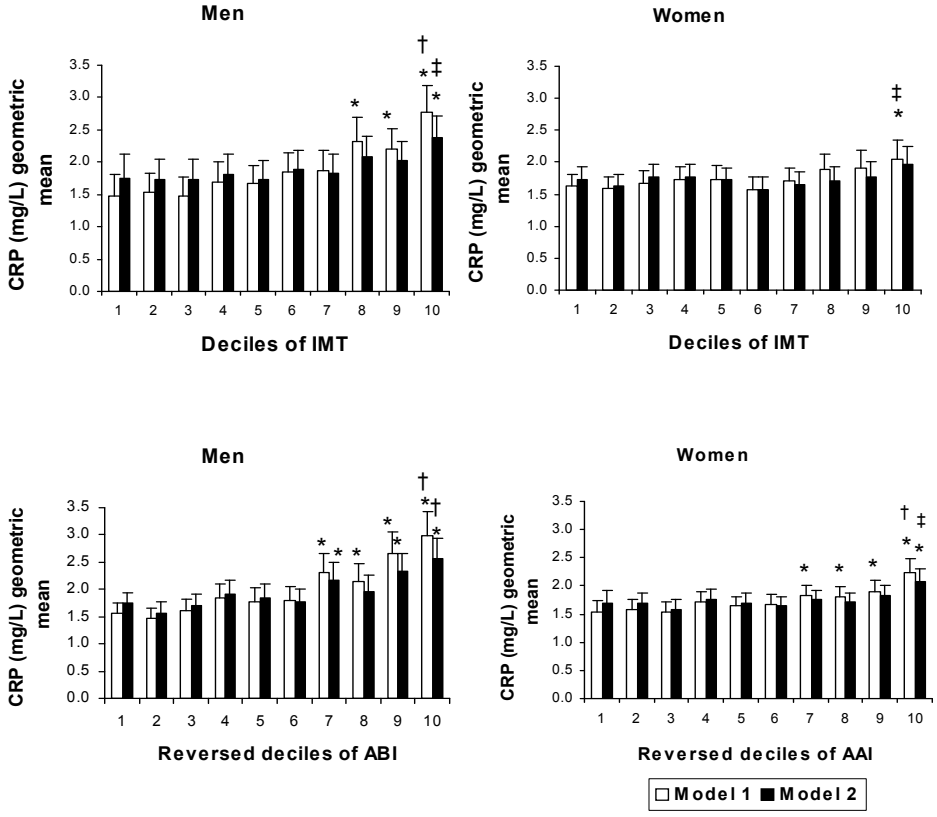
Categorical variables are presented as percentage. Continuous values are expressed as mean ± standard deviation. Median (25th, 75th percentiles) is presented in case of skewed distribution. *History of cardiovascular disease includes history of myocardial infarction, stroke and/or peripheral artery disease.

Figure 1. Geometric mean C-reactive protein level (mg/L) for categories of carotid plaque, intima-media thickness (IMT), aortic calcification, ankle-arm index (AAI) and coronary calcification.



Model 1: age and sex adjusted. Model 2: additionally adjusted for BMI, systolic and diastolic blood pressure, antihypertensive medication, total cholesterol, HDL cholesterol, cholesterol-lowering medication, smoking status, number of pack-years, serum glucose, anti-diabetic medication, hormone replacement therapy (for women) and cardiovascular history. *Significantly higher geometric mean C-reactive protein level as compared to the reference category (no atherosclerosis) ($p<0.05$). † p for trend <0.001. ‡ p for trend <0.05.

Figure 2. Geometric mean CRP level (mg/L) for deciles of intima-media thickness (IMT) and ankle-arm index (AAI) in men and women.



Model 1: age and sex adjusted. Model 2: additionally adjusted for BMI, systolic and diastolic blood pressure, antihypertensive medication, total cholesterol, HDL cholesterol, cholesterol-lowering medication, smoking status, number of pack-years, serum glucose, anti-diabetic medication, hormone replacement therapy (for women) and cardiovascular history. *Significantly higher geometric mean C-reactive level as compared to the reference category (no atherosclerosis) ($p<0.05$). † p for trend <0.001. ‡ p for trend <0.05.

Table 2. C-reactive protein in relation to progression of atherosclerosis during 6.4 years of follow-up

Degree of progression		Odds ratios (95% CI) Model 1			Odds ratios (95% CI) Model 2		
	N	CRP (mg/L)			CRP (mg/L)		
Carotid plaques		<1	1-3	>3	<1	1-3	>3
Mild	581	1.0	1.06 (0.84-1.33)	1.29 (1.00-1.67)	1.0	1.01 (0.80-1.27)	1.24 (0.94-1.63)
Moderate	318	1.0	1.14 (0.86-1.51)	1.10 (0.79-1.54)	1.0	1.03 (0.77-1.38)	1.02 (0.71-1.46)
Severe	229	1.0	1.36 (0.96-1.93)	1.99 (1.37-2.90)	1.0	1.12 (0.78-1.62)	1.72 (1.14-2.59)
Carotid IMT							
Mild	690	1.0	1.31 (1.03-1.68)	1.25 (0.94-1.66)	1.0	1.35 (1.04-1.75)	1.38 (1.01-1.88)
Moderate	690	1.0	1.07 (0.84-1.36)	1.06 (0.80-1.40)	1.0	1.01 (0.78-1.31)	1.05 (0.77-1.44)
Severe	229	1.0	1.21 (0.85-1.73)	1.41 (0.95-2.10)	1.0	0.99 (0.68-1.45)	1.16 (0.74-1.79)
Aortic calcification							
Mild	867	1.0	0.95 (0.76-1.19)	1.06 (0.82-1.38)	1.0	0.88 (0.69-1.12)	0.92 (0.68-1.24)
Moderate	731	1.0	1.13 (0.89-1.43)	1.37 (1.05-1.81)	1.0	1.01 (0.78-1.32)	1.14 (0.83-1.57)
Severe	174	1.0	1.38 (0.93-2.04)	1.36 (0.86-2.15)	1.0	1.35 (0.88-2.05)	1.37 (0.82-2.29)
AAI							
Mild	989	1.0	0.94 (0.76-1.15)	0.98 (0.78-1.24)	1.0	1.07 (0.86-1.33)	1.29 (0.99-1.69)
Moderate	989	1.0	0.94 (0.76-1.15)	0.96 (0.76-1.22)	1.0	1.07 (0.85-1.36)	1.32 (1.00-1.75)
Severe	329	1.0	1.04 (0.77-1.42)	1.42 (1.02-1.98)	1.0	1.21 (0.87-1.68)	1.99 (1.37-2.88)

Risk estimates are odds ratios (95% confidence intervals) for categories of high C-reactive protein. The category of C-reactive protein < 1 mg/L serves as a reference. Categories of no progression for carotid plaque, intima-media thickness (IMT), aortic calcification and ankle-arm index (AAI) comprise 1480, 689, 789 and 988 persons, respectively. Model 1: age and sex adjusted. Model 2: additionally adjusted for BMI, systolic and diastolic blood pressure, antihypertensive medication, total cholesterol, HDL-cholesterol, cholesterol-lowering medication, smoking status, number of pack-years, serum glucose, anti-diabetic medication, hormone replacement therapy (for women), cardiovascular history, extent of baseline atherosclerosis and follow-up time.

Discussion

We found an independent, graded association of CRP with extent and progression of carotid plaques and AAI. CRP was independently related to the highest level of carotid IMT, while the association with change in IMT was not significant. Although there was an independent, graded relation between CRP and aortic calcification, no independent association was found with progression of aortic calcification, nor with the amount of coronary calcification.

Relation of CRP with measures of atherosclerosis

Inflammation plays a role in all stages of atherosclerosis. In early stages it is involved in the formation and progression of atherosclerotic lesions in the intima of the arterial wall. In more advanced stages, inflammatory processes become more pronounced and are thought to play an important role in plaque vulnerability, rapid progression of plaques, and thrombotic complications.[22,33] We found no independent association between CRP and coronary calcification. Research on coronary plaque morphology shows that stable plaques are most often calcified, while vulnerable plaques are typically not.[34] The lack of an independent relation between CRP and coronary calcification, a feature of plaque stability, is in line with the finding that CRP plays an important role in plaque vulnerability.[24,35]

In our study, CRP was only related to the highest levels of IMT. This agrees with the view that intima-media-thickness represents early stages of atherosclerosis.[23]

With regard to carotid plaques and AAI, we found that CRP was most strongly related to advanced stages of atherosclerosis and to more severe categories of progression. This is in accordance with the view that the role of inflammation is more pronounced in advanced stages of atherosclerosis.

In the gender specific analyses, we observed a stronger relation of CRP with carotid IMT and AAI in men as compared to women. Whereas the difference could be at least partly explained by the fact that women had lower IMT levels than men, AAI levels were comparable between both sexes (data not shown).

Results of prior population-based studies

Only a few large population-based studies focused on CRP in relation to quantity of carotid plaque. An independent relation was found between CRP and grade of carotid artery stenosis [12] and between CRP and amount of carotid plaque.[14] The latter study found an association in men only. A Danish population-based study did not show an independent relation between CRP and number of carotid plaques.[21]

One population-based study investigated the relation between CRP and levels of carotid IMT and found an independent association between CRP and the highest decile of IMT.[21] Large population-based studies on the relation between quartiles of CRP and carotid IMT found associations that were lost after adjustment for cardiovascular risk factors.[8,12-14,19] One of these studies showed an independent relation between CRP and internal but not common carotid IMT.[12]

Studies on the relation between CRP and levels of AAI are scarce. Folsom et al. showed an independent, inverse relation between CRP and AAI in men only.[13] However, several large population-based studies showed an independent association between CRP and presence of peripheral artery disease (AAI<0.9).[7,9,10]

The only large, population-based study on the relation between CRP and amount of coronary calcification did not find an independent association.[18]

Relatively few studies have been performed on the association between CRP and progression of atherosclerosis. In a population-based study on carotid artery plaque progression, CRP was only related to early stages of plaque progression after 5 years, but not to advanced stages of progression. However, the number of participants with advanced progression was limited.[11] A large study on carotid IMT progression over a period of 2 years showed an association between CRP and IMT progression that was lost after adjustment for cardiovascular risk factors.[17] A study on the effect of CRP on decrease in AAI showed an independent association of borderline significance after 5 years and a clear independent association after 12 years.[5] Furthermore, a previous study on the relation between CRP and progression of atherosclerosis among a subgroup of 773 participants of the Rotterdam Study showed an independent relation between CRP and progression of carotid plaques, but not with change in aortic calcification and AAI over a mean period of 6.4 years.[6]

In summary, population-based studies on the relation between CRP and quantity of atherosclerosis, although limited in number, generally support independent relations with extent and progression of carotid plaques and AAI. Furthermore, CRP is found to be associated with the highest level of carotid IMT, but not with progression of IMT, or with the amount of coronary calcification. Our findings are generally in line with these studies.

Strengths and limitations

This is the largest population-based study to date in which the relation of CRP with extent and progression of atherosclerosis is examined in multiple vessel beds. However, some methodological issues need to be addressed. Firstly, analyses were conducted among different study populations. In analyses of progression of atherosclerosis, responders were younger (mean age difference 8.8 years), consisted of a higher percentage of men (42.5% versus 38.2%) and generally had lower levels of cardiovascular risk factors compared to the non-responders. Although the lower amount of cardiovascular risk factors may have somewhat limited the range of baseline levels of atherosclerosis, it is unlikely that it has affected the validity of the risk estimates. The population with measurements of coronary calcification consisted of 61% of the eligibles. A difference between responders and non-responders was found in the percentage of men (46.6% versus 37.8%), however no differences were present in levels of cardiovascular risk factors.[26]

Secondly, we attributed differences in the association between CRP and atherosclerosis to differences in applied measures of atherosclerosis, based on pathophysiological views as described above. However, we cannot exclude possible effects of differences in vessel beds.

Finally, in the study on progression we found the strongest relation between CRP and severe progression of atherosclerosis. However, categories of mild and moderate progression are most susceptible to misclassification. We cannot rule out that associations of CRP with these categories maybe diluted by misclassification.

Conclusion

This population-based study shows graded associations of CRP with extent and progression of atherosclerosis. However, the strength of the associations depends on the applied measure of athero-

sclerosis. We found an independent, graded association between CRP and extent and progression of carotid plaques and AAI. Furthermore, CRP was independently related to the highest level of carotid IMT, while the association with change in IMT was not significant. Although there was an independent, graded relation between CRP and aortic calcification, no independent association was found with progression of aortic calcification, nor with the amount of coronary calcification. Our findings are generally supported by previous studies on the relation between CRP and quantity of atherosclerosis. The inconsistency in the literature on the relation between CRP and atherosclerosis may, at least partly, be explained by differences in applied measures of atherosclerosis and lack of quantification.

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Chapter 2.3

A common polymorphism in the complement factor H gene and myocardial infarction

Abstract

Objectives. This study was designed to investigate the association between a common polymorphism (Tyr402His, rs1061170) in the complement factor H (CFH) gene and risk of coronary heart disease.

Background. The evidence that inflammation is an important mechanism in atherogenesis is growing. C- reactive protein (CRP), complement factors and complement regulatory factors have all been linked to coronary heart disease. The CFH gene is an important regulator of the alternative complement cascade. We investigated its association with coronary heart disease.

Methods. The study was embedded in the Rotterdam Study, a prospective population-based study among men and women aged 55 years and older. 5520 participants without history of coronary heart disease were genotyped for the Tyr402His polymorphism of the CFH gene. Cox proportional hazards analysis was used to determine risk of myocardial infarction for Tyr402His genotypes.

Results. Mean age among participants was 69.5 years (standard deviation 9.1 years). The overall frequency of the His allele was 36%; genotype frequencies were 41%, 45% and 14% for TyrTyr, TyrHis and HisHis, respectively. During a mean follow-up period of 8.4 years, 226 myocardial infarctions occurred. After adjustment for age, gender, established cardiovascular risk factors and CRP level, HisHis homozygotes had a hazard ratio of 1.77 (95% confidence interval 1.23-2.55) for myocardial infarction. Total cholesterol level, diabetes mellitus and smoking modified the effect. The Tyr402His polymorphism was not associated with established cardiovascular risk factors or CRP level.

Conclusions. Our data suggest that the CFH gene determines susceptibility to myocardial infarction. This finding underscores the importance of the alternative complement system in cardiovascular disease.

Introduction

Inflammation has been shown to play an important role in cardiovascular disease.[1] Both complement factors and complement regulatory factors have been linked to atherosclerosis.[2] Complement inhibitor factor H (CFH) is a plasma protein that is essential in the regulation of the alternative complement pathway. Recently, several studies have found that the Tyr402His (rs1061170) polymorphism in the CFH gene is strongly associated with age-related macular degeneration, with relative risks of 2.5-7.4 for homozygotes.[3-7]

Complement inhibitor factor H has been suggested to play a part in complement inhibition in atherosclerotic lesions,[8] and atherosclerosis has been implicated in the development of age-related macular degeneration.[9] Therefore, the association between the CFH gene polymorphism and age-related macular degeneration may at least in part be mediated by atherosclerosis. Research in coronary artery specimens suggests that interaction of CFH with proteoglycans may be the mechanism by which complement activation in the superficial layer of the coronary intima is controlled.[8] Consequently, we hypothesize that CFH may play a protective role in the development of coronary heart disease.

Complement inhibitor factor H is encoded by a single gene (HF1) on human chromosome 1q32. Several polymorphisms have been identified in the CFH gene, but their potential influence on the levels of expression or on the function of CFH is uncertain.[10] The Tyr402His polymorphism, representing a tyrosine-histidine change at amino acid 402, is particularly interesting, since this change is located within the cluster of positively charged amino acids implicated in the binding of heparin and C-reactive protein (CRP).[11] Binding to these factors augments the ability of CFH to down-regulate the effect of complement. The substitution of a positively charged histidine for a non-charged hydrophobic tyrosine in position 402 may alter the binding properties and have functional implications.[10] These changes may alter CFH's ability to suppress excess complement activation, ultimately leading to complement-related damage to arterial walls and vessel injury.[4]

We set out to investigate the association between the CFH gene Tyr402His polymorphism and the risk of myocardial infarction in the Rotterdam Study, a population-based cohort study in men and women aged 55 years and over.

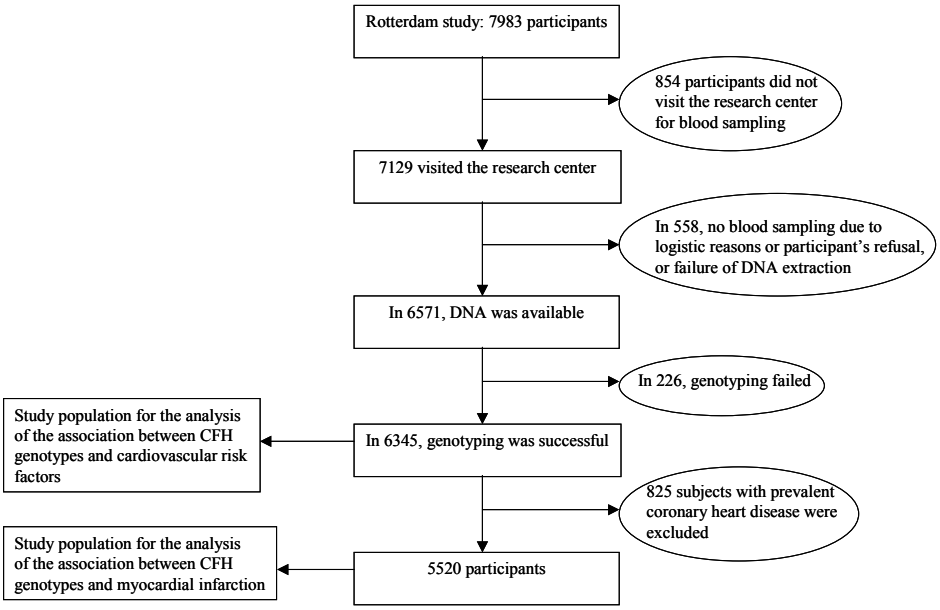
Methods

Study population

The present study is part of the Rotterdam Study, a population-based cohort study aimed at assessing the occurrence of and risk factors for chronic diseases in the elderly. Objectives and methods of the Rotterdam Study have been described in detail elsewhere.[12] The medical ethics committee of Erasmus Medical Center, Rotterdam, approved the study. Participants gave written informed consent and permission to retrieve information from treating physicians.

Figure 1 shows a flow chart describing the study population. The Rotterdam Study cohort includes 7983 men and women aged 55 years and over (78% of the eligible population), living in a well-defined suburb of the city of Rotterdam, The Netherlands. Baseline data were collected from 1990 until 1993. A trained interviewer visited all subjects at home and collected information using a computerized

Figure 1. Flow chart describing study population.



CFH = complement inhibitor factor H; DNA = deoxyribonucleic acid.

questionnaire. Additionally, established cardiovascular risk factors were measured at the research center in 7129 participants. DNA was available for 6571 subjects. Genotyping was successful in 6345 participants. After excluding participants with a history of myocardial infarction, percutaneous transluminal coronary angioplasty (PTCA) or coronary artery bypass grafting (CABG) at baseline, 5520 subjects were left for analysis.

Genotyping

The participants were genotyped for the Tyr402His (1277T>C) polymorphism of the CFH gene. This polymorphism has been described at http://www.ncbi.nlm.nih.gov/SNP under identification number rs1061170.

DNA was extracted with proteinase K and sodium dodecyl sulfate digestion at 37°C overnight and purified with phenol-chloroform extractions. The extracted DNA was then precipitated with NaCl at 4 mol/L and 2 volumes of cold absolute ethanol. DNA was solubilized in double-distilled water and stored at –20°C until used for DNA amplification. Genotypes were determined in 2-ng genomic DNA with the Taqman allelic discrimination assay (Applied Biosystems, Foster City, California). Primer and probe sequences were optimized by using the SNP assay-by-design service of Applied Biosystems (for details, see http://store.appliedbiosystems.com). Reactions were performed with the Taqman Prism 7900HT 384 wells format.

Assessment of covariates

The information obtained during the interview included current health status, medical history (including history of PTCA and CABG), drug use, and smoking status. At the research center, height and weight were measured and the body mass index was calculated (weight (kg)/height (m^2)). Blood pressure was measured at the right brachial artery using a random-zero sphygmomanometer with the participant in sitting position. Diabetes mellitus was defined as the use of blood glucose lowering medication or a random or post-load serum glucose level >= 11.1 mmol/l.[13] C-reactive protein was measured in serum, kept frozen at -20 °C, using a nephelometric method (Immage, Beckman Coulter, Fullerton, California). A 12-lead resting electrocardiogram (ECG) was recorded with an ACTA electrocardiograph (ESAOTE, Florence, Italy) at a sampling frequency of 500Hz, and stored digitally. All ECGs were processed by the Modular ECG Analysis System (MEANS[14]) to obtain ECG measurements and interpretations. Myocardial infarction found on ECG was based on criteria partly derived from the Minnesota code[15] A history of myocardial infarction was considered present in case of a self-report of myocardial infarction confirmed by ECG or additional clinical information, or the presence of an ECG characteristic of prior myocardial infarction.

Follow-up procedure

Follow-up started at the baseline examination and lasted until January 1st, 2002 for the present study. Information on fatal and non-fatal myocardial infarctions for the participants enlisted with the general practitioners (GPs) working in the study district (85% of the cohort) was obtained from these GPs. Computerized records were sent to the Rotterdam Study data center regularly. Subsequently, research assistants gathered information about these events at the GP offices. All medical records of the participants under the care of GPs outside the study area (15% of the cohort) were checked annually for possible events. Letters and, in case of hospitalization, discharge reports from medical specialists were obtained. With respect to the vital status of participants, information was also obtained regularly from the municipal health authorities in Rotterdam. After notification, cause and circumstances of death were established by questionnaire from the GPs.

Subsequently, two research physicians independently coded all reported events according to the International Classification of Diseases, 10th edition (ICD-10).[16] In case of disagreement, consensus was reached. Finally, a medical expert in cardiovascular disease, whose judgment was considered final, reviewed all events.

In identifying incident myocardial infarctions (ICD-10 code I21), all available information, which included ECG, cardiac enzyme levels, and the clinical judgment of the treating specialist, was used. Persons with incident silent myocardial infarctions were not identified.

Data analysis

Hardy-Weinberg equilibrium of the Tyr402His polymorphism was tested using a Chi square test. Differences in established cardiovascular risk factors for the three genotypes were examined by using analysis of covariance, adjusting for age and gender. For age, this analysis was only adjusted for gender, and for gender, this analysis was only adjusted for age. C-reactive protein serum levels were log-transformed because of their skewed distribution.

Cox proportional hazards analysis was used to determine the relative risks of myocardial infarction associated with Tyr402His genotypes. The proportional hazards assumption was tested by drawing log minus log plots of the survival function. In model 1, we adjusted for age and gender, in model 2, we adjusted for age, gender, body mass index, systolic blood pressure, diastolic blood pressure, total cholesterol, HDL-cholesterol, smoking, and diabetes mellitus, and in model 3, we adjusted for the covariates in model 2 and additionally for CRP. Age- and gender-adjusted survival curves were drawn showing event-free survival until the occurrence of myocardial infarction. These curves were evaluated at the mean of the covariates (age and gender) in the 5520 subjects used. For age, this was 69.1 years, for gender, this was 62% women.

Firstly, analyses were performed in all participants. Thereafter, to examine effect modification, subgroup analyses were performed in strata of age, gender, body mass index, systolic blood pressure, diastolic blood pressure, total cholesterol, HDL-cholesterol, smoking, diabetes mellitus and CRP level. For continuous variables, participants were divided into subgroups according to the median values of the variables. Similarly, models 1, 2 and 3, as described above, were used for these subgroup analyses. Interaction terms were tested for the Tyr402His polymorphism and each of the covariates we stratified on, adjusting for age and gender. For this purpose, the polymorphism was entered into the model as a continuous variable with three values, namely homozygotes for the common allele (value 0), heterozygotes (value 1) and homozygotes for the rare allele (value 2).

Values for cardiovascular covariates were missing in less than 4% of participants, except for CRP, which was missing in 7%. Missing values were handled by single imputation using the expectation-maximization (EM) algorithm based on age, gender, body mass index, systolic blood pressure, diastolic blood pressure, total cholesterol, HDL-cholesterol, smoking, diabetes mellitus and CRP.

All analyses were performed using SPSS 11.0 for Windows (SPSS Inc., Cary, North Carolina).

Results

Genotype distributions were in Hardy-Weinberg equilibrium. The overall frequency of the His allele was 36%; genotype frequencies were 41%, 45% and 14% for TyrTyr, TyrHis and HisHis, respectively. Table 1 shows baseline characteristics of all participants in which genotyping was successful. None of the studied characteristics, namely age, gender, body mass index, systolic and diastolic blood pressure, hypertension, total and HDL cholesterol, diabetes mellitus, smoking and CRP were associated with Tyr402His genotype.

During a mean of 8.4 (standard deviation 2.7) years of follow-up, 226 cases of myocardial infarction occurred among participants who had no history of myocardial infarction, CABG or PTCA at baseline. Follow-up information on myocardial infarction was complete for 5237 out of 5520 participants used for the analysis (94.9%). The potential number of person years that could have been contributed by these 5520 participants until myocardial infarction, death or the end of the follow-up period was 46516.7. We were able to observe 46173.5 person years (99.3%) until the date that a participant was last know to be alive, myocardial infarction, death or the end of the follow-up period.

In table 2, hazard ratios for myocardial infarction are displayed according to Tyr402His genotype. In reference to wild type, heterozygotes had a 14-16% increased risk of myocardial infarction, but

Table 1. Baseline characteristics of the total population.

	Total (n=6345)
Age (years)	69.5±9.1
Women (%)	60.0
Body mass index (kg/m^2)	26.3±3.7
Systolic blood pressure (mm Hg)	139±22
Diastolic blood pressure (mm Hg)	74±11
Total cholesterol (mmol/l)	6.6±1.2
HDL-cholesterol (mmol/l)	1.3±0.4
Diabetes mellitus (%)	10.4
Smokers (%)	
- Never	35.5
- Current	22.7
- Former	41.8
History of hypertension (%)	34.3
C-reactive protein (mg/l)*	1.87 (0.90-3.67)

Categorical variables are expressed as percentage. Values of continuous variables are expressed as mean ± standard deviation for total. * Median and interquartile range because of skewed distribution. HDL = high-density lipoprotein.

Table 2. Hazard ratios for myocardial infarction according to Tyr402His genotype.

		Hazard ratio (95% confidence interval)		
Genotype	**Events/ subjects**	**Model 1**	**Model 2**	**Model 3**
All				
TyrTyr	81/ 2251	1.00 (reference)	1.00 (reference)	1.00 (reference)
TyrHis	99/ 2509	1.14 (0.85-1.53)	1.16 (0.86-1.55)	1.16 (0.86-1.55)
HisHis	46/ 760	1.72 (1.20-2.47)	1.77 (1.23-2.54)	1.77 (1.23-2.55)
Men				
TyrTyr	46/ 863	1.00 (reference)	1.00 (reference)	1.00 (reference)
TyrHis	58/ 924	1.22 (0.83-1.79)	1.25 (0.85-1.84)	1.25 (0.84-1.84)
HisHis	28/ 293	1.82 (1.16-2.96)	1.94 (1.21-3.12)	1.95 (1.21-3.13)
Women				
TyrTyr	35/ 1388	1.00 (reference)	1.00 (reference)	1.00 (reference)
TyrHis	41/ 1585	1.03 (0.66-1.62)	1.02 (0.65-1.60)	1.02 (0.65-1.61)
HisHis	18/ 467	1.54 (0.87-2.71)	1.53 (0.86-2.70)	1.54 (0.87-2.73)

Model 1. Adjusted for age and gender; model 2. Adjusted for age, gender, body mass index, systolic blood pressure, diastolic blood pressure, total cholesterol, HDL-cholesterol, smoking, and diabetes mellitus; model 3. Adjusted for age, sex, body mass index, systolic blood pressure, diastolic blood pressure, total cholesterol, HDL-cholesterol, smoking, diabetes mellitus and C-reactive protein.

this did not reach statistical significance. After adjustment for age and gender (model 1), HisHis homozygotes had a hazard ratio of 1.72 (95% CI 1.20-2.47) for developing myocardial infarction. After additional adjustment for established cardiovascular risk factors (model 2), the hazard ratio slightly increased to 1.77 (95% CI 1.23-2.54). Further adjustment for CRP (model 3) did not change the estimate. When we repeated the analysis without excluding participants with coronary heart disease at baseline, the results did not change materially.

The hazard ratios in men and women are also displayed in table 2. After full adjustment (model 3), male HisHis homozygotes had a hazard ratio of 1.95 (95% CI 1.21-3.13) for developing myocardial infarction, whereas for female HisHis homozygotes this was 1.54 (95% CI 0.87-2.73). The latter value may not have reached statistical significance due to lack of power in women.

Event-free survival until the occurrence of myocardial infarction is displayed in figure 2. Homozygotes for the rare His allele had a significantly lower event free survival.

Figure 3 displays the effects of potential modifiers of the association between Tyr402His and myocardial infarction in an age- and gender-adjusted subgroup analysis. Overall, risk of myocardial infarction was increased for HisHis homozygotes in all subgroups. The risk of myocardial infarction was considerably higher in participants with total cholesterol levels above the median versus those with levels below the median (p for interaction 0.05), in diabetics versus non-diabetics (p for interaction

Figure 2. Age- and gender-adjusted event-free survival until incident myocardial infarction.

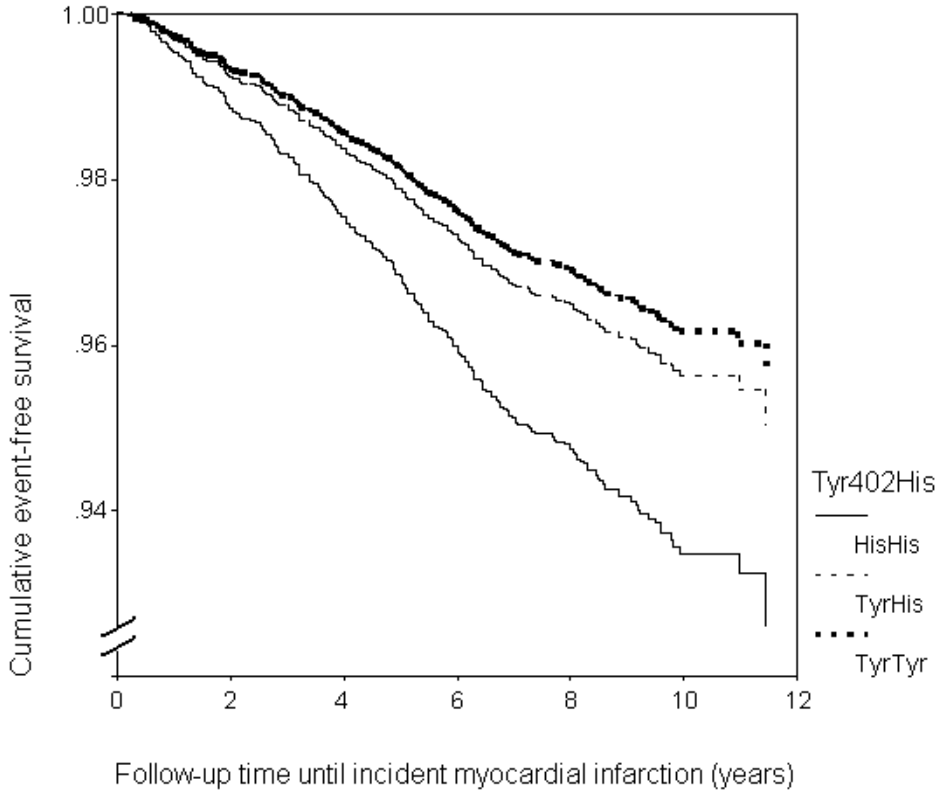
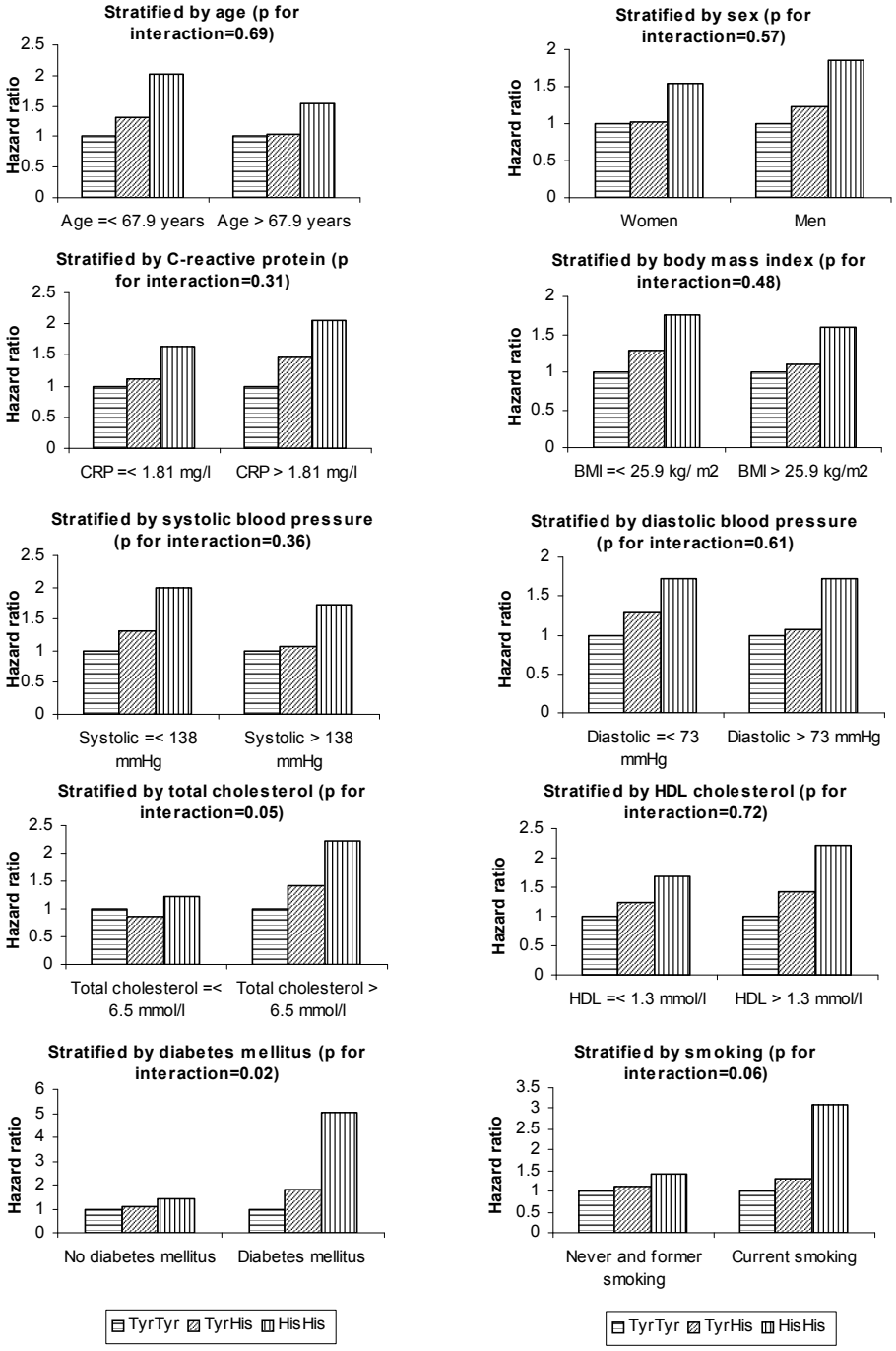


Figure 3. Age- and gender-adjusted hazard ratios for myocardial infarction according to Tyr402His genotype in subgroups, created according to median values for continuous variables.



BMI = body mass index; CRP = C-reactive protein; HDL = high-density lipoprotein.

0.02), and in current smokers versus former and never smokers (p for interaction 0.06). The risk for subjects with above median CRP levels was somewhat higher, but the interaction did not reach statistical significance (p for interaction 0.31). The remaining age- and gender-adjusted interaction coefficients of the Tyr402His polymorphism and the covariates upon which we stratified were not significant. Subgroup analysis with additional adjustment for age, gender, body mass index, systolic blood pressure, diastolic blood pressure, total cholesterol, HDL-cholesterol, smoking, diabetes mellitus and CRP did not materially alter the results.

Discussion

In the present study, the CFH gene Tyr402His polymorphism was associated with an increased risk of myocardial infarction in participants without a history of coronary heart disease. Total cholesterol levels, diabetes mellitus and smoking modified the effect. The CFH gene Tyr402His polymorphism was not associated with established cardiovascular risk factors, or with serum CRP levels. Adjustment for these factors did not essentially alter the risk of myocardial infarction. This study was performed within a large, prospective, population-based cohort, with lengthy follow-up time and a considerable number of incident myocardial infarction cases available for analysis.

Inflammation has been recognized as an important mechanism in coronary heart disease and other manifestations of atherosclerosis.[1] The complement system contributes to inflammation in the arterial intima, and thus may exert unfavourable effects on atherosclerosis.[2] The complement system contains several plasma and membrane-associated proteins that are organized in three activation pathways: the classical, the lectin and the alternative pathway. CFH is a plasma protein that plays an important part in the inhibition of the alternative pathway; it restricts the action of complement to activating surfaces by binding to C3b, accelerating the decay of the alternative pathway C3-convertase (C3bBb) and acting as a cofactor for the factor I-mediated proteolytic inactivation of C3b.[10] This mechanism allows for activation of the early complement cascade by opsonization and may thereby play a protective role; however, complement activation is limited to the C3 level, and does not lead to full complement activation with cell lysis and ensuing inflammation.[8]

Several studies provide evidence for a regulatory role of CFH in the development of atherosclerosis. CFH has been found to be associated with severity of coronary luminal narrowing in patients receiving coronary angiography.[17] In a study of human atherosclerotic lesions, CFH was observed in a large proportion of the lesions.[18] Experimental observations have raised the possibility that interaction between proteoglycans and CFH, which co-localize in the superficial layer of the intima, may inhibit complement activation in the superficial layer of the arterial intima.[8] Furthermore, it has been shown that CFH binds to CRP, which may help to inhibit the CRP-dependent alternative complement activation pathway induced by damaged tissue.[19,20] CRP has been found to be associated with both coronary heart disease and age-related macular degeneration.[21,22]

CFH is encoded by a single gene (HF1) that is part of the Regulator of Complement Activation gene cluster on human chromosome 1q32 that encodes several regulatory proteins of the complement system. Several polymorphisms have been identified in this gene, but their potential influence on the levels of expression or on the function of CFH are uncertain.[10] The Tyr402His polymorphism is located

within the cluster of positively charged amino acids implicated in the binding of heparin and CRP. Binding to either of these partners increases the affinity of CFH for the complement protein C3b,[20,23] augmenting its ability to down-regulate complement's effect. The substitution of a positively charged histidine for a non-charged hydrophobic tyrosine in position 402 may alter the binding properties and consequently have functional implications.

Recently, an association of the Tyr402His polymorphism with risk of vascular events could not be demonstrated in a nested case-control study within the Physicians' Health Study.[24] The authors report that they had the ability to detect, with 80% power, at an alpha of 0.05, a risk ratio of greater than 1.35 for the His variant for vascular events (myocardial infarction, ischaemic stroke, deep venous thrombosis/ pulmonary embolism, n=685). Therefore, and also based on the effect estimates and confidence intervals found, as stated by the authors, a modest risk of vascular events associated with the genotypes tested could not be fully excluded.

Several aspects of the present study warrant further consideration. One limitation is, that it remains to be investigated whether the Tyr402His variant is the true underlying variant and does not represent a marker in complete or partial linkage disequilibrium, either within the CFH gene itself or in flanking genes. Still, several arguments support the involvement of the CFH gene and in particular the Tyr402His polymorphism in explaining the risk of myocardial infarction. Firstly, the CFH gene is a credible candidate, since several studies provide evidence for a regulatory role of CFH in the development of atherosclerosis. Secondly, the substitution of a positively charged histidine for a non-charged hydrophobic tyrosine theoretically has functional implications for the CFH protein. Finally, haplotype reconstruction has implicated this polymorphism in relation to complement-mediated pathogenesis of age-related macular degeneration.[3,7] Despite these arguments, further research is warranted to disclose whether the Tyr402His variant is truly involved in explaining the risk of coronary heart disease. While our results are promising, the potential of other variants in the CFH gene to influence coronary heart disease risk should be further investigated.

Another limitation of the study is found in the follow-up. We performed a thorough follow-up procedure with regard to recognizable myocardial infarction, attaining 94.9% completeness. However, our follow-up did not contain incident silent myocardial infarction, although patients with silent myocardial infarction are in many aspects similar to those with recognized myocardial infarction.[25] The result is that incident silent myocardial infarction cases were missed and have been considered as non-cases in the analysis. Since this is non-differential misclassification of the outcome, it should not have influenced our results. We present relative risks, which remain the same. Only the risk difference between the genotypes could have changed due to this type of misclassification.[26]

The effect of the Tyr402His polymorphism on risk of myocardial infarction was modified by high total cholesterol levels, presence of diabetes mellitus and smoking. These factors all have been shown to be pathogens in vessel inflammation.[1,27,28] When the alternative complement pathway is activated by such pathogens, it is plausible that subjects with a genetic susceptibility in CFH such as the Tyr402His variant respond with a reduced complement inhibition and thereby increase their risk of vessel damage, coronary atherosclerosis and subsequently myocardial infarction. Since the Tyr402His polymorphism is located within the cluster of amino acids implicated in the binding of CRP, we also expected CRP level to modify the risk of myocardial infarction. Although the risk was slightly higher in participants with CRP levels above the median, the interaction with CRP was not significant.

In conclusion, we have found an association between the CFH gene Tyr402His polymorphism and myocardial infarction. This suggests that CFH may play an important role in atherosclerosis, and underscores the importance of the alternative complement system in cardiovascular disease.

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Chapter 2.4

Variation in the complement factor H and C-reactive protein genes and myocardial infarction

Abstract

Complement factor H (CFH) is an important regulator of the complement cascade. Binding of C-reactive protein (CRP) to CFH augments the ability of CFH to down-regulate the effect of complement in atherosclerotic lesions. The CFH Tyr402His polymorphism has been suggested to influence the ability of CFH to bind CRP. We hypothesized that the combined presence of unfavorable CRP and CFH genetic profiles is associated with risk of myocardial infarction. The Rotterdam Study is a population-based cohort study among 7983 men and women aged ≥ 55 years. The CFH Tyr402His (rs1061170) polymorphism was determined (His^{402} allele = 37%), and using three tagging polymorphisms (rs1130864, rs1205, rs3093068), CRP haplotypes were inferred (1=CTC, 2=TCC, 3=CCC, 4=CCG; frequencies 33%, 32%, 30% and 6%, respectively). Participants were grouped by both CFH genotype (TyrTyr [reference], TyrHis, HisHis) and CRP haplotype (haplotype 1 homozygotes [reference], haplotype 2 carriers, haplotype 3 carriers, haplotype 4 carriers), which resulted in a total of 12 groups. CFH His^{402} homozygotes that were also CRP haplotype 3 carriers had an age- and sex-adjusted hazard ratio of 5.9 (95% CI 2.1-16.5) to develop myocardial infarction compared to the reference group. In conclusion, this population-based study suggests that the combined presence of unfavorable CFH and CRP genetic profiles is associated with risk of myocardial infarction.

Introduction

Recently, the complement factor H (CFH) gene and the C-reactive protein (CRP) gene have received attention with regard to coronary heart disease risk. CFH is a plasma protein essential in the regulation of the alternative complement pathway[1] and has been suggested to play a part in complement inhibition in atherosclerotic lesions.[2] Binding of CRP to CFH augments the ability of CFH to down-regulate the effect of complement.[3,4] The His^{402} allele of the functional CFH Tyr402His polymorphism (rs1061170) has been suggested to influence the ability of CFH to bind CRP,[1] and has recently been found to be associated with risk of myocardial infarction in the Rotterdam Study.[5] CRP level is a consistent risk factor for cardiovascular disease.[6] Steady-state CRP level has been found to be influenced by CRP haplotypes.[7-9] However, the association between CRP haplotypes and coronary heart disease remains controversial. Few studies have been performed on this topic so far, and lack of power may play a part in their failure to show a consistent association.[8-10] In view of the biological effect of CRP on the ability of CFH to down-regulate the effect of complement, we investigated whether the combined presence of unfavorable genetic CFH and CRP profiles is associated with risk of myocardial infarction in the Rotterdam Study.

Methods

The Rotterdam Study is a population-based cohort study aimed at studying chronic diseases in the elderly. Objectives and methods of the Rotterdam Study have been described in detail elsewhere.[11] Briefly, the Rotterdam Study cohort includes 7983 men and women aged 55 years and over (78% of the eligible population), living in a well-defined suburb of the city of Rotterdam, The Netherlands. Baseline data were collected from 1990 until 1993. A trained interviewer visited all subjects at home and collected information using a computerized questionnaire, and established cardiovascular risk factors were measured at the research center in 7129 participants, as described in detail previously.[5] DNA was available for 6571 participants. The medical ethics committee of Erasmus Medical Center, Rotterdam, approved the study. Participants gave written informed consent and permission to retrieve information from treating physicians.

Follow-up started at the baseline examination and for the present study lasted until January 1, 2005. Information on fatal and non-fatal cardiovascular endpoints was obtained from general practitioners and letters and discharge reports from medical specialists.[5] Two research physicians independently coded all reported events according to the International Classification of Diseases, 10^{th} edition (ICD-10).[12] In case of disagreement, consensus was reached. A medical expert in cardiovascular disease, whose judgment was considered final, reviewed all events. In identifying incident myocardial infarctions (ICD-10 code I21), all available information, which included ECG, cardiac enzyme levels, and the clinical judgment of the treating specialist, was used.

Participants were genotyped for the Tyr402His (1277T>C, rs1061170) single nucleotide polymorphism (SNP) of the CFH gene as described previously.[13] This SNP was chosen because of its suggested functional properties, mentioned above. Furthermore, we determined CRP haplotypes by genotyping the participants for the haplotype tagging SNPs 1184 C>T (rs1130864), 2042 C>T (rs1205) and 2911

C>G (rs3093068).[9,13] We chose to determine CRP haplotypes in order to make use of the common variation across the entire CRP gene. CRP haplotypes were inferred by using the program PHASE, which implements a Bayesian statistical method for reconstructing haplotypes from population genotype data.[14]

For the analysis, participants were grouped according to both CFH genotype (TyrTyr [reference], TyrHis, HisHis) and CRP haplotype (haplotype 1 homozygotes [reference], haplotype 2 carriers, haplotype 3 carriers, haplotype 4 carriers), which resulted in 12 groups. Participants homozygous for both the common CFH Tyr genotype and the most frequent CRP haplotype 1 were used as the reference group. Cox proportional hazards analysis was used to determine the relative risks of myocardial infarction associated with combinations of Tyr402His genotype and CRP haplotype, adjusted for age and sex. The proportional hazards assumption was tested (by drawing log minus log plots of the survival function). All analyses were performed using SPSS 11.0 for Windows (SPSS Inc., Cary, North Carolina).

Results

Genotyping was successful for the 4 SNPs within the CRP and CFH genes in 5946 participants. After excluding participants with history of myocardial infarction, coronary artery bypass grafting or percutaneous transluminal coronary angioplasty, 5178 participants were left for analysis. A total of 277 myocardial infarctions occurred during a median follow-up time of 12 (inter-quartile range, 9-13) years. Chi-square tests showed that genotype distributions were in Hardy-Weinberg equilibrium in the full cohort. The population frequency of the His^{402} allele was 37%; genotype frequencies were 41%, 45% and 14% for TyrTyr, TyrHis and HisHis, respectively. The probability of the CRP haplotypes estimated by PHASE was ≥ 0.999 in all individuals. Haplotypes were coded as 1 to 4 in order of decreasing frequency in the population (coding from 1184 C>T, 2042 C>T and 2911 C>G: haplotype 1 = C-T-C, haplotype 2=T-C-C, haplotype 3=C-C-C, and haplotype 4=C-C-G). Frequencies of haplotypes 1, 2, 3 and 4 were 33%, 32%, 30% and 6%, respectively. These 4 haplotypes described 99.9% of our population.

Baseline characteristics of the study population are shown in table 1. Hazard ratios for developing myocardial infarction are displayed in table 2. The reference group had the lowest risk of myocardial infarction. Within CFH TyrTyr homozygotes, risk increased in carriers of CRP haplotype 2, 3 and 4. A similar increase in risk was seen within CFH HisHis homozygotes. Within CRP haplotype 2, haplotype 3 and haplotype 4 carriers, CFH HisHis homozygotes carried the highest relative risks. Although within the CRP haplotype 1-1 homozygotes, CFH HisHis homozygosity also resulted in a raised relative risk, this was not statistically significant. This may have been caused by lack of power. The risk pattern is illustrated in figure 1. Additional adjustment for body mass index, systolic and diastolic blood pressure, total and HDL-cholesterol, diabetes mellitus and smoking did not materially change the risk estimates.

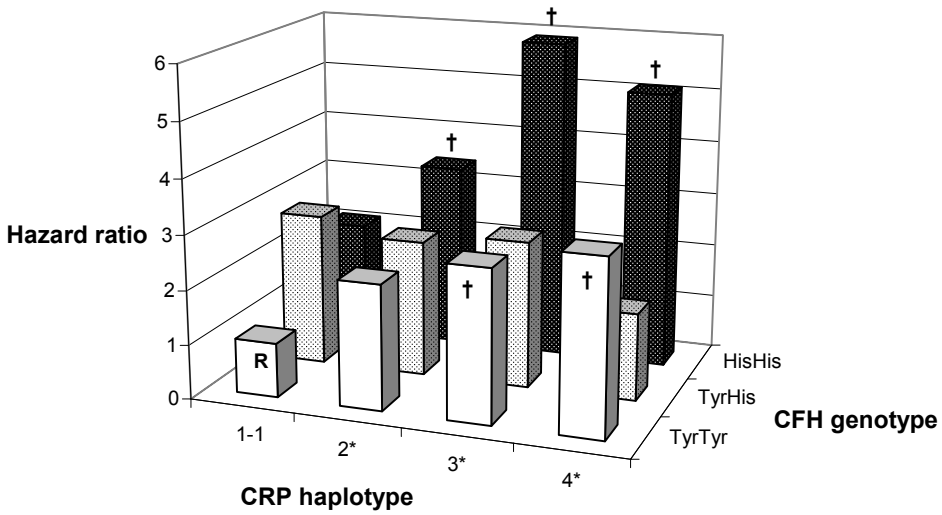
Table 1. Baseline characteristics (n=5178).

Variable	Value
Age (years)	69.1±9.2
Women	3293 (63.0%)
Body mass index (kg/m^2)	26.2±3.7
Systolic blood pressure (mm Hg)	139±22
Diastolic blood pressure (mm Hg)	74±11
Total cholesterol	
- (mmol/l)	6.6±1.2
- (mg/dl)	257±47
High-density lipoprotein cholesterol	
- (mmol/l)	1.4±0.4
- (mg/dl)	55±16
C-reactive protein (mg/l)*	1.80 (0.86-3.49)
Diabetes mellitus	508 (9.9%)
Smokers	
- Never	1879 (37.4%)
- Current	1134 (22.6%)
- Former	2012 (40.0%)
Hypertension (history)	1673 (33.2%)

Values of continuous variables are expressed as mean ± standard deviation. Categorical variables are expressed as count (percentage). Valid percentages may vary for some counts because of missing values in the variables.

* Median and interquartile range because of skewed distribution

Figure 1. Complement Factor H genotype, C-reactive protein haplotype and risk of myocardial infarction.



† P< 0.05; R = reference; 2* = haplotype 2 carriers; 3* = haplotype 3 carriers; 4* = haplotype 4 carriers. Individuals with haplotype 2 and 3 (2-3) were present in both the 2* and 3* group. Individuals with haplotype 2 and 4 (2-4) were present in both the 2* and 4* group. Individuals with haplotype 3 and 4 (3-4) were present in both the 3* and 4* group.

Table 2. Complement factor H genotype, C-reactive protein haplotype and risk of myocardial infarction.

Complement facor H genotype	C-reactive protein haplotype	Cases/ subjects	Hazard ratio (95% confidence interval)
TyrTyr	1-1	4/ 200	1.0 (reference)
	2*	52/ 1141	2.3 (0.8-6.4)
	3*	60/ 1097	2.8 (1.0-7.7)†
	4*	14/ 233	3.2 (1.0-9.7)†
TyrHis	1-1	13/ 245	2.8 (0.9-8.7)
	2*	60/ 1255	2.5 (0.9-6.8)
	3*	59/ 1163	2.7 (1.0-7.4)
	4*	8/ 277	1.6 (0.5-5.2)
HisHis	1-1	3/ 76	2.1 (0.5-9.5)
	2*	24/ 374	3.4 (1.2-9.8)†
	3*	39/ 355	5.9 (2.1-16.5)†
	4*	9/ 84	5.1 (1.6-16.5)†

2* = haplotype 2 carriers; 3* = haplotype 3 carriers; 4*=haplotype 4 carriers.

Individuals with haplotype 2 and 3 (2-3) were present in both the 2* and 3* group.

Individuals with haplotype 2 and 4 (2-4) were present in both the 2* and 4* group.

Individuals with haplotype 3 and 4 (3-4) were present in both the 3* and 4* group.

† $P < 0.05$

Discussion

These results suggest that the combined presence of unfavorable genetic CFH and CRP profiles is associated with myocardial infarction. To our knowledge, our study is the first to investigate this association. A combined effect of CFH and CRP genetic profiles has previously been found for age-related macular degeneration.[15] These findings support the biological effect of CRP on CFH previously suggested.[4] CFH has the ability to down-regulate the effect of complement in atherosclerotic lesions.[2] CRP is capable to enhance this effect: it has been shown that binding of CRP to CFH is the mechanism by which the CRP-dependent alternative pathway, induced by damaged tissue, is counterarrested.[4] The Tyr402His SNP is located within the cluster of amino acids implicated in the binding of CRP, and the His^{402} allele may diminish the binding properties for CRP because of the substitution of a positively charged histidine for a non-charged hydrophobic tyrosine.[1] The decrease in complement inhibition, combined with enhanced activation of the classical complement pathway by increased CRP levels, may underlie the large increase in risk of myocardial infarction.

Strengths of the present study include its large size, population-based design, coverage of 99.9% of the variation in the CRP gene, occurrence of 277 incident myocardial infarctions, and the thorough follow-up procedure. Nevertheless, some aspects of this study warrant further consideration. Firstly, although haplotyping was performed for the CRP gene, we only determined one SNP in the CFH gene. It remains to be investigated whether this SNP is the true underlying variant and does not represent a marker in complete or partial linkage disequilibrium. Still, the Tyr402His SNP is a credible candidate, because the substitution of a positively charged histidine for a non-charged hydrophobic tyrosine

could have functional implications for the CFH protein and furthermore, haplotype reconstruction has implicated this SNP in relation to complement-mediated pathogenesis of age-related macular degeneration.[16,17] Another aspect that merits attention is the fact that our findings are based on one single cohort. We did not have an independent reproducibility cohort at our disposal to consolidate the findings. The results would be strengthened if confirmed by another, independent, study. Our paper may stimulate other research groups to investigate this association.

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Chapter 3

Lipoprotein-associated phospholipase A2

Chapter 3.1

Epidemiology of lipoprotein-associated phospholipase A2

Introduction

Inflammation has been shown to play a central role in all phases of the atherosclerotic process.[1] Inflammatory pathways are implicated in early atherogenesis, in the progression of lesions, and in thrombotic complications. Clinical studies have shown associations of circulating markers of inflammation, such as C-reactive protein (CRP) and fibrinogen, with cardiovascular events.[2,3] Circulating inflammatory mediators may not only mark increased risk for cardiovascular events, but also, in some cases, may contribute to their pathogenesis. An inflammatory marker that has come under study recently with regard to cardiovascular disease is lipoprotein-associated phospholipase A2 (Lp-PLA2).

Lp-PLA2 belongs to the superfamily of phospholipase A2 enzymes.[4] It is a 45-kDa, Ca^{2+} independent protein, up-regulated in atherosclerotic plaques and strongly expressed in macrophages within the fibrous cap of rupture prone lesions.[5] It is called lipoprotein-associated PLA2 because of its tight association with lipoproteins. 80% of the enzyme in human plasma is located on low-density lipoprotein (LDL) and around 10% resides on high-density lipoprotein (HDL). In minor amounts, it associates with very low-density lipoprotein (VLDL) and lipoprotein(a).[6]

Lp-PLA2 has been suggested to have both pro-atherogenic and anti-atherogenic properties. When first identified, it was named platelet-activating factor acetylhydrolase (PAF-AH) owing to its ability to hydrolyze platelet-activating factor (PAF), a potent pro-inflammatory phospholipid. This ability suggests an anti-atherogenic role of Lp-PLA2, supported by research using mouse models.[7] However, in mice, Lp-PLA2 is predominantly associated with HDL, and therefore the proposed anti-atherogenic role of Lp-PLA2 is, at least in part, based on its association with the anti-atherogenic HDL. In addition to PAF hydrolysis, Lp-PLA2 can hydrolyze a broad spectrum of substrates including oxidized and polar phosphatidylcholines.[8] In humans, Lp-PLA2 is bound predominantly tot LDL cholesterol particles, and remains latent until the LDL-cholesterol particles undergo oxidative damage. Hereafter, Lp-PLA2 cleaves the oxidized phosphatidylcholine into lyso-phosphatidylcholine and free fatty acid metabolites.[9] These mediators have been suggested to elicit pro-atherogenic effects.[10] These pro-atherogenic effects may outweigh the anti-atherogenic effects in humans.

A substantial epidemiologic body of research is emerging on Lp-PLA2 and risk of cardiovascular disease in humans. First, several, mostly small, case-control studies have suggested that Lp-PLA2 may play a part in cardiovascular disease.[11-18] Hereafter, large cohort studies have examined Lp-PLA2 in relation to incident cardiovascular events. Furthermore, studies were performed on Lp-PLA2 and

measures of atherosclerosis. Finally, Lp-PLA2 genotypes have been used to shed more light on the relation between Lp-PLA2 and cardiovascular disease. What follows is an overview of epidemiological studies on Lp-PLA2 and cardiovascular outcomes.

Lp-PLA2 and incident cardiovascular events

West of Scotland Coronary Prevention Study (WOSCOPS)

Studies on the association between Lp-PLA2 and incident cardiovascular events are summarized in table 1. The first study was performed within WOSCOPS, a trial in which 6595 men who had cholesterol levels between 4.5 and 6.0 mmol /l, but who had no history of a myocardial infarction, were randomly assigned to receive 40 mg of pravastatin or placebo daily. The present study was a case-control study, including 580 men who had an incident coronary event (nonfatal myocardial infarction, death from coronary heart disease or revascularization), and 1160 controls matched on age and smoking status.[19] The study showed a relative risk of 1.18 (95% CI 1.05-1.33) per standard deviation (SD) of Lp-PLA2 level for having a coronary event after adjustment for age, systolic blood pressure, plasma triglycerides, LDL and HDL cholesterol, fibrinogen, white-cell count and CRP. The multivariable adjusted relative risk for having a coronary event for the highest versus the lowest quintile of Lp-PLA2 was significant and nearly doubled. There was no significant interaction with pravastatin use.

Women's Health Study (WHS)

The Women's Health Study is an ongoing randomized, double-blind, placebo-controlled trial of aspirin and vitamin E being conducted among 28,263 women aged 45 years and over with no history of cardiovascular disease or cancer. For the present study, 123 cases and 123 controls, matched for age and smoking status, were selected.[20] Cases were defined as study participants who provided a baseline blood sample and who subsequently had a cardiovascular event as defined by death due to coronary heart disease, non-fatal myocardial infarction or stroke. The mean follow-up period was three years. In univariate analyses, baseline levels of Lp-PLA2 were higher among cases than controls (mean 1.20 versus 1.05 mg/l, p= 0.016). However, after adjustment for random assignment to aspirin or vitamin E, LDL and HDL cholesterol, body mass index, history of hypertension, history of diabetes, parental history of myocardial infarction, frequency of exercise and current use of hormone replacement therapy, the effect was minimal and no longer statistically significant.

Atherosclerosis Risk in Communities (ARIC)

The ARIC study is a biracial cohort study of 15,792 men and women 45 to 64 years old, followed up for the subsequent development of a coronary heart disease event, including coronary heart disease related death. A case-cohort design was constructed, the final sample size for the analysis being 608 cases and 740 non-cases.[21] The age, race and sex adjusted mean level of Lp-PLA2 was higher in cases than in non-cases (404 versus 372 µg/l, p< 0.001). Lp-PLA2 levels in the highest tertile were associated with increased coronary heart disease risk in a model adjusted for age, sex and race (HR 1.78, 95% CI 1.33-2.38), however, after additional adjustment for LDL and HDL cholesterol, smoking status, systolic blood pressure, diabetes and CRP, the relative risk was attenuated and no longer significant. Further

Table 1. Lipoprotein-associated phospholipase A2 and cardiovascular events. RR = relative risk; HR = hazard ratio; SD = standard deviation.

Study	Authors, year	Subjects	Design	Cases	Non-cases	Determinant	Outcome	Association (multivariable adjusted)
WOSCOPS[19]	Packard et al., 2000	Hyperlipidemic men, mean age 56.8 years	Nested case control	580	1160	Lp-PLA2 level (mg/l)	Nonfatal myocardial infarction, death from coronary heart disease, revascularization	RR 1.18 (95% CI 1.05-1.33) per SD Lp-PLA2 level
WHS[20]	Blake et al., 2001	Apparently healthy women aged 45 years and over	Nested case control	123	123	Lp-PLA2 level (mg/l)	Nonfatal myocardial infarction, death from coronary heart disease, stroke	RR 1.17 (95% CI 0.45-3.05) for highest versus lowest quartile of Lp-PLA2 level
ARIC[21]	Ballantyne et al., 2004	Apparently healthy men and women aged 45-64 years	Case-cohort	608	740	Lp-PLA2 level (µg/l)	Incident coronary heart disease, including coronary heart disease related death	HR 1.15 (95% CI 0.81-1.63) for highest versus lowest tertile of Lp-PLA2 level. LDL cholesterol <130 mg/dl: HR 2.08 (95% CI 1.20-3.62) for highest versus lowest tertile of Lp-PLA2 level
ARIC[22]	Ballantyne et al., 2004	Apparently healthy men and women aged 45-64 years	Case-cohort	194	766	Lp-PLA2 level (µg/l)	Ischemic stroke	HR 1.93 (95% CI 1.14-3.27) for highest versus lowest tertile of Lp-PLA2 level
MONICA[23]	Koenig et al., 2004	Apparently healthy men aged 45 to 64 years	Cohort	97	837	Lp-PLA2 level (ng/ml)	Incident fatal or nonfatal acute myocardial infarction and sudden cardiac death	HR 1.21 (95% CI 1.01-1.45) per SD Lp-PLA2 level
Rotterdam Study[24]	Oei et al., 2005	Men and women aged 55 years and over	Case-cohort	308	1820	Lp-PLA2 activity (nmol *min^{-1} *ml^{-1})	Coronary heart disease	HR 1.20 (95% CI 1.04-1.39) per SD Lp-PLA2 activity
Rotterdam Study[24]	Oei et al., 2005	Men and women aged 55 years and over	Case-cohort	110	1820	Lp-PLA2 activity (nmol *min^{-1} *ml^{-1})	Ischemic stroke	HR 1.24 (95% CI 1.02-1.52) per SD Lp-PLA2 activity
	Brilakis et al., 2005 [25]	Men and women aged 26-76 years, undergoing clinically indicated coronary angiography	Cohort	61	405	Lp-PLA2 level (ng/ml)	Death, myocardial infarction, coronary revascularization, stroke	HR 1.30 (95% CI 1.06-1.59) per SD Lp-PLA2 level

analyses revealed that in individuals with low LDL cholesterol, elevated Lp-PLA2 was associated with a significantly higher risk for incident coronary heart disease, even after multivariable adjustment (HR 2.08, 95% CI 1.20-3.62). No significant associations were seen for individuals with higher LDL cholesterol.

The association between Lp-PLA2 and ischemic stroke was also evaluated in the ARIC study using a case-cohort design.[22] Mean Lp-PLA2 levels adjusted for sex, race and age were higher in the 194 cases than the 766 non-cases (443 vs 374 µg/l). In a model adjusted for age, sex, race, smoking status, systolic blood pressure, LDL and HDL cholesterol levels, antihypertensive medication and body mass index, Lp-PLA2 levels in the highest tertile were associated with a hazard ratio of stroke of 1.93 (95% CI 1.14-3.27).

Monitoring of trends and determinants in cardiovascular disease (MONICA) Augsburg Study

The MONICA study consisted of 4022 individuals sampled at random from a mixed urban/rural area. The study on Lp-PLA2 was based on 934 men aged 45 to 46 years, followed up for incident fatal or nonfatal acute myocardial infarction and sudden cardiac death.[23] Lp-PLA2 level was associated with an increase in coronary risk, with a hazard ratio of 1.21 (95% CI 1.01-1.45) per SD increase in Lp-PLA2 after adjustment for age, systolic blood pressure, total cholesterol/HDL cholesterol ratio, physical activity, body mass index, smoking, diabetes mellitus, alcohol intake, education and CRP.

Rotterdam Study

The Rotterdam Study is a prospective population-based cohort study comprising 7983 men and women aged 55 years or over. For the present investigation, a case-cohort design was used, with 308 coronary heart disease cases, 110 ischemic stroke cases and a random cohort of 1820 controls.[24] After adjustment for age, sex, body mass index, systolic blood pressure, non-HDL cholesterol, HDL cholesterol, diabetes, smoking, cholesterol-lowering medication, CRP, white blood cell count, and alcohol consumption, the hazard ratio of coronary heart disease was 1.20 (95% CI 1.04-1.39) per SD of Lp-PLA2 activity. The association was present over the entire range of cholesterol levels. For ischemic stroke, this hazard ratio was 1.24 (95% CI 1.02-1.52).

Lp-PLA2 and incident cardiovascular events in patients with coronary artery disease

Brilakis et al. evaluated the association between Lp-PLA2 level and incidence of major adverse events in 504 patients, aged 26-76 years, undergoing clinically indicated coronary angiography.[25] The most frequent indications for this angiography were acute coronary syndrome, an abnormal nuclear imaging study and dyspnoea upon exertion. Major adverse events were defined as death (including cardiac death), myocardial infarction, coronary revascularization and stroke. The hazard ratio for adverse events, adjusted for age, gender, smoking history, hypertension, total and HDL cholesterol, triglycerides, and log-CRP, was 1.30 (95% CI 1.06-1.59) per SD of Lp-PLA2, showing that Lp-PLA2 was an independent predictor.

Lp-PLA2 and coronary atherosclerosis

Several studies have examined the association between Lp-PLA2 and coronary artery disease ascertained by coronary angiography (table 2). Shohet et al. did so by means of two case-control studies.[26] First, Lp-PLA2 activity was compared between 72 patients with angiographic evidence of severe coronary atherosclerosis and 72 patients with angiographically normal coronary arteries. Cases and controls were matched for age and sex. Although Lp-PLA2 activity was slightly higher in cases, no significant differences could be demonstrated. To confirm these findings, a second study was undertaken in which 50 men with documented premature coronary artery disease and apparently healthy controls were matched for LDL cholesterol and for age. Again, although Lp-PLA2 activity was slightly higher in cases, it was not significantly different between cases and controls.

Caslake et al. performed a case-control study among 48 male subjects with coronary artery disease ascertained by coronary angiography, 46 male post myocardial infarction patients and 54 normal age matched controls.[27] Lp-PLA2 level was found to be associated with stenosis, independently of LDL and HDL cholesterol, smoking and systolic blood pressure.

Blankenberg et al. compared 496 coronary artery disease patients of both sexes suffering from stable angina pectoris or acute coronary syndrome, who had a lumen reduction > 30% in at least one major coronary artery, to 477 healthy control subjects.[28] They found that in the entire population, Lp-PLA2 activity was borderline associated with the presence of coronary artery disease, but the case/control difference seemed mainly present in women. However, when the coronary artery disease patients were divided according to stable or unstable angina, Lp-PLA2 activity appeared elevated in coronary artery disease patients suffering from acute coronary syndrome in both genders, even after controlling for age, body mass index (BMI), ever smoking, history of hypertension, LDL and HDL cholesterol and triglycerides. This association strengthened when excluding subjects receiving statin or ACE-inhibitor therapy, and doing so, there was a gradual increase in Lp-PLA2 activity among controls, stable angina pectoris patients and patients with acute coronary syndrome present in both genders.

In the above-mentioned study of Brilakis et al. in which adverse events were examined in 504 patients undergoing clinically indicated angiography, the association between Lp-PLA2 and angiographic coronary artery disease was also examined.[25] Although Lp-PLA2 levels were higher in patients with more extensive angiographic coronary artery disease, Lp-PLA2 was not independently predictive of angiographic coronary artery disease after adjusting for clinical and lipid variables.

Winkler et al. performed a case-control study in 2454 subjects with angiographically confirmed coronary artery disease and in 694 control subjects.[29] Lp-PLA2 activity was not associated with coronary artery disease in these subjects. However, after excluding subjects using lipid-lowering drugs (leaving 1630 subjects), Lp-PLA2 activity was associated with risk of coronary artery disease, with an odds ratio (OR) of 1.85 (95% CI 1.23-2.78) for the highest versus the lowest quartile of Lp-PLA2 activity after adjustment for aspirin, β-blockers, digitalis, age, gender, body mass index, smoking, diabetes, hypertension, CRP, fibrinogen, white blood cell count, serum amyloid A and LDL-cholesterol.

Finally, Khuseyinova et al. carried out a case-control study using 312 patients with a coronary stenosis of >= 50% of luminal diameter of at least one major coronary artery and 479 controls (occasional blood donors).[30] Mean Lp-PLA2 levels were found to be significantly higher in patients (296.1±122.5 ng/ml; mean±SD) compared to controls (266.0±109.8 ng/ml) ($p<0.0001$). After adjustment for age,

Table 2. Lipoprotein-associated phospholipase A2 and atherosclerosis. OR= odds ratio.

Authors, year	Subjects	Design	Cases	Non-cases	Determinant	Outcome	Association (multivariable adjusted)
Coronary atherosclerosis							
Shohet et al., 1999 [26]	Patients undergoing coronary angiography	Case-control	72	72	Lp-PLA2 activity (nmol *min^{-1} *ml^{-1})	Severe coronary disease (at least 1 vessel with >75% intraluminal obstruction)	Lp-PLA2 activity not significantly different (p>0.2)
Shohet et al., 1999 [26]	Men with documented premature coronary artery disease/ apparently healthy controls	Case-control	50	50	Lp-PLA2 activity (nmol *min^{-1} *ml^{-1})	Coronary artery bypass grafting, coronary angioplasty, angiographic evidence of >75% stenosis of at least 1 major coronary artery before the age of 60 in men and 65 in women	Lp-PLA2 activity not significantly different
Caslake et al., 2000 [27]	Men with stenotic disease on coronary angiography/ post myocardial infarction patients/ normal controls	Case-control	48/46	54	Lp-PLA2 level (ng/ml)	Stenotic disease/ myocardial infarction	General linear model: Lp-PLA2 level associated with coronary artery disease and post myocardial infarction status, p=0.01
Blankenberg et al., 2003 [28]	Coronary angiography patients with stable angina pectoris or acute coronary syndrome/ healthy control subjects	Case-control	496	477	Lp-PLA2 activity (nmol *min^{-1} *ml^{-1})	Coronary artery disease (lumen reduction >30%)	OR 1.8 (95% CI 1.01-3.2) for the highest versus the lowest quartile of Lp-PLA2 activity
Brilakis et al., 2005 [25]	Patients undergoing clinically indicated coronary angiography	Case-control	382	122	Lp-PLA2 level (ng/ml)	Coronary artery disease	No independent association
Winkler et al., 2005 [29]	Patients hospitalized for coronary angiography	Case-control	2454	694	Lp-PLA2 activity (U/l)	Coronary artery disease	OR 1.06 (95% CI 0.84-1.34) for highest versus lowest quartile of Lp-PLA2 activity. Non-users of lipid-lowering drugs: OR 1.85 (95% CI 1.23-2.78) for highest versus lowest quartile of Lp-PLA2 activity

Khuseyinova et al., 2005 [30]	Patients with coronary stenosis/ controls (occasional blood donors)	Case-control	312	479	Lp-PLA2 level (ng/ml)	Coronary stenosis of >= 50% of luminal diameter	OR 1.84 (95% CI 1.12-2.99) for the highest versus the lowest quartile of Lp-PLA2 level
Iribarren et al., 2005 [31]	Men and women aged 18-30	Nested case-control	266	266	Lp-PLA2 level (ng/ml) and Lp-PLA2 activity (nmol $*min^{-1}$ $*ml^{-1}$)	Presence of calcified coronary plaque ascertained by cardiac computed tomography	Highest versus lowest tertile: Lp-PLA_2 level, OR 1.28 (95% CI 1.03-1.60); Lp-PLA2 activity, OR 1.09 (95% CI 0.84-1.42)
Extracoronary atherosclerosis							
Campo et al., 2004 [32]	Men and women with primary hypercholesterolemia	Case-control	76	114	Lp-PLA2 activity (U/l)	Intima-media thickness > 1mm	No association
Santos et al., 2004 [33]	Patients referred for lower extremity arterial evaluation	Cross-sectional	247		Lp-PLA2 level (ng/ml)	Ankle-brachial index	Multiple regression model: Lp-PLA2 level associated with ankle-brachial index,p=0.05

gender, BMI, smoking status, alcohol intake, school education years, hypertension, diabetes, total and HDL cholesterol, the odds ratio of coronary artery disease was 1.84 (95% CI 1.12-2.99) for the highest versus the lowest quartile of Lp-PLA2 concentration.

A study on the association between Lp-PLA2 and coronary calcification ascertained by computed tomography was performed by Iribarren et al. It was a nested case-control study among participants of the Coronary Artery Risk Development in Young Adults (CARDIA) study, an ongoing investigation of heart disease risk factors and subclinical coronary artery disease among black and white men and women aged 18-30 years.[31] Cases (n=266) were those with and controls (n=266) those without evidence of calcified coronary plaque assessed by computed tomography. The age adjusted odds ratio of calcified coronary plaque per SD increment was 1.40 (95% CI 1.17 -1.67) and 1.39 (95% CI 1.14-1.70) for Lp-PLA_2 mass and activity, respectively. After adjusting for multiple covariates including LDL and HDL cholesterol, triglycerides, and CRP, a statistically significant association remained for Lp-PLA2 level (OR 1.28 (95% CI 1.03-1.60)) but not for activity (OR 1.09 (95% CI 0.84-1.42)). The reason for the differential effect of adjustment may have been the stronger correlation between enzymatic activity and LDL-cholesterol (r=0.52) than between enzymatic level with LDL-cholesterol (r=0.39).

Lp-PLA2 and extracoronary atherosclerosis

Campo et al. performed a study among 190 hypercholesterolemic Sicilian individuals, and found no association between Lp-PLA2 activity and carotid intima-media thickness (IMT).[32] Patients with abnormal carotid IMT (>1 mm) had a mean Lp-PLA2 activity of 471.3 U/l, whereas in controls this was 463.7 U/l, the difference not being statistically significant. Only unadjusted values of mean plasma Lp-PLA2 activity for subjects with normal and high carotid IMT were presented in this study. Furthermore, none of the established cardiovascular risk factors was found to be associated with IMT, most probably owing to small sample size.

Santos et al. investigated the association between Lp-PLA2 level and ankle-brachial index among 247 patients referred for lower extremity arterial evaluation.[33] In a multiple regression model that included univariate predictors of ankle-brachial index (age, hypertension, smoking, fasting plasma glucose) and statin use, Lp-PLA2 was a borderline-significant predictor of lower ankle-brachial index ($p = 0.05$).

Genetic polymorphisms affecting Lp-PLA2

Lp-PLA2 is encoded by a gene located at 6p12-p21.1. The gene is organized in 12 exons spanning at least 45 kb of DNA sequence.[34] In the Japanese, a point mutation, Val279Phe, has been found in exon 9. This mutation completely abolishes enzymatic activity of Lp-PLA2 in homozygotes, and lowers enzymatic activity in heterozygotes compared to wild type. 27% of the Japanese population was found to be heterozygous for the mutant allele and 4% was homozygous.[34] This mutation has been shown to be associated with ischemic stroke, coronary artery disease, atherosclerosis, and abdominal aortic aneurysm in Japanese, [35-39] and thus these results suggest that Lp-PLA2 may play a protective role in

cardiovascular disease. However, the findings of these studies could not be reproduced in a large-scale study in 2819 Japanese patients with myocardial infarction and 2242 Japanese controls.[40]

In white populations, the Val279Phe mutation has not been found. However, several common variants are present. In vitro, the Ala379Val variant resulted in a 2-fold decrease in the affinity of Lp-PLA2 for its substrate PAF, resulting in reduced degradation of PAF.[41] Abuzeid et al. performed a European case-control study, which compared 527 post-myocardial infarction men with 566 age-matched controls.[42] Homozygosity for the Val allele was independently associated with lower risk of myocardial infarction. Since the Val allele results in lower Lp-PLA2 activity, this study supported the pro-atherogenic, causal role of Lp-PLA2 in coronary heart disease. These findings do not concur with the findings of Ninio et al.[43] Using a prospective cohort of 1314 coronary artery disease patients and a group of 485 healthy controls, they found that the Val allele was associated with an increased Lp-PLA2 activity. Still, the Val allele was associated with a lower risk of future cardiovascular events and appeared less frequent in coronary artery disease cases than in controls. Campo et al. examined the Arg92His, Ile198Thr and Ala379Val variants in 190 hypercholesterolemic Sicilian individuals.[32] They found no associations of these variants with Lp-PLA2 activity, and no associations with carotid IMT > 1 mm. In summary, the findings from the genetic studies performed until now appear to be heterogeneous.

Discussion

Recently, a large amount of epidemiological research has emerged on the association between Lp-PLA2 and cardiovascular disease, overall suggesting an independent, pro-atherogenic role for Lp-PLA2 in cardiovascular disease. Several issues remain to be further addressed.

The first issue is, whether the effect of Lp-PLA2 on cardiovascular events is truly independent of LDL-cholesterol, to which it is bound. The results from WOSCOPS suggested that elevated levels of Lp-PLA2 are a strong risk factor for coronary heart disease that is independent of LDL-cholesterol. However, in the WHS, after adjusting for LDL-cholesterol, the effect of Lp-PLA2 was minimal and no longer statistically significant. A clear reason for the discrepancy of the results from the WHS with the results from WOSCOPS could not be found. Use of hormone replacement therapy in the WHS may have played a part, however, this was not likely, since prevalence of use did not differ among cases and controls, and no effect modification due to this factor was found. Furthermore, statistical power may have been low in the WHS due to small sample size (123 cases and 123 controls). Finally, the authors suggested the possibility that the predictive value of Lp-PLA2 may be limited to subjects with overt hyperlipidemia, as used in WOSCOPS. However, studies that followed also found associations between Lp-PLA2 and cardiovascular disease in non-hyperlipidemic subjects. The ARIC study results showed that, in individuals with low LDL cholesterol, elevated Lp-PLA2 was associated with a significantly higher risk for incident coronary heart disease, even after multivariable adjustment. In the total number of individuals, however, the association was not independent of LDL cholesterol. Differing results between this study and the two above-mentioned studies were suggested to be attributable to the markedly different study populations that were used. What followed was the MONICA study, again showing an independent association between Lp-PLA2 and coronary events. Since the average total cholesterol level in the MONICA study was lower than in WOSCOPS but higher than in ARIC, this study

suggested that Lp-PLA2 has the ability to predict coronary events across all levels of total cholesterol. The Rotterdam Study provided further evidence for this by finding associations independent of cholesterol, as did the study by Brilakis et al. Overall, most findings seem to support an independent role for Lp-PLA2 in cardiovascular risk prediction.

Another issue is, whether the effect of Lp-PLA2 on cardiovascular events is exerted through atherosclerosis, or whether other mechanisms may contribute. With regard to Lp-PLA2 and coronary atherosclerosis, the studies by Caslake et al., Blankenberg et al., Winkler et al. and Khuseyinova et al. suggest independent associations, implying that Lp-PLA2 indeed exerts its effect through atherosclerosis. However, the study by Brilakis et al. does not show an independent association with coronary atherosclerosis, although in the same study Lp-PLA2 was independently associated with cardiovascular events. An explanation for the discordance between the association of Lp-PLA2 with angiographic coronary artery disease and the association with adverse events in this study might be the concept that molecules that regulate inflammation will not necessarily correlate with plaque burden measures, and may represent other characteristics than atherosclerotic mass, such as the inflammatory activity within plaques or the degree of plaque destabilization and ongoing ulceration or thrombosis. This study raises the question whether Lp-PLA2 truly measures the extent of atherosclerosis, or whether it may be a marker of vulnerable plaque activity, which is further supported by the fact that Lp-PLA2 is strongly expressed in macrophages within the fibrous cap of rupture prone lesions.[5] To answer this, further investigation is required. The same issue has been raised with regard to C-reactive protein, which, in spite of its ability to predict cardiovascular events, does not seem to correlate equally well with atherosclerosis.[44]

Lp-PLA2 has been suggested to have both pro-atherogenic and anti-atherogenic properties. In humans, given that high levels of Lp-PLA2 are associated with cardiovascular events, evidence is building up that the pro-atherogenic properties of Lp-PLA2 outweigh the anti-atherogenic properties. Until now, genetic research appears to yield inconclusive results with regard to this matter. The issue may be further resolved by research using Lp-PLA2 inhibitors. Potent Lp-PLA2 inhibitors have already been discovered [10] and tested for their ability to lower enzyme activity in plasma and at vascular sites.[45] In the future, randomized controlled trial using these compounds may demonstrate the consequences of reducing Lp-PLA2 activity.

The final issue that needs to be investigated is a potential gender difference. The results from the WHS were not in agreement with studies showing an independent association between Lp-PLA2 and cardiovascular events, possibly due to gender differences. Lp-PLA2 has been found to be higher in men than in women.[21,24,25] Furthermore, animal models have shown that estrogen decreases Lp-PLA2 activity,[46] and Lp-PLA2 levels are higher in women not taking hormone replacement therapy than among women taking hormone replacement therapy.[20] Further research regarding Lp-PLA2 in populations stratified by gender, and also by other traditional risk factors such as age, is therefore supported.

In summary, studies regarding incident cardiovascular events suggest an independent, pro-atherogenic role for Lp-PLA2. Studies on coronary atherosclerosis seem to lean into the same direction. With regard to extracoronary atherosclerosis, less research has been performed. Genetic studies have yielded heterogeneous results so far. In the future, trials using Lp-PLA2 inhibitors may provide more insight into the mechanisms of action of Lp-PLA2 and its usefulness for cardiovascular risk reduction.

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Chapter 3.2

Lipoprotein-associated phospholipase A2 activity and coronary calcification

Abstract

Objectives. Although several studies have recently suggested that lipoprotein-associated phospholipase A2 (Lp-PLA2) is an independent predictor of coronary events, only one study has examined the association between Lp-PLA2 and coronary calcification, using young adults. We investigated the association between Lp-PLA2 activity and coronary calcification assessed by electron-beam computed tomography (EBT) in a population of older participants.

Methods and results. The Rotterdam Coronary Calcification Study is a population-based study in men and women aged ≥55 years. Coronary calcification assessed by EBT was quantified in a calcium score according to Agatston's method. Lp-PLA2 activity measured in samples collected 7 years before scanning (n=520) was associated with coronary calcification in men after adjustment for age. The odds ratio per standard deviation of Lp-PLA2 activity of having a total calcium score >1000 was 1.6 (95% confidence interval 1.1-2.4), as compared to a total calcium score ≤100. After adjustment for non-HDL and HDL-cholesterol, this association disappeared. In women, the association was less consistent. For Lp-PLA2 measured concurrently to scanning (n=703), no association was found with coronary calcification.

Conclusions. Lp-PLA2 activity is moderately associated with coronary calcification after adjustment for age. The effect of Lp-PLA2 on coronary calcification may be exerted through its effect on LDL-cholesterol.

Introduction

Lipoprotein-associated phospholipase A2 (Lp-PLA2) has lately gained interest as a predictor of cardiovascular disease. Several recent studies have found an independent association between Lp-PLA2 and coronary events.[1-4] Basic science implies that the effect of Lp-PLA2 on coronary heart disease is exerted through effects of the enzyme on the development of atherosclerosis. Lp-PLA2 hydrolyzes oxidatively modified low-density lipoprotein (LDL) by cleaving oxidized phosphatidylcholines, generating lysophosphatidylcholine and oxidized free fatty acids. Lysophosphatidylcholine and oxidized free fatty acids are both chemoattractants for monocytes and may account for a part of the pro-inflammatory capacities of oxidized LDL-cholesterol.[5]

Case-control studies in high-risk participants report independent associations of Lp-PLA2 with angiographically documented coronary artery disease.[6-9] One case-control study among patients undergoing clinically-indicated coronary angiography found that although Lp-PLA2 mass was associated with severity of angiographic coronary artery disease, the association did not persist after adjustment for lipids and other cardiovascular risk factors.[10]

Only one study has been conducted on Lp-PLA2 and coronary calcification. In a nested case-control study among young adults participating in the CARDIA study, an independent association was found of Lp-PLA2 mass with calcified coronary plaque.[11] No studies have been reported on Lp-PLA2 and coronary calcification in older adults. In a population-based cohort study in men and women aged ≥55 years, we investigated whether Lp-PLA2 activity is associated with coronary calcification detected by electron-beam computed tomography (EBT).

Methods

Study population

The Rotterdam Coronary Calcification Study is designed to study determinants and consequences of coronary calcification, detected by EBT. The study is embedded in the Rotterdam Study, a population-based study aimed at addressing the occurrence of and risk factors for chronic disease in the elderly, which started with a baseline visit between 1990 and 1993. All inhabitants of a suburb of Rotterdam, aged ≥55 years, were invited (response 78%). The rationale and design of the Rotterdam Study have been described elsewhere.[12] Follow-up visits took place in 1993 to 1994 and 1997 to 1999. From 1997 onwards, participants through 85 years of age completing the third phase of the Rotterdam Study were invited to participate in the Rotterdam Coronary Calcification Study and to undergo an EBT scan. They were scanned from 1997 to 2000. Of the 3371 eligible participants, scans were obtained for 2063 participants (response 61%). Due to several causes, i.e. metal clips from cardiac surgery, severe artifacts and registration errors (ECG, acquisition), image acquisition data could not be reconstructed or analyzed in 50 participants. Thus, calcium scores were available for 2013 participants.

Measurements of Lp-PLA2 activity performed at the third phase of the Rotterdam Study, and thus concurrently to EBT scanning, were available in a random sample of 703 participants. Of these 703, baseline measurements of Lp-PLA2 activity could be performed in 520 participants. The median duration between risk factor assessment and EBT scanning was 7 years (1990-1993) and 50 days (1997-1999).

The Medical Ethics Committee of Erasmus MC approved the study, and all participants gave informed consent.

Measurement of Lp-PLA2 activity

Plasma aliquots prepared from blood samples were collected and stored at -80°C, and Lp-PLA2 activity was measured with a high throughput radiometric activity assay as described in detail previously.[1] Lp-PLA2 activity was expressed as nanomoles of platelet-activating factor hydrolyzed per minute per milliliter of plasma samples. Prior to analysis of plasma samples from the Rotterdam Coronary Calcification Study, a pre-study validation was conducted to determine the reliability of the Lp-PLA2 activity assay. The coefficient of variation (CV) for intra-assay precision ranged from 3.51-8.96%. The CV for inter-assay precision ranged from 8.48 -15.08%. Three cycles of freeze-thaw of frozen plasma did not result in appreciable loss of activity. The assay was therefore considered suitable for analysis of the Rotterdam Coronary Calcification Study samples, which were tested in duplicate. Samples were re-tested if the replicate CV was > 25%. The range of detection was 8-150 nmol/min per ml.

Coronary calcification

We assessed coronary calcification in the epicardial coronary arteries detected on EBT scans. Imaging was performed with a C-150 Imatron scanner (Imatron, South San Francisco, CA, USA). Before the participants were scanned, they exercised breath holding. From the level of the root of the aorta through the heart, 38 images were obtained with 100 ms scan time and 3 mm slice thickness. We acquired images at 80% of the cardiac cycle, using electrocardiogram triggering, during a single breathhold. The scanner was calibrated on a daily basis using a water phantom. Quantification of coronary calcification was performed with AccuImage software (AccuImage Diagnostics Corporation, South San Francisco, CA, USA) displaying all pixels with a density of over 130 Hounsfield Units. Trained scan readers were blinded to the clinical data of the participants. The presence of calcification was defined as a minimum of two adjacent pixels (area=0.52 mm^2) with a density over 130 Hounsfield Units. We placed a region of interest around each high-density lesion in the epicardial coronary arteries. The peak density in Hounsfield Units and the area in mm^2 of the individual coronary calcifications were calculated. A calcium score was obtained by multiplying each area of interest with a factor indicating peak density within the individual area, as proposed by Agatston et al.[13] We added the scores for individual calcifications, resulting in a calcium score for the entire epicardial coronary system.

Assessment of covariates

The Rotterdam Coronary Calcification Study is embedded in the ongoing Rotterdam Study. Therefore, information was available on risk factors assessed 7 years before EBT scanning (1990-1993) and concurrently to EBT scanning (1997-1999). Apart from blood sampling methods, protocols for the interview and clinical examination were identical at both examinations. Information obtained in the home interview included current health status, medical history, drug use, and smoking. Clinical measures were obtained during a visit to the research center. Height and weight were measured and the body mass index was calculated (weight (kg)/length (m^2)). Blood pressure was measured at the right brachial artery using a random-zero sphygmomanometer with the participant in sitting position.

In 1990–1993 non-fasting blood samples were obtained while in 1997–1999 blood samples were obtained after an overnight of fasting. Laboratory techniques were identical for both periods: serum total cholesterol was determined by an enzymatic procedure,[14] and high-density lipoprotein (HDL) cholesterol was measured similarly after precipitation of the non-HDL fraction. Non-HDL cholesterol was computed by subtracting HDL cholesterol from total cholesterol. LDL cholesterol was determined in fasting blood samples in 120 randomly selected participants (1990-1993) using an enzymatic method (Roche, Mannheim, Germany). The correlation coefficient between non-HDL cholesterol and LDL-cholesterol was high ($r=0.97$, $p<0.001$). Glucose was determined enzymatically by the Hexokinase method. Diabetes was defined as the use of anti-diabetic medication and/or non-fasting glucose levels > =11.1 mmol/l (1990–1993) and/or fasting glucose levels >=7.0 mmol/l (1997–1999).[15] Using a nephelometric method (Immage®, Beckman Coulter), C-reactive protein was measured in blood samples, which were kept frozen at -20 °C (1990-1993) and at –80°C (1997-1999).

Statistical analysis

The analysis was performed similarly for the subcohort of 520 participants (1990-1993) and the subcohort of 703 participants (1997-1999). Firstly, we tested for differences between the populations for analysis and the remainder of the Rotterdam Coronary Calcification Study by using a t-test for continuous and a chi-square test for nominal variables. The Mann Whitney test was used for C-reactive protein and total calcium score, because their distributions were skewed.

Since male sex is an important determinant of coronary artery calcification,[16] all analyses were conducted separately in men and women. We performed linear regression analysis to assess the relationship between Lp-PLA2 activity and total calcium score measured on continuous scales. The distribution of the residuals was highly skewed when we used the total calcium score for this purpose. After log-transformation of the total calcium score, the residuals were normally distributed with a constant variance. Therefore, log (total calcium score +1) was used for linear regression analysis. We used Lp-PLA2 activity as the independent variable and the log total calcium score as the dependent variable. We computed the increase in log total calcium score per (sex-specific) standard deviation increase of Lp-PLA2 activity. Correlations of Lp-PLA2 activity with several cardiovascular risk factors have been demonstrated previously in the Rotterdam Study.[1] Therefore, we adjusted the analysis: in model 1, we adjusted for age; in model 2, we additionally adjusted for non-HDL cholesterol and HDL-cholesterol; in model 3, we also added body mass index, systolic blood pressure, diabetes mellitus, smoking, C-reactive protein, cholesterol lowering medication, and in women hormone replacement therapy.

Subsequently, we set out to investigate whether mean log(calcium scores) differ for tertiles of Lp-PLA2 activity. For this purpose, we divided Lp-PLA2 activity into sex-specific tertiles (cut-offs in men and women 41 and 50 nmol/min per ml and 36 and 46 nmol/min per ml respectively for the 1990-1993 cohort, and 40 and 48 nmol/min per ml and 34 and 42 nmol/min per ml respectively for the 1997-1999 cohort), and performed analysis of covariance. Geometric means of the calcium scores and their confidence intervals were computed. We used the above-mentioned three models for adjustment.

Finally, to obtain relative risks of having a high calcium score per (sex-specific) standard deviation of Lp-PLA2 activity, multinomial regression was used. For this purpose, coronary calcium score was

divided into four categories, namely 0 to 100, 101-500, 501-1000 and above 1000. Again, the above three models were used for adjustment.

Values for cardiovascular risk factors were missing in less than 2% of participants in both subcohorts, except for C-reactive protein, which was missing in 5% (1990-1993) and diabetes mellitus, which was missing in 8% (1997-1999). Missing values were handled by imputing the mean for normally distributed variables, the median for skewed variables, and the value with the highest prevalence for nominal variables. Analyses were performed with SPSS 11.0 for Windows (SPSS, Inc, Chicago, IL, USA).

Results

The characteristics of the study population are shown in table 1. The characteristics of both subcohorts were similar to the characteristics of the remaining population of the Rotterdam Coronary Calcification Study with a few minor exceptions. Participants in the 1990-1993 subcohort had a slightly lower systolic blood pressure (133 mm Hg versus 134 mm Hg) and a slightly higher HDL-cholesterol (1.38 mmol/l versus 1.35 mmol/l). Participants in the 1997-1999 subcohort had a slightly lower dia-

Table 1. Study population characteristics 7 years before scanning (1990-1993) and concurrently to scanning (1997-1999).

	7 years before scanning		Concurrently to scanning	
Variable	**Subcohort (n=520)**	**Total (n=2013)**	**Subcohort (n=703)**	**Total (n=2013)**
Age (years)	63.8±5.3	64.2±5.5	71.5±5.7	71.3±5.7
Men (%)	45	46	47	46
Women using hormone replacement therapy (%)	16	21	19	22
Body mass index (kg/m^2)	26.3±3.4	26.3±3.4	27.0±3.8	27.0±3.9
Systolic blood pressure (mm Hg)	133±19	134±20	142±21	143±21
Diastolic blood pressure (mm Hg)	73±11	74±11	75±11	76±11
Non-HDL cholesterol (mmol/l)	5.4±1.2	5.4±1.2	4.5±0.9	4.4±1.0
HDL-cholesterol (mmol/l)	1.38±0.42	1.35±0.38	1.38±0.39	1.38±0.39
Diabetes (%)	6	6	13	12
Smokers (%)				
- Current	23	22	16	16
- Past	48	48	54	54
C-reactive protein (mg/l)*	1.6 (0.8-3.1)	1.6 (0.8-3.1)	2.5 (1.2-4.6)	2.4 (1.3-4.5)
Cholesterol lowering medication (%)	3.7	3.2	13.4	14.5
History of myocardial infarction (%)	8.2	9.5	10.0	11.5
Calcium score*	119 (12-567)#		139 (14-581)	134 (13-578)

Categorical variables are expressed as percentage. Values of continuous variables are expressed as mean ± standard deviation.

*Expressed as median and interquartile range due to the skewed distribution.

#Calcium score measured in 1997-2000 for the subcohort of 520 participants.

Table 2. Regression coefficients for the increase in log(calcium score) per standard deviation increase of lipoprotein-associated phospholipase A2 activity.

		Regression coefficient (95% confidence interval)	P-value
Lp-PLA2 measured in samples, collected 7 years before scanning			
Men	Model 1	0.30 (0.03, 0.58)	0.03
	Model 2	-0.02 (-0.33, 0.29)	0.91
	Model 3	-0.06 (-0.38, 0.25)	0.70
Women	Model 1	0.23 (-0.30, 0.32)	0.08
	Model 2	0.01 (-0.03, 0.50)	0.96
	Model 3	0.07 (-0.24, 0.37)	0.67
Lp-PLA2 measured concurrently to scanning			
Men	Model 1	0.07 (-0.32, 0.19)	0.51
	Model 2	-0.07 (-0.15, 0.30)	0.62
	Model 3	0.06 (-0.19, 0.31)	0.64
Women	Model 1	0.01 (-0.22, 0.24)	0.91
	Model 2	-0.07 (-0.33, 0.20)	0.63
	Model 3	0.03 (-0.24, 0.29)	0.85

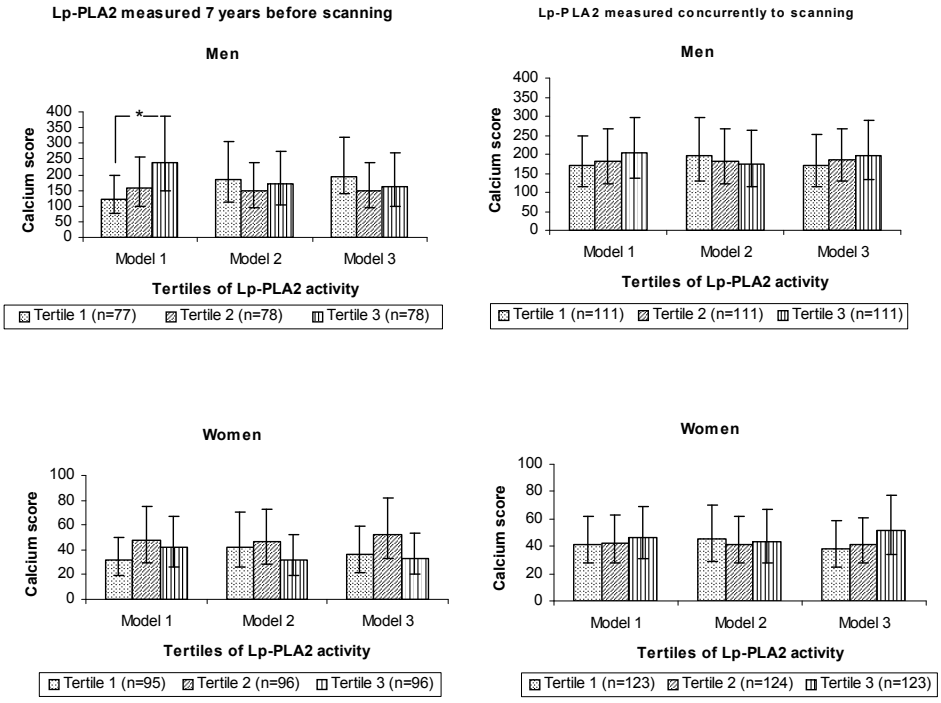
Model 1: adjusted for age; model 2: adjusted for age, non-HDL cholesterol and HDL-cholesterol; model 3: adjusted for age, non-HDL cholesterol, HDL-cholesterol, body mass index, systolic blood pressure, diabetes mellitus, smoking, C-reactive protein, cholesterol lowering medication and in women also for hormone replacement therapy.

stolic blood pressure (75 mmHg versus 76 mmHg). Mean Lp-PLA2 activity in the 1990-1993 subcohort was 44 (standard deviation 11) nmol/min per ml plasma, in the 1997-1999 subcohort this was 42 (standard deviation 11) nmol/min per ml plasma.

Table 2 provides regression coefficients describing the increase in log calcium score per standard deviation increase of Lp-PLA2 activity. After adjustment for age, there was a significant association between Lp-PLA2 activity measured in samples collected 7 years before EBT scanning and coronary calcification in men. In women, the regression coefficient was of a similar magnitude, but the association did not reach statistical significance. When additional adjustment for non-HDL and HDL-cholesterol was performed, the association disappeared. Subsequent adjustment for other cardiovascular risk factors did not materially change the estimates. In contrast, there was no association between Lp-PLA2 activity measured concurrently to EBT scanning and coronary calcification. Geometric means of calcium scores for tertiles of Lp-PLA2 activity are displayed in figure 1. For Lp-PLA2 activity measured in samples collected 7 years before EBT scanning, the calcium score in the highest tertile of Lp-PLA2 activity was higher than the calcium score in the lowest tertile after adjustment for age in men. This association was borderline significant (p=0.052). After adjustment for cholesterol, this difference disappeared. Subsequent adjustment for other cardiovascular risk factors did not further change the estimates. For Lp-PLA2 activity measured concurrently to EBT scanning, there were no significant differences in calcium scores in tertiles of Lp-PLA2 activity using all three models.

For Lp-PLA2 activity measured in samples collected 7 years before EBT scanning, after adjustment for age, in men, the odds ratio per standard deviation of Lp-PLA2 activity of having a total calcium score in the highest category was 1.6 (95% confidence interval 1.1-2.4), as compared to a total calcium

Figure 1. Geometric means and 95% confidence intervals of total calcium score for tertiles of Lipoprotein-associated phospholipase A2 activity.



* P-value = 0.052. Model 1: adjusted for age; model 2: adjusted for age, non-HDL cholesterol and HDL-cholesterol; model 3: adjusted for age, non-HDL cholesterol, HDL-cholesterol, body mass index, systolic blood pressure, diabetes mellitus, smoking, C-reactive protein, cholesterol lowering medication, and in women also for hormone replacement therapy.

score in the lowest category (table 3). For the second-highest category, this was 1.7 (95% CI 1.1-2.6). This association disappeared after additional adjustment for cholesterol. In women, no association could be demonstrated. For LpPLA2 activity measured concurrently to EBT scanning, no association was found with coronary calcification in both sexes.

Table 3. Odds ratios and 95% confidence intervals for severity of coronary calcification per standard deviation of lipoprotein-associated phospholipase A2 activity.

		Calcium score			
		0-100	101-500	501-1000	>1000
Lp-PLA2 measured in samples, collected 7 years before scanning					
Men	OR (model 1)	1.0 (reference)	1.2 (0.9-1.8)	1.7 (1.1-2.6)	1.6 (1.1-2.4)
	OR (model 2)	1.0 (reference)	0.9 (0.6-1.4)	1.3 (0.8-2.3)	1.2 (0.8-1.8)
	OR (model 3)	1.0 (reference)	0.9 (0.6-1.4)	1.4 (0.8-2.3)	1.1 (0.7-1.7)
Women	OR (model 1)	1.0 (reference)	1.2 (0.9-1.6)	1.0 (0.7-1.5)	1.1 (0.6-1.7)
	OR (model 2)	1.0 (reference)	1.0 (0.7-1.4)	0.8 (0.5-1.3)	0.9 (0.5-1.6)
	OR (model 3)	1.0 (reference)	1.0 (0.7-1.4)	0.7 (0.4-1.3)	1.0 (0.5-1.8)
Lp-PLA2 measured concurrently to scanning					
Men	OR (model 1)	1.0 (reference)	1.0 (0.7-1.3)	1.2 (0.9-1.7)	1.2 (0.9-1.6)
	OR (model 2)	1.0 (reference)	0.8 (0.6-1.1)	1.1 (0.7-1.6)	1.1 (0.7-1.5)
	OR (model 3)	1.0 (reference)	0.8 (0.6-1.2)	1.1 (0.8-1.7)	1.1 (0.8-1.6)
Women	OR (model 1)	1.0 (reference)	1.0 (0.8-1.3)	0.9 (0.7-1.3)	0.8 (0.5-1.2)
	OR (model 2)	1.0 (reference)	1.0 (0.8-1.4)	0.9 (0.6-1.4)	0.8 (0.5-1.3)
	OR (model 3)	1.0 (reference)	1.1 (0.8-1.5)	0.9 (0.6-1.5)	0.9 (0.5-1.6)

Model 1: adjusted for age; model 2: adjusted for age, non-HDL cholesterol and HDL-cholesterol; model 3: adjusted for age, non-HDL cholesterol, HDL-cholesterol, body mass index, systolic blood pressure, diabetes mellitus, smoking, C-reactive protein, cholesterol lowering medication and in women also for hormone replacement therapy.

Discussion

Although Lp-PLA2 activity has been found to be independently associated with cardiovascular events in the Rotterdam Study,[1] its association with coronary calcification seems less consistent. Lp-PLA2 activity measured in samples collected 7 years before EBT scanning was associated with coronary calcification after adjustment for age in men. In women, the association did not reach statistical significance. After adjustment for non-HDL cholesterol and HDL-cholesterol, the association disappeared. Lp-PLA2 activity measured concurrently to EBT scanning was not associated with coronary calcification.

The biological role of Lp-PLA2 in atherogenesis has been studied extensively. By hydrolyzing oxidized phospholipids, the enzyme is capable of generating two bioactive lipids, lysophosphatidylcholine and oxidized fatty acids, within oxidized LDL. These products are both chemoattractants for monocytes, suggesting that Lp-PLA2 has a pro-inflammatory role in atherogenesis.[5] However, Lp-PLA2, also known as platelet-activating factor acetylhydrolase (PAF-AH), is also suggested to have anti-inflammatory properties[17] by hydrolyzing platelet-activating factor, which plays a role in the activation of platelets, monocytes and macrophages.[18] The positive association found between Lp-PLA2 and incident cardiovascular disease[1-4] implies that the pro-inflammatory effects outweigh the anti-inflammatory effect of the enzyme. The moderate association of Lp-PLA2 activity with coronary calcification in men, demonstrated in this study, supports this notion, the disappearance of the association after adjustment for cholesterol suggesting that the effect of Lp-PLA2 on coronary calcification may be exerted through its effect on LDL-cholesterol. In women, the analysis using calcium score

as a continuous variable rendered a regression coefficient of a similar magnitude as in men, but it did not reach statistical significance. Failure to demonstrate the association in women in the analysis using calcium score as a categorical variable, may have been caused by the fact that less women had calcium scores in the high categories.

In the present study, Lp-PLA2 activity was measured in plasma samples drawn 7 years before EBT scanning and samples drawn concurrently to EBT scanning. Previously, it has been demonstrated within the Rotterdam Coronary Calcification Study that established cardiovascular risk factors measured 7 years before EBT scanning are strongly associated with the amount of coronary calcification, while some of these associations attenuate when risk factors are measured simultaneously to EBT scanning.[16] This concurs with the observation that the predictive value of cardiovascular risk factors attenuates with increasing age,[19-21] and may explain why an association was found between Lp-PLA2 activity measured 7 years before EBT scanning and coronary calcification, while this association was not found for Lp-PLA2 activity measured concurrently to EBT scanning. Another explanation may be that the strength of the association increases with increasing time between assessment of Lp-PLA2 activity and the measurement of coronary calcification. A point of mention here is that, in the presently used subcohorts, total cholesterol level measured 7 years before scanning was independently associated with coronary calcification, while for total cholesterol measured concurrently to scanning, the association disappeared after multivariable adjustment (data not shown).

Although the independent association of Lp-PLA2 with coronary events has recently been demonstrated in several population-based studies,[1-4] the association between Lp-PLA2 and coronary atherosclerosis has mostly been examined in case-control studies with high-risk subjects. In a case-control study among male subjects experiencing symptoms of angina, Lp-PLA2 mass was found to be independently associated with positive angiograms.[7] This study was performed in a relatively small number of high-risk subjects, which were younger than the subjects in our population. Furthermore, the severity of disease was not recorded in the study, and angiograms were defined as positive when stenotic disease was present, which contrasts with the continuous measures of asymptomatic coronary calcification in our study. Similarly, Lp-PLA2 activity was independently associated with presence of coronary artery disease in a case-control study with men and women with presence of an angiographically determined stenosis >30% in at least one major coronary artery experiencing acute coronary syndrome or stable angina and healthy control subjects,[8] in a case-control study in patients with angiographic evidence of coronary artery disease and in age- and gender-matched blood donors,[6] and among patients hospitalized for coronary angiography who were not using lipid-lowering drugs.[9] Conversely, in a study enrolling 504 patients undergoing clinically indicated coronary angiography, Lp-PLA2 mass was found to be associated with severity of angiographically determined coronary artery disease, but after adjustment for clinical and lipid variables, this association disappeared.[10] Interestingly, in the same study, an independent relationship was found between Lp-PLA2 and cardiovascular events. Although this was also a study in high-risk subjects and the participants used were younger than the participants in the Rotterdam Study, these results seem to be in accordance with the results of the Rotterdam Study, which also showed that Lp-PLA2 activity was independently associated with cardiovascular events[1] but was not independently associated with coronary calcification. Regarding this issue, it needs to be considered that molecules that regulate inflammation will not necessarily correlate with plaque burden measures, as illustrated by inflammatory markers such as

C-reactive protein. Although C-reactive protein is associated with coronary events, it has been a less consistent predictor of the extent of atherosclerotic disease, and may measure other characteristics than atherosclerotic mass, such as the activity of lymphocyte and macrophage populations within plaque or the degree of plaque destabilization and ongoing ulceration or thrombosis.[22] The same may apply to Lp-PLA2.

To our knowledge, only one study has been reported on the association of Lp-PLA2 with coronary calcification. In a nested case-control study among CARDIA participants, an independent association was found of Lp-PLA2 with calcified coronary plaque in young adults.[11] Subjects in the highest tertile of Lp-PLA2 mass had a relative risk of 2.15 for coronary artery calcification compared to subjects in the lowest tertile of Lp-PLA2 mass. For subjects in the highest tertile of Lp-PLA2 activity, the relative risk was 2.40 compared to the lowest tertile. After adjustment for cholesterol, the association of Lp-PLA2 mass with calcified coronary plaque remained materially the same, and the association of Lp-PLA2 activity with calcified coronary plaque attenuated. The reason for the differential effect of adjustment may be the stronger correlation between enzymatic activity and LDL-cholesterol (r=0.52) than between enzymatic mass with LDL-cholesterol (r=0.39). The finding of an independent association between Lp-PLA2 measured concurrently to scanning and coronary calcification in the CARDIA study contrasts with our findings. The disappearance of the relation between Lp-PLA2 measured 7 years before scanning and coronary calcification after adjustment for cholesterol in our study may be due to the different age range of the participants (young adults in the CARDIA study, elderly in the Rotterdam Study). The complete absence of an association between Lp-PLA2 measured concurrently to scanning and coronary calcification in our study may be due to the fact that the age range was even higher by this time. Whether the difference in age was the main factor that contributed to the observed discrepancy in findings remains to be resolved.

In conclusion, the disappearance of the moderate association of Lp-PLA2 activity with coronary calcification after adjustment for cholesterol in this study suggests that the effect of Lp-PLA2 on coronary calcification may be exerted through its effect on LDL-cholesterol. The absence of the association while measuring Lp-PLA2 activity concurrently to scanning may be due to attenuation of the predictive value of cardiovascular risk factors with increasing age or may indicate a stronger relation when Lp-PLA2 is measured longer before the assessment of coronary calcification. Future studies are needed to further elucidate the role of Lp-PLA2 in the development of cardiovascular disease.

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Chapter 3.3

Lipoprotein-associated phospholipase A2 activity and extracoronary atherosclerosis

Abstract

Objective. Lipoprotein-associated phospholipase A2 (Lp-PLA2) may be a new and independent predictor of cardiovascular events. The effect of Lp-PLA2 may be exerted through effects of the enzyme on the development of atherosclerosis. Therefore, we investigated the association between Lp-PLA2 activity and measures of extracoronary atherosclerosis.

Methods and results. Lp-PLA2 activity was determined in a random sample of 1820 participants from the Rotterdam Study, a population-based cohort study in men and women ≥55 years. Common carotid intima-media thickness, carotid plaques, ankle-arm index and aortic calcification were examined. Atherosclerosis status could be assigned in 1609 participants. The age-adjusted odds ratio of having atherosclerosis at any site for the highest versus the lowest tertile of Lp-PLA2 activity was 1.86 (95% CI 1.01-3.43) in men and 1.60 (95% CI 1.08-2.37) in women. After additional adjustment for cholesterol, these associations attenuated or even disappeared. The odds ratios of having atherosclerosis at specific sites (carotid arteries and aortic-iliac-femoral tract) followed a similar pattern.

Conclusions. Although Lp-PLA2 has been found to be independently associated with cardiovascular events, the association with measures of subclinical extracoronary atherosclerosis found in this study strongly attenuated or even disappeared after adjustment for cholesterol.

Introduction

Recently, several studies suggested that lipoprotein-associated phospholipase A2 (Lp-PLA2) may be a new and independent risk factor for cardiovascular disease.[1-5] Lp-PLA2 hydrolyzes oxidatively modified low-density lipoprotein (LDL) by cleaving oxidized phosphatidylcholines, generating lysophosphatidylcholine and oxidized free fatty acids. Lysophosphatidylcholine and oxidized free fatty acids are both chemoattractants for monocytes and may account for a part of the proinflammatory capacities of oxidized LDL-cholesterol.[6]

Although experimental studies imply that the effect of Lp-PLA2 on cardiovascular disease is exerted through effects of the enzyme on the development of atherosclerosis, little population-based research has been reported on this topic. Several studies have examined the association between Lp-PLA2 and angiographically documented coronary atherosclerosis in case control settings using high-risk subjects, most of them finding an independent association,[7-10] and one of them finding that the association disappeared after adjusting for clinical and lipid variables.[11] Furthermore, in a nested case-control study among young adults participating in the population-based CARDIA study, an independent association was found of Lp-PLA2 mass with calcified coronary plaque assessed by computed tomography.[12]

In a study among 190 hypercholesterolemic Sicilian individuals, no association was found between Lp-PLA2 activity and carotid intima-media thickness (IMT).[13] However, this was a relatively small study, showing only unadjusted values of mean plasma Lp-PLA2 activity for subjects with normal and high carotid IMT. In a study among 247 patients referred for lower extremity arterial evaluation, Lp-PLA2 was a borderline significant predictor of lower ankle-arm index (AAI) after adjustment for conventional cardiovascular risk factors and statin use.[14] So far, no population-based studies have investigated whether Lp-PLA2 activity is associated with measures of extracoronary atherosclerosis. We investigated whether Lp-PLA2 activity is associated with atherosclerosis at different sites of the vascular tree in the Rotterdam study.

Methods

Study population

The Rotterdam Study is a prospective population-based cohort study comprising 7983 men and women ≥55 years of age. Its overall aim is to investigate the incidence of and risk factors for chronic disabling diseases. From 1990 to 1993, all inhabitants of a suburb of the city of Rotterdam aged 55 years and over were invited to participate in an extensive home interview and two visits to the research center. The overall response was 78%. The Medical Ethics Committee of Erasmus MC approved the Rotterdam Study and written informed consent was obtained from all participants. A more detailed description of the Rotterdam Study and the collection of data have been given previously.[15]

For the present study, a random sample of 1820 participantts was drawn from the source population, and in this subcohort, baseline measurements of Lp-PLA2 activity were conducted. Within this group, measurements of IMT were available for 1430 participants, and assessment of carotid plaques, AAI and aortic calcification for 1435, 1624 and 1259 participants, respectively. Missing measurements

were mainly attributable to logistic reasons. Values for cardiovascular risk factors were missing in less than 6% of participants.

Measurement of Lp-PLA2 activity

Plasma aliquots prepared from non-fasting blood samples were collected at baseline and stored at -80°C, and Lp-PLA2 activity was measured with a high throughput radiometric activity assay. Briefly, plasma samples were aliquoted into 96-well microtiter plates and mixed with a substrate solution consisting of 0.4 µM [^{3}H]-platelet-activating factor (Specific Activity 21.5 Ci/mmol, Perkin Elmer Life Sciences) and 99.6 µM C16-platelet-activating factor (Avanti Polar Lipids Inc) in assay buffer (100mM HEPES, 150mM NaCl, 5mM EDTA, pH7.4). The reactions were allowed to proceed at room temperature for 5 min before sequestering of the phospholipid substrates by an ice-cold fatty acid-free bovine serum albumin solution at a final concentration of 16.1 mg/ml. The BSA-lipid complexes were then precipitated with ice-cold trichloroacetic acid at a final concentration of 7.8% and pelleted by centrifugation at ~6,000 g for 15 min at 4°C. Aliquots of the supernatant containing the reaction products were transferred to another microplate (Perkin Elmer) and the radioactivity counted in a Topcount liquid scintillation counter (Perkin Elmer Life Sciences) on addition of Microscint-20 scintillation cocktail (Perkin Elmer Life Sciences). Lp-PLA2 activity was expressed as nanomoles of platelet-activating factor hydrolyzed per minute per ml of plasma samples.

Before analysis of plasma samples from the Rotterdam Study, a pre-study validation was conducted to determine the reliability of the Lp-PLA2 activity assay. Six plasma samples were tested in triplicate, and the coefficient of variation (CV) for intra-assay precision ranged from 3.51-8.96%. To assess inter-assay precision, six plasma samples were tested on three occasions, and CV ranged from 8.48 to15.08%. Three cycles of freeze-thaw of frozen plasma did not result in appreciable loss of activity. The assay was therefore considered suitable for analysis of the Rotterdam Study samples, which were tested in duplicate. Samples were re-tested if the replicate CV was > 25%. The range of detection was 8-150 nmol/min/ml.

Measures of atherosclerosis

Ultrasonography of both carotid arteries was performed with a 7.5 MHz linear-array transducer and a duplex scanner (ATL UltraMark IV, Advanced Technology Laboratories, Bethel, Washington, USA). Measurements of the common carotid IMT involved regions of the common carotid arteries proximal to the carotid bulb, starting at a distance of 1 cm from the bulb. IMT was determined as the average of mean near- and far-wall measurements, computing the average of left and right common carotid IMT.[16] We considered carotid IMT below 1 mm as absence of atherosclerosis according to this measurement.

The internal carotid artery, carotid bifurcation, and common carotid artery were examined both left and right for the presence of plaques. Plaques were defined as a focal widening relative to adjacent segments, with the protrusion into the lumen composed either of only calcified deposits or a combination of calcification and non-calcified material.[16] The anterior and posterior wall were evaluated for the presence of a plaque. Carotid plaques were dichotomized into presence or absence of carotid plaques.

Using a random zero sphygmomanometer, sitting blood pressure was measured at the right upper arm. The average of two measurements obtained at one occasion was used. Systolic blood pressure at the ankles (posterior tibial artery) was measured in supine position with a random zero sphygmomanometer and an 8 MHz continuous wave Doppler probe (Huntleigh 500D, Huntleigh Technology, Bedfordshire, UK). The ratio of the systolic blood pressure at the ankle to the systolic blood pressure at the arm was computed to obtain the AAI. For the analyses, we used the lowest value of two legs. Values of the AAI larger than 1.50 were considered invalid.[17] We considered AAI above 0.90 as absence of atherosclerosis according to this measurement.

Aortic calcification was diagnosed by radiographic detection of calcified deposits in the abdominal aorta on a lateral abdominal film.[18] Aortic calcification was dichotomized into presence or absence of aortic calcification.

Based on the above four measurements, we assigned atherosclerosis status to the participants. Atherosclerosis status could be assigned to 1609 participants, which were subsequently used for the analysis. Participants without atherosclerosis were defined as those with either three or four measures of atherosclerosis available, which all showed absence of atherosclerosis. Presence of atherosclerosis was defined as presence of atherosclerosis at any site measured.

Furthermore, presence of atherosclerosis was classified according to the two vessel beds involved, being the carotid arteries and the aortic-iliac-femoral tract. Atherosclerosis in the carotid arteries was defined as IMT > 1 mm or presence of carotid plaques, and atherosclerosis in the aortic-iliac-femoral tract was defined as AAI < 0.9 or presence of aortic calcification. Atherosclerosis in both the carotid arteries and the aortic-iliac-femoral tract was defined as IMT > 1 mm or presence of carotid plaques concomitant with AAI < 0.9 or presence of aortic calcification.

Assessment of covariates

At baseline, covariates were ascertained using standard procedures as described previously.[4] C-reactive protein was measured using a nephelometric method (Immage, Beckman Coulter) in serum which was kept frozen at -20 °C. This system has a within-run precision < 5.0%, a total precision < 7.5% and a reliability coefficient of 0.995. To compute the correlation between total cholesterol and LDL-cholesterol, we determined LDL-cholesterol in a random sample of 42 subjects using an enzymatic method (Roche, Mannheim, Germany). The correlation coefficient between total cholesterol and LDL-cholesterol was high ($r=0.91$, $p<0.001$).

Statistical analysis

First, we tested for differences between the subcohort used for analysis and the remainder of the Rotterdam Study, by using a t-test for continuous and a chi-square test for dichotomous variables. Because the distribution of C-reactive protein was skewed, the Mann Whitney test was used for this variable. Second, age-adjusted (except for age) and sex-adjusted (except for sex) correlation coefficients were computed for the association of age, sex and cardiovascular risk factors with Lp-PLA2.

Lp-PLA2 activity was divided into tertiles using cut-off values of 39 and 48 nmol/min/ml. Because gender differences may exist for Lp-PLA2 activity, we also did sex-specific analyses, using sex-specific tertiles (cut-offs 37 and 47 nmol/min/ml for women and 41 and 51 nmol/min/ml for men). We used binary logistic regression to examine the association between tertiles of Lp-PLA2 activity and presence

of any sign of atherosclerosis in men and women. In model 1, we adjusted for age. In model 2, total cholesterol and HDL-cholesterol were added. In model 3, body mass index, systolic blood pressure, diabetes, smoking, cholesterol lowering medication and C-reactive protein were entered additionally for men, and for women, hormone replacement therapy was also added. We used multinomial logistic regression to examine the association between tertiles of Lp-PLA2 activity and presence of atherosclerosis in the carotid arteries, the aortic-iliac-femoral tract, or both, in men and women. Again, three models were constructed as described above.

Missing values for cardiovascular risk factors were handled by imputing the mean for normally distributed variables, the median for skewed variables, and the value with the highest prevalence for nominal variables. A sensitivity analysis was performed to investigate whether the results changed when only subjects with complete information on all covariates were used.

Results

The characteristics of the subcohort used for the analysis were similar to the baseline characteristics of the remaining population of the Rotterdam Study with a few minor exceptions. Subjects in the subcohort were slightly younger (68.8 versus 71.0 years of age), had a slightly lower mean systolic blood pressure (138 versus 140 mm Hg) and diastolic blood pressure (73 versus 74 mm Hg), a slightly higher total cholesterol level (6.7 versus 6.6 mmol/l) and a somewhat lower prevalence of myocardial infarction (11% versus 14%). Table 1 shows the baseline characteristics of the participants according to sex and atherosclerosis status.

Lp-PLA2 activity was positively associated with male sex (Spearman correlation coefficient r=0.16), body mass index (r=0.074), systolic blood pressure (r=0.070), and total cholesterol (r=0.42). An inverse association was present with HDL cholesterol (r= -0.28). Lp-PLA2 activity was not significantly associated with age, diabetes, smoking, and CRP.

In table 2, the association between Lp-PLA2 activity and presence of atherosclerosis in all participants and in men and women separately is displayed. A total of 303 participants were classified as not having atherosclerosis, 1306 participants were classified as having atherosclerosis. After adjusting for age and sex, the overall odds ratio of having atherosclerosis was 1.77 (95% CI 1.26-2.50) for the highest compared with the lowest tertile of Lp-PLA2 activity. In men, the age-adjusted odds ratio was 1.86 (95% CI 1.01-3.43), and in women, it was 1.60 (95% CI 1.08-2.37). After adjustment for total and HDL cholesterol, the effect disappeared; what was left was a non-significant, attenuated odds ratio of 1.40 (95% CI 0.70-2.77) in men. Additional adjustment for body mass index, systolic blood pressure, diabetes, smoking, cholesterol lowering medication, C-reactive protein and for women, hormone replacement therapy, did not materially alter the risk estimates. The middle versus the lowest tertile of Lp-PLA2 did not show any association with atherosclerosis.

Table 3 shows odds ratios of presence of atherosclerosis in the carotid arteries, the aortic-iliac-femoral tract, or both, according to tertiles of Lp-PLA2 activity. The age- and sex-adjusted odds ratio for having aortc-iliac-femoral atherosclerosis was 1.97 (95% CI 1.34-2.90) for the highest versus the lowest tertile of Lp-PLA2 activity in all participants. In men, the corresponding age-adjusted odds ratio was 1.95 (95% CI 0.98-3.87), and in women, it was 1.70 (95% CI 1.08-1.13). These associations

Table 1. Baseline characteristics of the study population.

		No atherosclerosis		Atherosclerosis	
	All	Men	Women	Men	Women
	(n=1609)	(n=83)	(n=220)	(n=543)	(n=763)
Age (years)	68.8±8.7	63.6±6.9	64.1±7.0	69.0±8.1	70.6±9.1
Hormone replacement therapy (%)			20		14
Body mass index (kg/m^2)	26.2±3.6	25.2±2.6	26.5±3.6	25.5±3.1	26.6±3.9
Systolic blood pressure (mm Hg)	138±22	132±18	128±20	139±22	141±22
Diastolic blood pressure (mm Hg)	73±11	75±11	71±10	74±11	73±11
Total cholesterol (mmol/l)	6.7±1.2	6.3±1.2	6.7±1.1	6.4±1.1	7.0±1.3
HDL-cholesterol (mmol/l)	1.3±0.35	1.3±0.3	1.5±0.4	1.2±0.3	1.4±0.4
Diabetes mellitus (%)	10	7	3	10	12
Smokers (%)					
- Current	24	22	12	29	24
- Past	41	60	33	62	26
Cholesterol lowering medication (%)	2.2	0.0	0.4	3.0	2.5
C-reactive protein (mg/l)*	1.78 (0.93-3.58)	1.54 (0.70-3.49)	1.41 (0.65-2.73)	1.90 (0.97-3.95)	1.82 (1.00-3.57)
Lp-PLA2 activity (nmol/min/ml plasma)	45±12	44±10	41±10	48±11	44±12
History of myocardial infarction (%)	11	1	3	18	9
Carotid intima-media thickness (mm)	0.80±0.17	0.74±0.11	0.69±0.10	0.86±0.18	0.80±0.16
IMT > 1 mm (%)	10	0	0	18	10
Ankle-arm index	1.05±0.23	1.22±0.14	1.13±0.12	1.05±0.24	1.00±0.24
AAI < 0.90 (%)	19	0	0	21	26
Carotid plaques† (%)	59	0	0	78	72
Aortic calcification‡ (%)	71	0	0	86	84

Categorical variables are expressed as percentage. Values of continuous variables are expressed as mean ± standard deviation. *Expressed as median and interquartile range because of skewed distribution; †percentage of subjects with ≥1 carotid plaques; ‡percentage of subjects with aortic calcification.

Table 2. Odds ratios (ORs) and 95% confidence intervals for presence of atherosclerosis for tertiles of Lp-PLA2 activity in all subjects, men and women.

	Total (n=1609)	Men (n= 626)	Women (n=983)
Second vs first tertile Lp-PLA2			
OR (model 1)	1.02 (0.75-1.38)	1.10 (0.63-1.93)	1.24 (0.85-1.80)
OR (model 2)	0.71 (0.51-0.99)	0.92 (0.51-1.66)	0.82 (0.54-1.23)
OR (model 3)	0.71 (0.50-1.00)	0.92 (0.51-1.69)	0.83 (0.54-1.28)
Third vs first tertile Lp-PLA2			
OR (model 1)	1.77 (1.26-2.50)	1.86 (1.01-3.43)	1.60 (1.08-2.37)
OR (model 2)	1.00 (0.67-1.48)	1.40 (0.70-2.77)	0.78 (0.49-1.25)
OR (model 3)	1.04 (0.69-1.57)	1.42 (0.71-2.86)	0.83 (0.54-1.28)

Model 1, adjusted for age (and sex when appropriate); model 2, adjusted for age (and sex when appropriate), total cholesterol level and HDL-cholesterol level; model 3, adjusted for age (and sex when appropriate), total cholesterol level, HDL-cholesterol level, body mass index, systolic blood pressure, diabetes, smoking, cholesterol lowering medication, C-reactive protein, and for women also hormone replacement therapy.

Table 3. Odds ratios (ORs) and 95% confidence intervals for severity of carotid plaques for tertiles of Lp-PLA2 activity in men and women.

	No atherosclerosis	Carotid atherosclerosis	Aortic-iliac-femoral atherosclerosis	Carotid and aortic-iliac-femoral atherosclerosis
Total	n=303	n=299	n=446	n=561
Second vs first tertile Lp-PLA2				
OR (model 1)	1.00 (ref)	0.73 (0.49-1.08)	1.15 (0.81-1.65)	1.13 (0.79-1.61)
OR (model 2)	1.00 (ref)	0.55 (0.36-0.84)	0.81 (0.55-1.18)	0.75 (0.51-1.10)
OR (model 3)	1.00 (ref)	0.55 (0.36-0.84)	0.80 (0.54-1.19)	0.76 (0.50-1.13)
Third vs first tertile Lp-PLA2				
OR (model 1)	1.00 (ref)	1.31 (0.87-1.99)	1.97 (1.34-2.90)	1.97 (1.34-2.90)
OR (model 2)	1.00 (ref)	0.84 (0.52-1.36)	1.11 (0.71-1.73)	1.01 (0.65-1.58)
OR (model 3)	1.00 (ref)	0.86 (0.53-1.40)	1.15 (0.73-1.82)	1.10 (0.69-1.75)
Men	n=83	n=117	n=167	n=259
Second vs first tertile Lp-PLA2				
OR (model 1)	1.00 (ref)	1.00 (0.51-1.95)	1.29 (0.68-2.42)	1.02 (0.55-1.89)
OR (model 2)	1.00 (ref)	0.97 (0.48-1.96)	1.04 (0.53-2.04)	0.81 (0.42-1.55)
OR (model 3)	1.00 (ref)	0.93 (0.45-1.90)	1.08 (0.55-2.13)	0.83 (0.42-1.61)
Third vs first tertile Lp-PLA2				
OR (model 1)	1.00 (ref)	1.50 (0.73-3.08)	1.95 (0.98-3.87)	2.04 (1.06-3.95)
OR (model 2)	1.00 (ref)	1.46 (0.65-3.28)	1.39 (0.64-3.00)	1.36 (0.65-2.86)
OR (model 3)	1.00 (ref)	1.40 (0.62-3.17)	1.39 (0.64-3.03)	1.39 (0.65-2.96)
Women	n=220	n=182	n=279	n=302
Second vs first tertile Lp-PLA2				
OR (model 1)	1.00 (ref)	0.79 (0.48-1.29)	1.43 (0.93-2.22)	1.42 (0.91-2.22)
OR (model 2)	1.00 (ref)	0.55 (0.33-1.94)	0.96 (0.60-1.53)	0.89 (0.55-1.44)
OR (model 3)	1.00 (ref)	0.58 (0.33-0.99)	0.94 (0.57-1.54)	0.92 (0.55-1.55)
Third vs first tertile Lp-PLA2				
OR (model 1)	1.00 (ref)	1.24 (0.76-2.03)	1.70 (1.08-1.13)	1.82 (1.15-2.88)
OR (model 2)	1.00 (ref)	0.67 (0.38-1.20)	0.84 (0.49-1.44)	0.81 (0.47-1.39)
OR (model 3)	1.00 (ref)	0.69 (0.37-1.22)	0.86 (0.49-1.51)	0.86 (0.48-1.55)

Model 1, adjusted for age (and sex when appropriate); model 2, adjusted for age (and sex when appropriate), total cholesterol level and HDL-cholesterol level; model 3, adjusted for age (and sex when appropriate), total cholesterol level, HDL-cholesterol level, body mass index, systolic blood pressure, diabetes, smoking, cholesterol lowering medication, C-reactive protein, and for women also hormone replacement therapy.

disappeared after adjustment for total and HDL-cholesterol. The odds ratios for carotid atherosclerosis were somewhat lower and did not reach statistical significance. Again, these were attenuated by adjustment for cholesterol. A strong association was found with having both carotid and aortc-iliac-femoral atherosclerosis; the overall age- and sex-adjusted odds ratio for the highest versus the lowest tertile of Lp-PLA2 activity was 1.97 (95% CI 1.34-2.90). For men and women, the age-adjusted odds ratios were 2.04 (95% CI 1.06-3.95) and 1.82 (95% CI 1.15-2.88), respectively. Again, the associations disappeared after adjustment for total and HDL-cholesterol.

Performing the analysis using only subjects with complete information on all covariates did not materially change the results.

Discussion

Lp-PLA2 activity has been found previously to be independently associated with cardiovascular events in the Rotterdam Study.[4] In the present study, Lp-PLA2 activity was associated with extracoronary atherosclerosis at different sites of the arterial tree after adjustment for age and sex. However, after adjustment for total cholesterol and HDL-cholesterol, the associations between Lp-PLA2 activity and measures of atherosclerosis strongly attenuated or even disappeared.

The inconsistency between the association of Lp-PLA2 with clinical and subclinical atherosclerosis merits attention. First, the atherosclerosis measurements need to be evaluated. This study was performed within the Rotterdam Study, a large population-based study in subjects ≥55 years of age. We used several techniques to measure atherosclerosis. Ultrasound was used to measure the IMT of the common carotid artery and to detect plaques in the common carotid artery, bifurcation and internal carotid artery. We took x-ray films to assess the amount of aortic calcification, which has been shown to be a highly specific technique for the measurement of aortic intimal atherosclerosis,[19] and we used AAI as a measure of lower extremity atherosclerosis. The measures of carotid, aortic and lower extremity atherosclerosis have all shown to be associated with cardiovascular risk factors and risk of cardiovascular events and are considered to be measures of generalized atherosclerosis.[16,20-23] In a previous study we found that C-reactive protein is independently associated with all these measures of atherosclerosis.[24] This suggests that measures of atherosclerosis were determined appropriately in our study.

Second, it needs to be considered that molecules that regulate inflammation will not necessarily correlate with plaque burden measures, as illustrated by inflammatory markers such as C-reactive protein. Although C-reactive protein is associated with coronary events, it has not been a good predictor of the extent of atherosclerotic disease, and may measure other characteristics than atherosclerotic mass, such as the activity of lymphocyte and macrophage populations within plaque or the degree of plaque destabilization and ongoing ulceration or thrombosis.[25] The same may apply to Lp-PLA2. In a study enrolling 504 patients undergoing clinically indicated coronary angiography, Lp-PLA2 mass was found to be associated with severity of angiographically determined coronary artery disease.[11] However, after adjustment for clinical and lipid variables, this association disappeared. In the same study, LpPLA2 was independently associated with coronary events. Furthermore, Lp-PLA2 has been found to be independently associated with cardiovascular events in the West of Scotland Coronary Prevention Study (WOSCOPS), Atherosclerosis Risk in Communities (ARIC), Monitoring of Trends and Determinants in Cardiovascular Disease (MONICA), and Rotterdam studies,[1-5] and univariately associated in the Women's Health Study.[26] Also, Lp-PLA2 was found to be independently associated with a positive history of coronary artery disease in a small randomized controlled trial.[27]

Lp-PLA2 is bound to LDL-cholesterol and therefore highly correlated with LDL-cholesterol levels. In the present study, no LDL-cholesterol levels were available and therefore we adjusted for total cholesterol levels. Because of the high correlation between LDL and total cholesterol in a random sample

in the present study and because the correlation between Lp-PLA2 and total cholesterol in our study was even higher than that of Lp-PLA2 with LDL-cholesterol in the WOSCOPS[1] and the ARIC study,[2] we believe that we adjusted sufficiently for LDL-cholesterol. Furthermore, whereas residual confounding would lead to an overestimation of the effect, we no longer observed an association between Lp-PLA2 activity and measures of atherosclerosis after adjustment for total and HDL-cholesterol, showing that adjustment for total cholesterol exerts its effect on the risk estimates.

Few studies have been reported on the association between Lp-PLA2 and extracoronary atherosclerosis. In a study among 190 hypercholesterolemic Sicilian individuals, no association was found between Lp-PLA2 activity and a carotid IMT >1 mm.[13] However, this was a small study in high-risk subjects, and only unadjusted values of mean plasma Lp-PLA2 activity for patients with normal and high carotid IMT were presented. Sex-specific values were not given. Furthermore, none of the established cardiovascular risk factors was found to be associated with IMT in this study, most likely because of small sample size. In a study among 247 patients referred for lower extremity arterial evaluation, Lp-PLA2 was a borderline significant predictor of lower AAI after adjustment for conventional cardiovascular risk factors and statin use.[14]

Several studies have examined the association between Lp-PLA2 and angiographically documented coronary atherosclerosis in case control settings using high-risk subjects, most of them finding an independent association,[7-10] and one of them finding that the association disappeared after adjusting for clinical and lipid variables.[11] Furthermore, in a nested case-control study among young adults participating in the population-based CARDIA study, an independent association was found of Lp-PLA2 mass with calcified coronary plaque assessed by computed tomography.[12] These studies have been conducted in various populations and were different in design, and are therefore not strictly comparable to our study. Discrepancies between these studies and our study may be attributable to different sites of the atherosclerosis measurements (coronary versus extracoronary), use of high-risk subjects in the case-control studies and differences in the age range of the participants.

In conclusion, although Lp-PLA2 activity has been found to be independently associated with cardiovascular events in the Rotterdam Study, the association with measures of subclinical extracoronary atherosclerosis found in this study strongly attenuated or even disappeared after adjustment for cholesterol. Future studies are needed to further elucidate the role of Lp-PLA2 in the stages of the atherosclerotic process and the development of cardiovascular disease.

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Chapter 4

Other markers

Chapter 4.1

Fibrinogen gene haplotypes and cardiovascular disease

Abstract

Fibrin network structure has been correlated with coronary disease. Fibrinogen γ and α (FGG and FGA) gene haplotypes (chromosome 4q28) may be associated with fibrin network structure, and thereby with rigidity of the fibrin clot and sensitivity of the fibrin clot to the fibrinolytic system. Through these mechanisms they may influence risk of cardiovascular disease. We set out to investigate the relation between combined fibrinogen FGG and FGA gene haplotypes, representing the common variation of the fibrinogen FGG and FGA genes, coronary events and measures of coronary and extracoronary atherosclerosis. The study was embedded in the Rotterdam Study, a prospective population-based study among men and women aged ≥ 55 years. Common haplotypes were studied using seven tagging SNPs across a 30-kb region with the FGG and FGA genes. Incident coronary events were registered, and carotid intima-media thickness, carotid plaques, ankle-arm index, aortic calcification and coronary calcification were assessed. Seven haplotypes with frequencies >1% covered 97.5% of the genetic variation. In 5,667 participants without history of coronary heart disease, 733 coronary heart disease cases occurred during a median follow-up time of 11.9 years. Fibrinogen gene haplotypes were not associated with coronary events. Fibrinogen gene haplotypes did not show a consistent association with measures of coronary and extracoronary atherosclerosis. In conclusion, fibrinogen FGG and FGA gene haplotypes are not associated with coronary events, coronary atherosclerosis or extracoronary atherosclerosis. Confirmation of these findings by future population-based studies is warranted.

Introduction

Fibrinogen is an important coagulation factor, acting as an adhesive protein essential for platelet aggregation as well as forming insoluble fibrin fibers in the final stage of the blood coagulation cascade.[1] Several prospective epidemiological studies have demonstrated an independent association between fibrinogen level and cardiovascular disease. This has recently been confirmed in a large, individual participant meta-analysis.[2]

Fibrinogen may contribute to the progression of atherosclerosis through several potential pathophysiological mechanisms, whose roles are still unclear.[3] One of the mechanisms that have been gaining attention lately is the effect of changes in the structure of the fibrin network, because a correlation has been demonstrated between fibrin structure and coronary disease.[4,5] A possible underlying mechanism is the relationship between fibrin structure and hypofibrinolysis.[6] Fibrin structure is in part determined by genetic influences.[7,8] Therefore, genetic variants altering fibrinogen structure and function and consequently fibrin structure may provide an opportunity to further investigate the involvement of fibrinogen in atherogenesis.

The fibrinogen molecule consists of two sets of three different peptide chains- Aα, Bβ and γ chains- encoded by three genes, fibrinogen γ (FGG), α (FGA) and β (FGB), located in a region of approximately 50 kb on chromosome 4q.[9] Various single nucleotide polymorphisms (SNPs) have been identified in the fibrinogen genes, which may result in heterogeneity in circulating fibrinogen and fibrin clot structure. A weak association of the Ala^{312} allele of the Thr312Ala (rs6050) SNP in the FGA gene with low fibrin gel porosity and fibre-mass:length ratio has been reported in survivors of myocardial infarction.[10] Later, this allele has been found to be associated with clots having large fibrin fibres and increased stiffness.[11] This allele has also been associated with increased post-stroke mortality and pulmonary embolism.[12,13] The T allele of the 10034C/T (rs2066865) SNP in the FGG gene may affect the sensitivity of the fibrin clot to the fibrinolytic system by altering the proportion of the γ′ chains as a result of more frequent cleavage of pre-mRNAs and increased splicing, and an FGG haplotype containing this allele has been found to be associated with risk of deep venous thrombosis.[14] Haplotypes based on FGG SNP rs1049636 and FGA SNP rs2070011 have been associated with risk of myocardial infarction in a case-control setting.[15] The rs2070011 SNP in FGA is an independent determinant of fibrin gel porosity and modulates the relation between plasma fibrinogen concentration and fibrin clot porosity.[16]

The above findings suggest that fibrinogen FGG and FGA haplotypes may affect the formation of the fibrin network structure and consequently the sensitivity of the fibrin clot to the fibrinolytic system. Through these mechanisms they may influence risk of coronary heart disease. We set out to investigate the association between common variations in the fibrinogen FGG and FGA genes and coronary heart disease in the Rotterdam Study, a large, prospective, population-based cohort study.

Methods

Study population and baseline data collection

The Rotterdam Study is a population-based cohort study aimed at assessing the occurrence of and risk factors for chronic diseases in the elderly. Objectives and methods have been described in detail

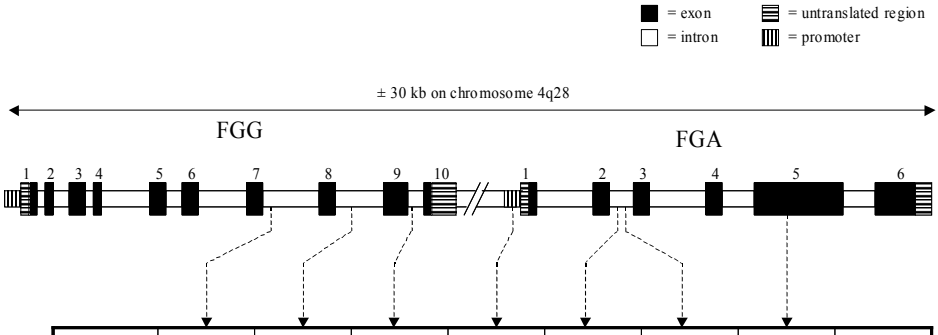
elsewhere.[17] The cohort includes 7,983 men and women aged 55 years and over (78% of the eligible population), living in a well-defined suburb of the city of Rotterdam, The Netherlands. The medical ethics committee of Erasmus Medical Center, Rotterdam, approved the study. Participants gave written informed consent and permission to retrieve information from treating physicians.

Baseline data were collected from 1990 until 1993, as described previously.[18] All participants were visited by a trained interviewer. Additionally, in 7,129 participants, established cardiovascular risk factors were measured at the research center. Fibrinogen measurements were done in a random sample of 2,968 participants. Platelet poor plasma was frozen in liquid nitrogen and stored at –80°C. Fibrinogen levels were derived from the clotting curve of the prothrombin time assay using Thromborel S as a reagent on an automated coagulation laboratory (ACL 300, Instrumentation Laboratory). The coefficient of variation was 5%.

Genotyping

The Seattle SNPs Program for Genomic Applications[19] has identified various SNPs in the fibrinogen genes based on 23 unrelated individuals of European descent from the CEPH pedigrees and has constructed haplotypes based on this dataset. We combined the four haplotypes of the FGG gene and the five haplotypes of the FGA gene and determined tagging SNPs needed to describe this combined FGG-FGA haplotype structure. All participants were genotyped for seven tagging SNPs (figure 1). We numbered the SNPs in relation to the transcription start site, in accordance with the Human Genome Organisation guidelines (www.gene.ucl.ac.uk/nomenclature/guidelines.html). In the Seattle SNPs program, SNP numbering was based on GenBank accession number AF350254 (FGG) and AF361104 (FGA), containing the fibrinogen gene sequences of Seattle SNPs. Our SNP number 4288 corresponds

Figure 1. Schematic depiction of haplotypes based on FGG and FGA in the study population (n=6514).



Haplotype	4288G>A, rs2066860	6326G>A, rs2066861	7792T>C, rs1049636	-58G>A, rs2070011	1374G>A, rs2070014	1526T>C, rs2070016	4253A>G, rs6050, Thr312Ala	Frequency
1	G	G	T	G	G	T	A / Thr^{312}	26.4%
2	G	**A**	T	**A**	G	T	**G / Ala^{312}**	25.6%
3	G	G	**C**	G	**A**	T	A / Thr^{312}	16.7%
4	G	G	T	G	G	**C**	A / Thr^{312}	12.2%
5	G	G	**C**	**A**	G	T	A / Thr^{312}	11.6%
6	**A**	G	T	G	G	T	A / Thr^{312}	3.7%
7	G	G	T	**A**	G	T	**G / Ala^{312}**	1.3%

to Seattle number 5836, 6326 to 7874, 7792 to 9340, -58 to 2224, 1374 to 3655, 1526 to 3807, and 4253 to 6534. These SNPs have also been described at http://www.ncbi.nlm.nih.gov/SNP under the appropriate rs numbers (figure 1).

Genotypes were determined in 2-ng genomic DNA with the Taqman allelic discrimination assay (Applied Biosystems, Foster City, CA, USA). Reactions were performed with the Taqman Prism 7900HT in 384-wells format. Haplotypes present in the population were inferred by means of the haplo.em function of the program Haplo Stats (http://cran.r-project.org/src/contrib/Descriptions/haplo.stats.html), which computes maximum likelihood estimates of haplotype probabilities.[20,21] Only haplotypes with frequencies >1% were used in the analysis. These seven haplotypes were coded as haplotype numbers 1 through 7 in order of decreasing frequency in the population.

Measures of extracoronary and coronary atherosclerosis

Several non-invasive measurements of extracoronary atherosclerosis were conducted at baseline. A detailed description of the procedures has been given previously.[22] Briefly, ultrasonography of both the left and the right carotid artery was performed according to the protocol of the Rotterdam Study and mean common carotid intima-media thickness (IMT) was determined. The left and right common carotid arteries, bifurcations and internal carotid arteries were evaluated for the presence of atherosclerotic lesions (plaques), resulting in a plaque score between 0 and 6. The ratio of systolic blood pressure at the ankles to the systolic blood pressure at the arm was computed to obtain the ankle-arm index (AAI). Aortic calcification was diagnosed by radiographic detection of calcified deposits in the abdominal aorta. The extent of aortic calcification was scored according to the length of the involved area of the posterior wall, with scores 0-5 corresponding to 0, <1, 1-2.4, 2.5-4.9, 5.0-9.9 and ≥10 cm.

From 1997 onwards, participants through 85 years of age completing the third phase of the Rotterdam Study were invited to participate in the Rotterdam Coronary Calcification Study and to undergo electron-beam computed tomography (EBCT) scans to assess coronary calcification in the epicardial coronary arteries, as described in detail previously.[23] The calcium score was obtained as proposed by Agatston et al.[24]

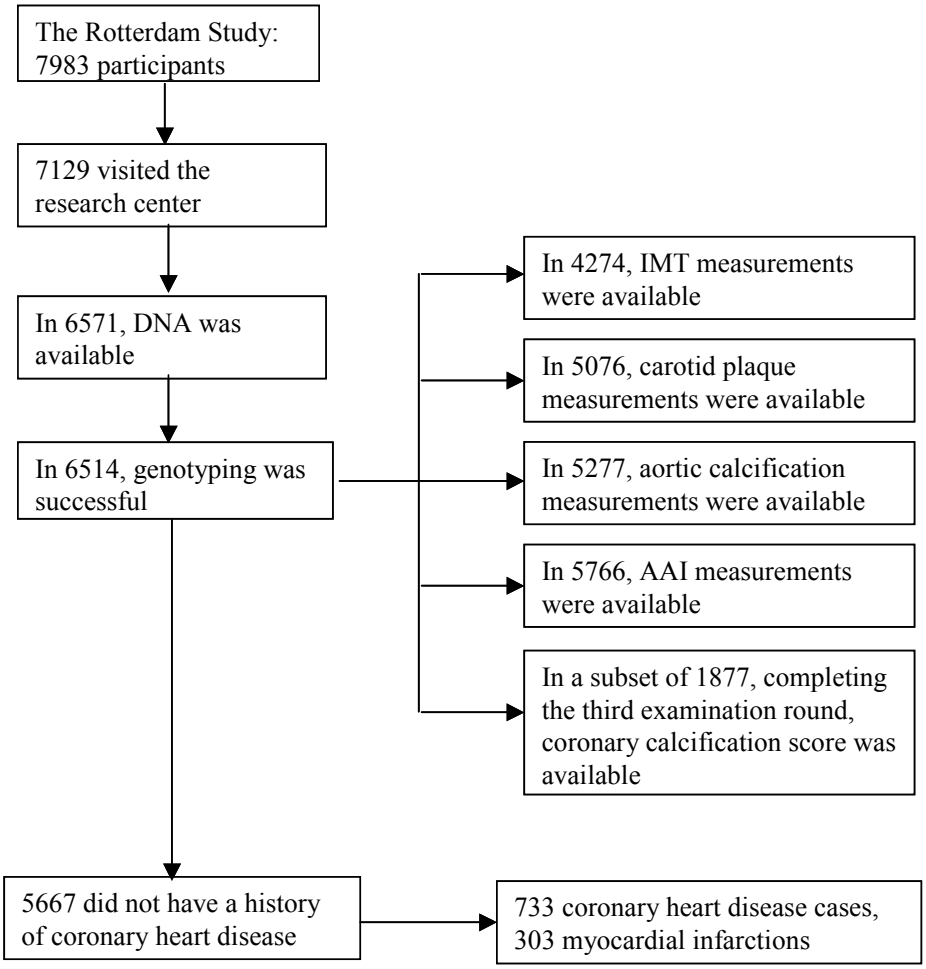
Follow-up procedure

Follow-up started at the baseline examination and for the present study lasted until January 1st, 2005. Information on fatal and non-fatal cardiovascular endpoints was obtained from general practitioners and letters and discharge reports from medical specialists.[18] Reported events were coded according to the International Classification of Diseases, 10th edition (ICD-10).[25] We defined incident coronary heart disease as myocardial infarction, coronary artery bypass grafting (CABG), percutaneous transluminal coronary angioplasty (PTCA) and cardiac death. In identifying myocardial infarctions, all available information, which included ECG, cardiac enzyme levels, and the clinical judgment of the treating specialist, was used. We defined cardiac death as death from myocardial infarction or other ischemic heart disease (ICD-10: I20-I25), sudden cardiac death (I46), sudden death undefined (R96), or death from heart failure (I50).

Population for analysis

Figure 2 shows a flow chart describing the population for analysis. After excluding participants with coronary heart disease at baseline (history of myocardial infarction, PTCA or CABG), 5,667 participants were left for the analysis of the association between fibrinogen gene haplotypes and incident coronary events. Measurements of IMT, carotid plaques, AAI and aortic calcification at baseline were available in limited numbers of participants, mainly because of logistic reasons such as limited availability of research assistants (figure 2). Coronary calcification was measured in a subset of participants completing the third phase of the Rotterdam Study. Genotyping was successful in 1,877 of these participants.

Figure 2. Flow chart describing the study population.



Data analysis

Hardy-Weinberg equilibriums of the SNPs were tested using Chi square tests. Associations between individual SNPs and coronary heart disease and myocardial infarction were examined using Cox proportional hazards models in SPSS 11.0.

Associations between fibrinogen gene haplotypes and age, sex, body mass index (BMI), systolic blood pressure, diastolic blood pressure, total cholesterol, HDL-cholesterol, diabetes mellitus, and fibrinogen plasma level were examined using the haplo.glm function of the program Haplo Stats (http://cran.r-project.org/src/contrib/Descriptions/haplo.stats.html).[20,21,26] Haplo.glm is based on a generalized linear model and computes the regression of a trait on haplotypes and other covariates. Haplotype 1 had the highest frequency and served as the reference category. The haplo.score function of Haplo Stats was used to test the association between fibrinogen gene haplotypes and smoking, which was entered as an ordinal variable (never, former, current). Details on the background and theory of score statistics can be found in Schaid et al.[26] We adjusted the analyses for age and sex. Using haplo.score, we computed simulation P-values for each haplotype to account for multiple testing. The number of simulations was set at 1,000.

The association between fibrinogen-gene haplotypes and coronary heart disease and myocardial infarction was investigated by using the haplo.glm function. Again, haplotype 1 served as the reference category. First, we adjusted for age and sex, and second, we additionally adjusted for body mass index, systolic blood pressure, diastolic blood pressure, total cholesterol, HDL-cholesterol, smoking, and diabetes mellitus. The analysis was repeated without excluding participants with coronary heart disease at baseline. The analysis was also repeated examining FGG and FGA haplotypes separately.

The associations between fibrinogen haplotypes and IMT, carotid plaques, AAI, aortic calcification and coronary calcification were examined using haplo.score. This non-parametric approach was chosen because the distributions of AAI and coronary calcification were skewed and difficult to normalize, and carotid plaques and aortic calcification were ordinal variables, which can be dealt with by haplo.score. Subsequently, atherosclerosis measures were dichotomized; IMT > 1 mm, carotid plaque score ≥ 4, AAI < 0.9, aortic calcification ≥ 5 cm and coronary calcification score > 1,000 were used as the outcomes in regression analysis using haplo.glm. Again, two models were used for adjustment.

Values for cardiovascular covariates were missing in less than 4% of participants. These missing values were handled by single imputation using the expectation-maximization algorithm in SPSS 11.0. Haplo.em, haplo.score and haplo.glm were all implemented in the Haplo Stats software using the R language. All tests were two-sided.

Results

Table 1 shows baseline characteristics of the study population. Mean age was 69.5 years and 59% was female. Among participants without history of coronary heart disease and with successful genotyping, median follow-up time was 11.9 years (interquartile range 7.9-12.9 years) and incident coronary heart disease occurred in 733 participants during follow-up, including 303 myocardial infarctions.

DNA was available in 6,571 participants. Failure of genotyping for individual SNPs occurred in 2.9 to 4.7%. Genotype distributions for the seven haplotype tagging SNPs were in Hardy-Weinberg equi-

Table 1. Baseline characteristics of the study population (n=6514).

Variable	
Age (years)	69.5±9.2
Women	3871 (59%)
Body mass index (kg/m^2)	26.3±3.7
Systolic blood pressure (mm Hg)	139±22
Diastolic blood pressure (mm Hg)	74±11
Total cholesterol (mmol/l)	6.6±1.2
HDL-cholesterol (mmol/l)	1.3±0.4
Diabetes mellitus	669 (10%)
Smokers	
- Never	2244 (34%)
- Current	1441 (22%)
- Former	2649 (41%)
History of myocardial infarction	760 (13%)

Categorical variables are expressed as count (percentage). Valid percentages may vary for some counts because of missing values in the variables. Values of continuous variables are expressed as mean ± standard deviation.

librium (table 2). Individual SNPs were not significantly associated with coronary heart disease and myocardial infarction (data not shown). Notably, this was also the case for the Thr312Ala SNP in FGA. Heterozygotes and Ala^{312} homozygotes had hazard ratios of 1.00 (95% CI 0.86-1.17) and 0.96 (95% CI 0.72-1.29), respectively, of developing coronary heart disease. For myocardial infarction, the relative risk was somewhat increased, but not significant; it was 1.15 (95% CI 0.91-1.47) and 1.19 (95% CI 0.77-1.83), for heterozygotes and Ala^{312} homozygotes, respectively.

The FGG and FGA SNPs are located on chromosome 4q28. According to the HapMap website (http://www.hapmap.org), they lie in two linkage disequilibrium blocks spanning approximately 30 kb of genomic DNA. However, linkage disequilibrium between these two blocks is very high, with a D'of 0.94. Therefore, we combined haplotypes of both genes. Haplotype reconstruction resulted in twenty-four FGG-FGA haplotypes in the Rotterdam Study population. Seven haplotypes had frequencies >1%, and together these seven haplotypes covered 97.5% of the genetic variation. The structures of these haplotypes and their frequencies are displayed in figure 1.

Haplotypes 2 and 5 were associated with lower BMI, with beta coefficients of –0.28 (95% CI, –0.50, -0.06; p=0.01) kg/m^2 per allele copy and –0.18 (95% CI –0.35, -0.01; p=0.03) kg/m^2 per allele copy, respectively. Haplotype 3 was associated with lower diastolic blood pressure, with a beta coefficient of –0.62 (95% CI, -1.08, -0.17; p=0.007) mmHg per allele copy. In view of the number of variables we examined, this significance level was not particularly high; the associations lost significance after adjustment for multiple comparisons. Haplotypes were not associated with age, sex, total cholesterol, HDL cholesterol, diabetes mellitus, history of myocardial infarction, or smoking (data not shown). The regression model became unstable when systolic blood pressure was examined. Therefore we used haplo.score, and no association was found with systolic blood pressure (data not shown).

Haplotype 4 was associated with higher fibrinogen plasma level. Haplotype 4 resulted in a fibrinogen plasma level of 3.21 (95% CI 3.15-3.27) g/l, as compared to a level of 3.13 g/l for haplotype 1

Table 2. Frequencies of SNPs in the study population (n=6514).

Gene	SNP	Genotype	n (%)	Minor allele frequency	P-value HWE Chi square
FGG	4288G>A, rs2066860	GG	5897(92.6)	A = 3.7%	0.31
		GA	464 (7.3)		
		AA	6 (0.1)		
	6326G>A, rs2066861	GG	3457(54.6)	A = 26.0%	0.46
		GA	2460 (38.8)		
		AA	417 (6.6)		
	7792T>C, rs1049636	TT	3159(49.5)	C = 29.4%	0.15
		TC	2694(42.2)		
		CC	526 (8.2)		
FGA	-58G>A, rs2070011	GG	2346(36.8)	A = 39.1%	0.27
		GA	3075 (48.3)		
		AA	951 (14.9)		
	1374G>A, rs2070014	GG	4348(68.7)	A = 17.2%	0.77
		GA	1795 (28.3)		
		AA	190 (3.0)		
	1526T>C, rs2070016	TT	4829 (75.6)	C = 12.8%	0.93
		TC	1418 (22.8)		
		CC	103 (1.6)		
	4253A>G, rs6050 (Thr312Ala)	AA	3272 (52.2)	G (Ala^{312})= 27.6%	0.61
		AG	2521 (40.3)		
		GG	470 (7.5)		

(reference) (p=0.02). Other fibrinogen haplotypes were not significantly associated with fibrinogen plasma level (data not shown).

In table 3, age-and sex-adjusted odds ratios for coronary heart disease and for myocardial infarction are displayed for different fibrinogen haplotypes. For both outcomes, the odds ratios for all haplotypes were around one. Additional adjustment for cardiovascular risk factors did not materially change the point estimates, and neither did repeating the analysis without excluding participants with coronary heart disease at baseline. When FGG and FGA haplotypes were analyzed separately, also, no associations were found with coronary events (data not shown).

Table 4 displays the associations between fibrinogen haplotypes and IMT, carotid plaques, AAI, aortic calcification and coronary calcification. Haplotype 3 was associated with lower IMT (p=0.03). However, haplotype 3 was not associated with other measures of atherosclerosis. Haplotype 4 was associated with lower amount of aortic calcification (p=0.01). Although the direction of the associations of this haplotype with the other measures of atherosclerosis was consistent, none of the other associations was significant. Of note is, that the protective direction of the associations of haplotype 4 with measures of atherosclerosis was not in accordance with the association of haplotype 4 with higher fibrinogen plasma level. Remaining haplotypes did not show significant associations with measures of atherosclerosis.

Table 3. Associations between fibrinogen haplotypes and coronary events, adjusted for age and sex (n=5667).

Haplotype	Frequency	Odds ratio (95% confidence interval)	
		Coronary heart disease	Myocardial infarction
Haplotype 1	26.5%	1.00 (reference)	1.00 (reference)
Haplotype 2	25.6%	1.01 (0.86-1.18)	1.06 (0.85-1.31)
Haplotype 3	16.6%	0.93 (0.78-1.11)	0.83 (0.64-1.09)
Haplotype 4	12.3%	1.01 (0.83-1.23)	0.90 (0.88-1.20)
Haplotype 5	11.6%	1.14 (0.95-1.39)	1.01 (0.76-1.34)
Haplotype 6	3.6%	1.06 (0.77-1.45)	1.19 (0.77-1.85)
Haplotype 7	1.3%	0.95 (0.57-1.59)	1.68 (0.92-3.08)

Table 4. Fibrinogen haplotypes and measures of extracoronary and coronary atherosclerosis, adjusted for age and sex.

	Intima-media thickness		Carotid plaques		Ankle-arm index		Aortic calcification		Coronary calcification	
Haplo-type	Score statistic	P-value	Score statistic	P-value	Score statistic	P-value	Score statistic	P-value	Score statistic	P-value
1	0.67	0.50	-0.60	0.57	-0.13	0.89	-0.08	0.93	0.38	0.72
2	1.02	0.30	0.17	0.87	-0.34	0.72	-0.20	0.82	0.16	0.86
3	-2.15	0.03	0.07	0.94	1.56	0.11	0.22	0.82	0.23	0.83
4	-0.84	0.39	-1.52	0.13	0.31	0.76	-2.54	0.01	-1.09	0.28
5	0.59	0.57	1.28	0.18	-1.38	0.16	1.46	0.14	-0.28	0.80
6	0.08	0.93	0.85	0.38	0.43	0.66	1.16	0.24	0.29	0.76
7	1.52	0.14	1.55	0.13	0.33	0.72	1.12	0.27	-0.99	0.31

P-values in the table were obtained using haplo.score, after 1000 simulations.

Using IMT > 1 mm, severe carotid plaques, AAI < 0.9 and severe aortic calcification as outcomes in haplo.glm did not result in significantly raised or lowered odds ratios, using either model for adjustment (data not shown). Using coronary calcification score > 1000 as the outcome, haplotype 4 resulted in an odds ratio of 0.70 (95% CI 0.49-0.99), p=0.05. This association was borderline significant after multivariable adjustment and was in accordance with the direction of the association seen in table 4.

Discussion

In this population-based study, fibrinogen FGG-FGA haplotypes were not associated with coronary events. Furthermore, fibrinogen haplotypes did not show a consistent association with measures of coronary and extracoronary atherosclerosis.

Strengths of the present study include its population-based nature, coverage of 97.5% of the variation in the FGG and FGA genes, occurrence of 733 incident coronary events, and availability of several

non-invasive measures of coronary and extracoronary atherosclerosis. Nevertheless, several aspects of this study warrant further consideration. First, because we wanted to examine structural aspects of fibrinogen in our study, and FGG and FGA, not FGB, are expected to be involved in this matter, we did not determine FGB haplotypes. Second, we chose to combine FGG and FGA haplotypes in the analysis although linkage disequilibrium between these two regions was not complete, D' being 0.94. Third, effects of gene haplotypes on multifactorial diseases may generally be expected to be of modest magnitude. Therefore, although 733 coronary events were available for analysis, we cannot exclude the possibility that statistical power in our study was insufficient to uncover a potential association. Power calculation for the present study shows that, with a power of 80% and an alpha of 0.05, in reference to haplotype 1 (the most common haplotype, frequency 26.4%), we were able to demonstrate a relative risk of coronary heart disease (n=733) of at least 1.29 and a relative risk of myocardial infarction (n=303) of at least 1.49 (both for haplotype 2, frequency 25.6%). The true association may be smaller than this. Fourth, not all participants had complete information on all 4 measures of extracoronary atherosclerosis presented in this study. Although we cannot exclude the possibility that health-related issues have also played a role, missing data were predominantly because of logistic reasons and are unlikely to have affected our results. Furthermore, coronary calcification was assessed in a subset of participants through 85 years of age that reached the third phase of the Rotterdam Study (1997-1999). We need to bear in mind that these participants had somewhat different characteristics because of the time that had elapsed since the baseline examination in 1990-1993.[23] However, we consider it unlikely that this could have affected the association between fibrinogen haplotypes and coronary calcification.

Fibrinogen level is a consistent risk factor for cardiovascular disease.[2] Lately, structural aspects of fibrinogen have gained attention as risk factors for cardiovascular disease.[27] Collet et al. have demonstrated that fibrin network architecture is the most important factor in determining rates of fibrinolysis.[6] This could provide an explanation for the finding that men who had suffered from myocardial infarction at young age have a clot structure with decreased fibrin gel porosity.[4,5] Similar findings have been documented in conditions such as peripheral vascular disease, diabetes and hypercholesterolemia, which on their turn are associated with coronary artery disease.[28,29]

Investigation of fibrinogen genes has mostly focused on FGB SNPs, some of which have been found to be associated with fibrinogen level and coronary artery disease.[30-32] However, fibrin structure is also in part determined by genetic influences. This is supported by a twin study,[7] and a study that has demonstrated that healthy male relatives of patients with premature coronary artery disease have a prothrombotic clot structure characterized by reduced permeability.[8] Several SNPs in the fibrinogen FGG and FGA genes have been shown to result in heterogeneity in fibrin clot structure and have been associated with both venous and arterial thrombosis. Firstly, some evidence of an association of the Ala^{312} allele of the Thr312Ala (rs6050) SNP in the FGA gene with low fibrin gel porosity and fibre-mass:length ratio has been reported in survivors of myocardial infarction.[10] Later, this allele has been found to be associated with clots having large fibrin fibres and increased stiffness.[11] This allele has also been associated with increased post-stroke mortality and pulmonary embolism.[12,13] In the present study, when analyzed separately, the Ala^{312} allele resulted in an increased, but not significant, hazard ratio for myocardial infarction, namely 1.19 (95% CI 0.77-1.83), for Ala^{312} homozygotes. Furthermore, our haplotypes 2 and 7, which contained the Ala^{312} allele, were not associated with adverse outcomes.

Secondly, the T allele of the 10034C/T (rs2066865) SNP in the FGG gene may affect the sensitivity of the fibrin clot to the fibrinolytic system by altering the proportion of the γ′ chains, and an FGG haplotype containing this allele has been found to be associated with risk of deep venous thrombosis by Uitte de Willige et al. in the Leiden Thrombophilia Study.[14] They noted that the FGA Thr312Ala (rs6050) SNP is in strong, but not complete, linkage disequilibrium with the FGG haplotype associated with thrombosis (D′ =0.97). Uitte de Willige et al were unable to identify the rs6050 SNP as an independent risk factor, and suggested that the risk associated with this polymorphism may be caused by linkage disequilibrium with the unfavorable FGG haplotype they identified. In the present study, we did not determine the rs2066865 SNP. According to the Seattle SNPs haplotype pattern, this SNP was present in our haplotype 2. As mentioned above, this haplotype was not associated with cardiovascular disease. In view of their above-mentioned results, Uitte de Willige et al. studied the association between FGG gene haplotypes and myocardial infarction in the "Study of Myocardial Infarctions Leiden"(SMILE), a population-based case-control study.[33] However, they had to conclude that none of the four common FGG gene haplotypes, including the haplotype that increased thrombosis risk, has a strong effect on the risk of myocardial infarction, and they stated that this was in line with studies on other prothrombotic factors that affect the risk of venous thrombosis but have small or no effects on arterial disease.

Furthermore, Mannila et al. studied the association between fibrinogen gene haplotypes and myocardial infarction in the Stockholm Coronary Artery Risk Factor (SCARF) study, a case-control study of factors predisposing to premature myocardial infarction in 377 postinfarction patients and 387 healthy individuals.[15] Their main finding was that haplotypes based on the FGG SNP rs1049636 and FGA SNP rs2070011 were associated with risk of myocardial infarction. Limitations of the study included the retrospective design and the fact that only parts of the FGG, FGA and FGB genes were examined. In a subsequent study, Mannila et al. further strengthened their findings by reporting that the rs2070011 SNP appeared to be an independent determinant of fibrin gel porosity and that it modulated the relation between plasma fibrinogen concentration and fibrin clot porosity.[16] In the present study, the rs1049636 and rs2070011 SNPs showed no associations with adverse outcomes when analyzed separately. Furthermore, our haplotype 5 contains the rare alleles of both these SNPs, and this haplotype did not show any association with events nor with measures of atherosclerosis.

In short, our results are concordant with the results of Uitte de Willige et al.,[33] showing no significant associations between fibrinogen haplotypes and myocardial infarction. We could not reproduce the findings of Mannila et al. that haplotypes based on rs2070011 and rs1049636 are associated with risk of myocardial infarction.[15] This discrepancy may have arisen because of differences in study population (mean age in the Rotterdam Study was 69.5 years versus 53.0 in the SCARF study, 60% versus 18% was female) and the fact that the SCARF study was designed to study determinants of premature myocardial infarction, whereas the Rotterdam Study was designed to examine myocardial infarction in a general population. Whether these differences were the main factors that contributed to the observed discrepancy in findings remains to be resolved.

In conclusion, in this large, prospective, population-based cohort study, FGG and FGA fibrinogen haplotypes were not associated with coronary events, coronary calcification or extracoronary atherosclerosis. Confirmation of these findings by future population-based studies and subsequent pooling

of the results of these studies may render adequate statistical power to provide a final answer in this matter.

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Chapter 4.2

Heat shock protein 27 and cardiovascular disease

Abstract

Aims. Heat shock protein 27 (HSP27) has been hypothesized to be a potential biomarker of atherothrombosis. However, no prospective studies have yet been performed to investigate the association between HSP27 plasma level and incident cardiovascular events among initially healthy individuals.
Methods and results. Plasma levels of HSP27 were evaluated at baseline among 255 initially healthy participants in the Women's Health Study who subsequently developed myocardial infarction, stroke, or cardiovascular death during a follow-up period of up to 5.9 years (cases) and among an equal number of age and smoking matched women who remained free of cardiovascular disease over the same time period (controls). Overall, HSP27 plasma levels were inversely associated with age (Spearman correlation coefficient r= -0.258, $P < 0.001$), but not with other established cardiovascular risk factors. Conditional logistic regression analysis showed no significant association of baseline HSP27 plasma level with future cardiovascular disease; the odds ratio for the upper versus the lower tertile of HSP27 level at baseline was 0.99 (95% confidence interval 0.62-1.57, P for trend = 0.99).
Conclusion. In this prospective study of initially healthy women, baseline HSP27 plasma level was not associated with incident cardiovascular events.

Introduction

Heat shock proteins (HSPs) are molecular chaperones that protect against stress stimuli including heat shock, oxidized low-density lipoprotein (LDL), mechanical stress, oxidants and cytokine stimulation.[1] Their primary function is to fulfill chaperoning activity: as new proteins are being produced by ribosomes, HSPs assist in correct folding of polypeptide chains into functional protein, and after a stress event, HSPs assist in refolding or degradation of damaged or denatured proteins.[2]

HSPs are divided into several families according to molecular weight, including the 110, 90, 70, 60, and 40 kDa families, the small HSPs such as HSP27, and the HSP10 family. HSPs have been implicated in the pathogenesis of several disease processes. In relation to atherosclerosis, HSPs from the HSP60 and HSP70 families have been most widely investigated.[1,2] Recently, however, cardiovascular attention has also focused on HSP27, which is known to have chaperoning activity, to inhibit F-actin polymerization, to protect against apoptosis and to be involved in the presentation of oxidized proteins to the proteosome degradation machinery.[3] Specifically, using atherosclerotic carotid endarterectomy samples and control endarteries, we demonstrated that HSP27 secretion correlates negatively with atherosclerotic plaque complexity by comparing the complicated versus the non-complicated adjacent area from the same specimen and control endarteries. We also reported reduced HSP27 plasma levels in atherosclerotic patients compared with healthy subjects.[4] Park et al. used the same strategy but examined the tissue compartment, and also reported that HSP27 expression is increased in the normal-appearing vessel adjacent to atherosclerotic plaque compared to both the plaque core area and the reference arteries.[5] By contrast, however, they reported that HSP27 plasma level was increased in acute coronary syndrome patients compared to normal reference subjects.

Taken together, these two recent studies raise the possibility that HSP27 may serve as a marker for atherothrombosis. To further evaluate this hypothesis, we performed a prospective, nested case-control study to examine whether baseline levels of HSP27 among initially healthy individuals are associated with future cardiovascular event rates.

Methods

Study design

We used a prospective nested case-control design within the Women's Health Study, a recently completed randomized, double-blind, placebo-controlled trial of low-dose aspirin and vitamin E in the primary prevention of cardiovascular disease and cancer in US female healthcare professionals. Eligible participants were apparently healthy women, aged 45 years or older, who were free of self-reported cardiovascular disease or cancer at study entry (1992–1995). Baseline characteristics of participants of the Women's Health Study have been described in detail previously.[6] At the time of enrollment, participants gave written informed consent, completed questionnaires on demographics, medical history, medication, and lifestyle factors, and were asked to provide a blood sample. The study was approved by the institutional review board of the Brigham and Women's Hospital (Boston, Mass). The study complies with the Declaration of Helsinki.

Participants were prospectively followed for a composite end point of first-ever major cardiovascular event (non-fatal myocardial infarction, non-fatal stroke, or cardiovascular death). Medical records were obtained for all women in whom a cardiovascular end point was reported to occur and were reviewed in a blinded fashion by an end-points committee of physicians. Myocardial infarction was confirmed if symptoms met World Health Organization criteria and if the event was associated with abnormal levels of cardiac enzymes or diagnostic electrocardiograms. A confirmed stroke was defined as a new neurologic deficit of sudden onset that persisted for at least 24 hours. Death was confirmed to be from cardiovascular causes on the basis of an examination of autopsy reports, death certificates, medical records and information obtained from the next of kin or other family members.

For each cardiovascular disease case, a control matched by age, smoking status and length of follow-up was chosen among those subjects who remained free of cardiovascular disease at the time the index event occurred in the case participant. The control participants were selected from those who remained event-free up to the date that the dataset was closed for selection of study participants. For the present investigation, 255 incident cardiovascular disease case-control pairs were identified. Out of the total of 255 case subjects, 111 were diagnosed with myocardial infarction, another 111 were diagnosed with stroke, and 33 were confirmed to have died from cardiovascular causes. In this nested case-control analysis, the maximum length of follow-up was 5.9 years.

Blood collection and laboratory evaluation

EDTA blood samples were obtained at the time of enrollment and stored in vapor phase liquid nitrogen (–170°C). For each of the 255 case and control subjects in this study, samples were thawed and analyzed in a core laboratory certified by the National Heart, Lung, and Blood Institute/Centers for Disease Control and Prevention Lipid Standardization Program.

HSP27 plasma levels were measured with an enzyme-linked immunosorbent assay (ELISA) from Calbiochem (San Diego, CA). This assay employs the quantitative sandwich enzyme immunoassay technique. Briefly, a monoclonal antibody specific for HSP27 was pre-coated onto a microtitre plate. Samples, standards and controls were incubated along with a polyclonal HSP27 antibody in the microtitre plate. After incubation and a wash step, a horseradish peroxidase enzyme/IgG antibody conjugate was added. After another incubation and wash to remove unbound substances, an enzyme substrate was added and color was generated that was proportional to the amount of HSP27 present in the sample. Assays were run in duplicate and were repeated if the replicate coefficient of variation was >10%. The interassay coefficient of variation of the assay at concentrations of 2.2 and 26.3 ng/mL was 10.8 and 9.3%, respectively. The range of detection was 1 to 800 ng/mL.

Total and high-density lipoprotein (HDL) cholesterol were assayed directly with reagents from Genzyme Corporation (Cambridge, Mass) and Roche Diagnostics (Indianapolis, Ind) with the use of a Hitachi 911 autoanalyzer. CRP was measured with a high-sensitivity immunoturbidimetric assay on the Hitachi 917 autoanalyzer (Roche Diagnostics, Indianapolis, Ind.), with the use of reagents and calibrators from Denka Seiken (Tokyo, Japan). Fibrinogen was measured with an immunoturbidimetric assay, which is a mass-based assay with international standards (Kamiya Biomedical, Seattle, Wash.). sICAM-1 was measured using an enzyme-linked immunosorbent assay (R & D Systems, Minneapolis, Minn.).

Statistical analysis

We first evaluated differences in baseline characteristics between case and control groups using paired t-tests for normally distributed, continuous variables, Wilcoxon signed-ranks tests for continuous variables with skewed distributions, McNemar tests for dichotomous variables, and marginal homogeneity tests for variables with more than two categories.

Second, we examined associations between HSP27 plasma level and baseline characteristics of the control participants. Specifically, we calculated means, medians and proportions of the baseline characteristics according to tertiles of HSP27 level at baseline. We used ln-transformed, continuous plasma level of HSP27 as the independent variable and tested for trends by using linear regression for continuous variables, logistic regression for dichotomous variables, and multinomial regression for variables with more than two categories. Furthermore, we computed Spearman correlation coefficients between HSP27 plasma level and baseline characteristics using both the cases and the control subjects.

To address the predictive value of baseline HSP27 levels, we calculated the relative risk of future cardiovascular events associated with HSP27 plasma level by logistic regression analysis, conditional on the matching on age, smoking (never, former, current) and length of follow-up. HSP27 plasma level was divided into tertiles based on the distribution in the control subjects and the lowest tertile was used as the reference category. Analysis for trend was performed by entering ln transformed HSP27 plasma level into the model as a continuous variable. Furthermore, we investigated the presence of a threshold effect by dichotomizing HSP27 plasma level based on the 50^{th}, 75^{th}, and 90^{th} percentiles in the control subjects and entering it into the model. We used several levels of adjustment. Model 1 was matched on age, smoking and length of follow-up. In model 2, we additionally adjusted for BMI, systolic blood pressure, hypertension, total cholesterol, HDL cholesterol, hyperlipidemia and diabetes mellitus. In model 3, we added C-reactive protein, exercise, alcohol intake, menopausal status, and hormone replacement therapy to the variables in model 2.

To examine effect modification, we stratified the above analysis on age (below and above the median), BMI (< 25, ≥ 25-30, ≥ 30 kg/m^2), hypertension, hyperlipidemia, diabetes mellitus, smoking (never, former, current), CRP (≤ 3 and > 3 mg/l), fibrinogen (below and above the median) and sICAM-1 (below and above the median). In these latter analyses, matching was broken to obtain enough power for the stratified analyses. Interaction terms were computed by entering the variables into the conditional logistic regression model as continuous variables (with values of 1, 2, 3 for variables with 3 categories and values 1, 2, 3, 4 for variables with 4 categories).

All analyses were conducted with SPSS 13.0 for Windows (SPSS Inc., Cary, North Carolina). A two-tailed probability value of 0.05 was considered a statistically significant result.

Results

Baseline characteristics of cases and controls are shown in Table 1. As anticipated, cases had a significantly higher prevalence of established cardiovascular risk factors. Notably, CRP, fibrinogen and sICAM-1 levels were also significantly higher in cases compared to controls. The distribution of HSP27

Table 1. Baseline characteristics of cases and controls.

Variable	Controls (n=255)	Cases (n=255)	P-value
Age (y)	61.0±8.7	61.0±8.7	MV
Body mass index (kg/m^2)	25.5±4.4	27.0±5.2	0.001
Systolic blood pressure (mmHg)	127±14	135±16	< 0.001
Diastolic blood pressure (mmHg)	78±9	80±9	0.004
Hypertension (%)	30.6	55.7	< 0.001
Total cholesterol (mg/dl)	214±37	224±43	0.009
LDL cholesterol (mg/dl)	126±32	133±38	0.04
HDL cholesterol (mg/dl)	54±15	48±15	< 0.001
Hyperlipidemia (%)	36.9	46.3	0.04
Diabetes mellitus (%)	2.0	13.7	< 0.001
Smoking (%)			MV
- Never	42.0	42.0	
- Past	35.7	35.7	
- Current	22.4	22.4	
C-reactive protein (mg/l)	2.3 (0.9-4.0)*	3.2 (1.6-6.1)*	< 0.001
Fibrinogen (mg/dl)	375 (324-417)*	390 (333-452)*	0.02
sICAM (ng/ml)	354 (310-399)*	378 (326-447)*	0.002
Heat shock protein 27 (ng/ml)	18.6 (11.6-36.2)*	19.6 (9.7-39.3)*	0.58

Categorical variables are expressed as percentage. Continuous variables are expressed as mean ± standard deviation. P-values were obtained by paired samples t-test, Wilcoxon signed-ranks test, McNemar test or Marginal Homogeneity, whichever was appropriate.

MV=matching variable

*Median and inter-quartile range because of skewed distribution.

plasma level was right-skewed and median levels were similar in cases and controls (19.6 vs 18.6 ng/ml, p=0.58) (Table 1).

Table 2 displays baseline characteristics of controls according to tertiles of HSP27 plasma level. Age was inversely associated with HSP27 plasma level; mean age decreased from 63.4 in the lowest tertile of HSP27 to 58.0 in the highest tertile of HSP27 (P for trend < 0.001). No significant trends were present in the remaining baseline characteristics. Spearman correlation coefficients are shown in table 3. HSP27 plasma level was significantly correlated with age (r= -0.258, P<0.001). Diabetes mellitus showed a borderline significant correlation (r= -0.087, P=0.051). Correlation coefficients between the remaining characteristics and HSP27 plasma level were small and did not reach statistical significance. Adjustment for age did not materially change the estimates (data not shown), although it made the correlation with diabetes mellitus reach statistical significance (r= -0.098, P=0.027).

Odds ratios for cardiovascular disease are displayed in Table 4. No trend was found when ln transformed HSP27 plasma level was entered into the logistic regression model (odds ratio 1.00, 95% confidence interval 0.83-1.21, P = 0.99). The odds ratio of developing cardiovascular disease for the highest versus the lowest tertile of HSP27 plasma level was 0.99 (95% confidence interval 0.62-1.57). The risk estimates did not change materially when adjusted for other cardiovascular risk factors (Table 4, Models 2 and 3). We found no threshold effect when we dichotomized HSP27 plasma level based

Table 2. Baseline characteristics according to tertiles of HSP27 plasma level among control subjects.

	Tertiles of HSP27			
Variable	1 (n=85) ≤ 13.6 ng/ml	2 (n=85) >13.6 to 28.7 ng/ml	3 (n=85) > 28.7 ng/ml	P for trend
Age (y)	63.4±9.1	61.6±8.4	58.0±7.7	< 0.001
Body mass index (kg/m^2)	25.2±3.7	25.9±4.9	25.4±4.6	0.89
Systolic blood pressure (mmHg)	129±16	127±13	124±12	0.32
Diastolic blood pressure (mmHg)	78±10	78±9	77±9	0.37
Hypertension (%)	32.9	34.1	24.7	0.81
Total cholesterol (mg/dl)	210±39	216±36	217±36	0.65
LDL cholesterol (mg/dl)	124±32	127±32	128±33	0.66
HDL cholesterol (mg/dl)	51±13	55±16	55±17	0.42
Hyperlipidemia (%)	40.0	38.8	31.8	0.22
Diabetes mellitus (%)	3.5	1.2	1.2	0.41
Smoking (%)				
- Never	47.1	40.0	38.8	Reference
- Past	28.2	41.2	37.6	0.37
- Current	24.7	18.8	23.5	0.38
C-reactive protein (mg/l)	2.4 (1.0-4.1)*	2.3 (0.8-4.1)*	2.2 (0.9-4.0)*	0.71†
Fibrinogen (mg/dl)	353 (321-414)*	376 (323-417)*	386 (329-420)*	0.52†
sICAM (ng/ml)	347 (307-393)*	351 (314-390)*	368 (309-422)*	0.42†

Categorical variables are expressed as percentage. Continuous variables are expressed as mean ± standard deviation. P for trend was obtained by linear, logistic or multinomial regression, whichever was appropriate. *Median and inter-quartile range because of skewed distribution. †Variables were ln transformed to test for trend.

Table 3. Correlation between HSP27 plasma level and baseline characteristics for all subjects.

Variable	Spearman correlation coefficient	P-value
Age	-0.258	<0.001
Body mass index	-0.009	0.84
Systolic blood pressure	-0.051	0.25
Diastolic blood pressure	-0.008	0.86
Hypertension	-0.016	0.72
Total cholesterol	0.015	0.73
LDL cholesterol	0.020	0.66
HDL cholesterol	0.064	0.16
Hyperlipidemia	0.004	0.92
Diabetes mellitus	-0.087	0.05
Smoking	-0.013	0.77
C-reactive protein	-0.021	0.64
Fibrinogen	0.009	0.85
sICAM	0.011	0.82

Table 4. HSP27 plasma level and risk of cardiovascular disease.

HSP27 (ng/ml)	Odds ratio (95% confidence interval)		
	Model 1	Model 2	Model 3
Tertile 1	Reference	Reference	Reference
Tertile 2	0.67 (0.43-1.04)	0.67 (0.39-1.16)	0.53 (0.29-0.98)
Tertile 3	0.99 (0.62-1.57)	1.14 (0.63-2.06)	1.06 (0.55-2.05)
Continuous, ln transformed	1.00 (0.83-1.21)	1.02 (0.81-1.29)	0.96 (0.74-1.24)

Model 1: Matched on age, smoking (never, former, current) and length of follow-up

Model 2: Model 1, additionally adjusted for BMI, systolic blood pressure, hypertension, total cholesterol, HDL cholesterol, hyperlipidemia, diabetes mellitus

Model 3: Model 2, additionally adjusted for exercise, alcohol use, menopausal status, hormone replacement therapy, C-reactive protein.

on the 50th, 75th, and 90th percentiles in the control subjects and entered it into the model (data not shown).

When we entered interaction terms into the model, we found a possible interaction of HSP27 level and hyperlipidemia (P for interaction= 0.043). Stratification on hyperlipidemia resulted in a higher risk of cardiovascular disease associated with HSP27 level in hyperlipidemic subjects. However, the odds ratios did not reach statistical significance. No interactions were found with other characteristics, including lipid profile.

Discussion

Contrary to our *a priori* hypothesis, in this prospective study, we found no association between baseline HSP27 plasma level and risk of future cardiovascular events among initially healthy women. Further, although HSP27 was inversely associated with age, it was not associated with other established cardiovascular risk factors.

HSPs represent the response of cells of the vessel wall to various stressors, including atherosclerosis risk factors.[7] They fulfill chaperoning activity, and they appear to be important in preventing damage and in cellular repair processes after injury.[8] HSPs have also been shown to regulate cell apoptosis.[9] On the other hand, HSPs may also play a part in atherogenesis.[1,2] When cells are dying, intracellular HSPs are released into intercellular spaces to form soluble HSPs. HSP60 and 70 have been shown to bind to Toll-like receptor 4/CD14 complex, which is a soluble HSP receptor, resulting in expression of adhesion molecules by endothelial cells, in proliferation of smooth muscle cells, and in induction of several pro-inflammatory cytokines by macrophages. These inflammatory processes all contribute to the development of atherosclerosis.[1] Furthermore, HSPs may serve as a link between infections and the atherosclerotic process. Infection with agents that contain homologous HSP proteins, such as for instance Chlamydia pneumoniae, could induce an anti-self response against HSPs expressed by endothelial cells of stressed arteries through molecular mimicry in susceptible individuals.[2]

In the past years, HSPs have been gaining interest in atherosclerosis research. HSPs from the HSP60 and HSP70 families and antibodies against these HSPs have been most widely investigated. Correlations have been reported between several of these factors and cardiovascular risk factors such as LDL cholesterol and hypertension, and psychosocial measures such as socio-economic status and

psychological stress.[10-12] Furthermore, several of these factors have been associated with carotid disease and ischemic stroke,[10,13,14] with presence and severity of coronary atherosclerosis,[15,16] and with restenosis after percutaneous transluminal coronary angioplasty.[17] They have also been associated with aortic disease and peripheral vascular disease.[18-20] More recently, increased concentrations of circulating HSP70 have been associated with low risk of coronary artery disease and with decreased intima-media thickness in hypertensive patients.[21,22]

However, little has been published on HSP27 in relation to atherosclerosis.[3] With regard to the cardiovascular system, HSP27 has been reported to be differentially expressed in left ventricular samples from normal and failing dog myocardium.[23] Mice overexpressing HSP27 were protected from lethal ischemia/reperfusion injury compared to their negative littermates.[24] Increased expression of HSP27 protected against ischemic injury in adult rat cardiomyocytes.[25] HSP27 was reported to act as an anti-apoptotic protein against doxorubicin, a chemotherapeutic drug that has may cause dilative cardiomyopathy and congestive heart failure.[26] Also, expression of a specific diphosphorylated form of HSP27 was present in healthy blood vessels as opposed to vessels with cardiac allograft vasculopathy in patients who had undergone cardiac transplantaion.[27] Recently, it was reported that intracellular HSP27 allowed protection against plasmin-induced anoikis in human vascular smooth muscle cells and was inversely localized with apoptotic cells within culprit atherosclerotic carotid plaques.[28] The role of HSP27 in the extracellular compartment remains unclear; exogenously added HSP27 was shown to prevent neutrophil apoptosis,[29] but did not have any effect on plasmin-induced apoptosis in vascular smooth muscle cells.[28]

To our knowledge, two clinical studies of HSP27 and atherosclerosis have previously been reported. In the first, we used atherosclerotic carotid and femoral endarterectomy samples and mammary and radial control endarteries to demonstrate that HSP27 secretion is decreased in atherosclerotic plaques compared with control arteries.[4] Furthermore, secretion was barely detectable in complicated plaques. To confirm the hypothesis that plasma protein content can reflect arterial wall secretion, in the same study we measured soluble HSP27 level in the plasma of patients with carotid stenosis and healthy controls, and showed that HSP27 plasma levels were decreased in atherosclerotic patients. In the second study, Park et al. compared HSP27 expression in carotid plaque core areas, normal-appearing areas from the same vessel specimens, and nonatherosclerotic renal and internal mammary reference arteries. Confirming the results obtained by us,[4,28] they reported that HSP27 expression is increased in the normal-appearing vessel adjacent to atherosclerotic plaque compared to both the plaque core area and the reference arteries.[5] Contrary to the higher HSP27 expression, the phosphorylation of HSP27, which downregulates chaperone action and resistance against oxidative stress,[3] was decreased in the normal-appearing areas. Furthermore, in the same study Park et al. reported that HSP27 plasma levels were increased in acute coronary syndrome patients compared to normal reference subjects. They also demonstrated that plasma level of HSP27 was significantly correlated with serum level of total cholesterol ($r=0.254$, $P< 0.05$), and was not correlated with age, gender, smoking, diabetes mellitus and hypertension.

In contrast with the two above-mentioned reports, no association was found between HSP27 plasma level and cardiovascular disease in the present study. To evaluate this discrepancy, strengths as well as limitations of our study should be considered. The present study is part of a large prospective cohort whose methods have been evaluated repeatedly.[30,31] In the nested case-control sample

we used, cases had a higher prevalence of traditional risk factors such as hypertension and hyperlipidemia, as expected. Levels of blood biomarkers such as CRP and fibrinogen were also significantly higher in cases compared to controls. Consequently, we expect that a true difference in HSP27 level between cases and controls, if present, would have been demonstrated. The quality of the HSP27 assay should also be addressed. The precision of the assay was monitored; the interassay coefficient of variation ranged from 9.3% to 10.8%, and assays were run in duplicate and repeated if the replicate coefficient of variation was >10%. In view of these points, we consider it unlikely that the results of the present study are methodologically flawed.

Given this situation, the possibility that HSP27 plasma level might not closely reflect the secretion of HSP27 from atherosclerotic plaques should be considered as an alternative explanation for our results. If we address the hypothesis that HSP27 may be degraded by culprit atherosclerotic plaque,[28] it is unlikely that variations in HSP27 plasma level could be detected until the disease is advanced, as demonstrated in patients with a mean age of nearly 70 years with carotid atherosclerosis.[4] In this regard, we have recently observed increased plasma levels of proteolytic markers of neutrophil activation in these patients with carotid atherosclerosis.[32]

Another possibility is that HSP27 plasma level rises in the acute phase of ischemic events, as shown for HSP70,[33] in which case raised plasma level would not precede cardiovascular disease. This would help to explain the results found by Park et al., who used acute coronary syndrome patients and drew blood within 24 hours from presentation to the emergency department.[5] The study design we used, with blood collection at baseline, does not allow demonstration of such an association.

Strengths of our study include the prospective study design, inclusion of 255 cases of cardiovascular disease and the availability of detailed information on cardiovascular risk factors and several interrelated biomarkers, which made a profound analysis of the biomarker of interest possible. As such, apart from adjusting the analysis of the association between HSP27 plasma level and cardiovascular events for potential confounders, we were able to investigate whether HSP27 is associated with CRP and fibrinogen, inflammatory markers that may serve as measures for presence of infection, which on its part could play a role in the induction of HSPs. We were also able to test the hypothesis that HSPs may result in expression of adhesion molecules by endothelial cells by investigating whether HSP27 is associated with sICAM-1.

Our study included female health care professionals who were mostly white and apparently healthy at study initiation. Therefore, the results may not be applicable to other populations such as those with advanced atherosclerosis or acute coronary syndrome, or men, especially since HSP27 has been shown to have an estrogen response element in its promoter.[34,35] Nonetheless, the present study does not support an association between baseline HSP27 plasma level and incident cardiovascular disease. Furthermore, it does not support associations between HSP27 plasma level and established cardiovascular risk factors, with the exception of an inverse correlation with age. Confirmation of these findings by other prospective studies is warranted.

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Part II

Inflammation and heart failure

Chapter 5

Inflammation and risk of heart failure

Chapter 5.1

Cardiovascular risk factors and echocardiographic parameters

Abstract

Background. Insight into echocardiographic parameters in the general population may facilitate early recognition of ventricular dysfunction, reducing the population morbidity and mortality of heart failure. We examined the distribution of structural, systolic and diastolic echocardiographic parameters and their associations with cardiovascular risk factors in the Rotterdam Study.

Methods and Results. The Rotterdam Study is a population-based cohort study in men and women aged ≥ 55 years. Participants with prevalent heart failure, myocardial infarction and atrial fibrillation and flutter were excluded. Structural, systolic and diastolic parameters were assessed using two-dimensional, M-mode and Doppler echocardiography. Echocardiograms were available in 4425 participants. Structural parameters were generally larger in men, and most consistently associated with age, body mass index and blood pressure in both sexes. Prevalence of moderate and poor left ventricular systolic function was 2.8 and 1.1 %, respectively, in men, and 1.7 and 0.4%, respectively, in women. Age, body mass index and blood pressure were most consistently associated with systolic function. Doppler peak E and peak A velocities were higher in women than in men, and E/A ratio was lower in women than in men. Age and diastolic blood pressure were most consistently associated with E/A ratio in both sexes.

Conclusions. Ventricular systolic and diastolic dysfunction was present in asymptomatic individuals. Selected established cardiovascular risk factors were associated with structural, systolic and diastolic parameters. The value of echocardiography in the identification of individuals with preclinical ventricular dysfunction warrants further investigation.

Introduction

Heart failure is an important health problem. Its prevalence is increasing, in part because of improved treatment of patients with heart failure and increased survival of patients with myocardial infarction.[1] Since heart failure is primarily a condition in the elderly, the aging of the population also contributes to its increasing prevalence. Although in most patients, abnormalities of systolic and diastolic dysfunction coexist,[1]it has been shown that persons with normal ejection fraction can have impaired diastolic function.[2] Also of note is, that persons with a very low ejection fraction may be asymptomatic; this has been termed preclinical systolic dysfunction.[3]

It has been recognized that therapeutic interventions introduced even before the appearance of left ventricular dysfunction or symptoms can reduce the population morbidity and mortality of heart failure.[1,4] This underlines the importance of early recognition of preclinical ventricular dysfunction. Echocardiography, which has become a common method to assess ventricular function, may be useful to gain more insight into this matter. To its advantage, it is noninvasive and comprehensive. As a consequence, echocardiography has been used in several population-based studies to examine the prevalence and determinants of systolic and diastolic dysfunction in the community[3,5-9] and to investigate the predictive value of echocardiographic parameters for heart failure, cardiovascular disease and mortality.[3,7,10-13]

The growing importance of insight into echocardiographic characteristics of the general population has prompted us to perform echocardiography in the Rotterdam Study, a population-based study in men and women aged 55 years and over. The purpose of the present report is to describe the distribution of structural, systolic and diastolic echocardiographic parameters in our study population and to examine the associations of established cardiovascular risk factors with these echocardiographic parameters.

Methods

Study population

The study was performed within the framework of the Rotterdam Study, a population-based cohort study aimed at assessing the occurrence of and risk factors for chronic diseases in the elderly.[14] Baseline visits of the Rotterdam Study took place in 1990-1993. All inhabitants of a suburb of Rotterdam aged 55 years and over were invited and 7983 agreed to participate (response 78%). Follow-up visits took place in 1993 to 1994 and 1997 to 1999. In 2000-2001, the cohort was extended with 3011 participants from the same suburb (response 67%), also aged 55 years and over. For the present study, data collection took place from 2002-2005. Within this period, the participants from the original cohort completed their fourth center visit (n=3550), and the participants of the extended cohort completed their second center visit (n=2389). Of these, 3052 and 2235, respectively, underwent cardiac echocardiography. The large majority of missing echocardiograms was explained by incidental absence of echocardiographers. Participants with prevalent heart failure (n=304), prevalent myocardial infarction, (n=561), and atrial fibrillation (n=124) or atrial flutter (n=13) at the time of echocardiography were excluded from the analysis. This led to the exclusion of a total of 862 persons and consequently

resulted in a total of 4425 participants available for analysis. The medical ethics committee of Erasmus Medical Center, Rotterdam, approved the study. Participants gave written informed consent and permission to retrieve information from treating physicians.

Assessment of cardiovascular risk factors and prevalent disease

A trained interviewer visited all participants at home and collected information on current health status, medical history, drug use, and smoking, using a computerized questionnaire. Participants were categorized as current, past and never smokers. Clinical measures were obtained during a visit at the study center. Height and weight were measured and the body mass index (BMI) was calculated (weight (kg)/height (m)^2). Blood pressure was measured at the right brachial artery using a random-zero sphygmomanometer with the participant in sitting position. Serum total cholesterol and high-density lipoprotein (HDL) cholesterol were obtained using an automatic enzymatic procedure (Hitachi 911, Roche CHOD PAP). Diabetes mellitus was defined as the use of antidiabetic medication or a fasting glucose level of ≥7 mmol/l.[15] Presence of symptomatic heart failure at the time of echocardiography was assessed by using a validated score similar to the definition of heart failure of the European Society of Cardiology, as described previously,[16] and the information needed was obtained by screening of all medical records of general practitioners (GPs) in retrospect for the occurrence of heart failure and obtaining letters and discharge reports from medical specialists when a case was found. A history of myocardial infarction was considered present when myocardial infarction was detected by screening GP medical records and reviewing letters and discharge reports from medical specialists, or when an ECG characteristic of prior myocardial infarction was detected during a follow-up visit. Presence of atrial fibrillation or atrial flutter at the time of echocardiography was assessed with electrocardiography by applying the Modular ECG Analysis System (MEANS), which has been extensively evaluated,[17,18] and is characterized by a high sensitivity (96.6%) and a high specificity (99.5%) in coding arrhythmias.[19] For a small sample of participants, ECGs were not available at the time this report was written because of logistic problems and were approximated by using ECGs from the previous examination (year 2000-2001).

Echocardiography

For each participant, an echocardiogram was obtained. The first 2188 echocardiograms were performed with a commercially available ultrasonography system (AU3 Partner, Esaote Biomedica, with a 3.5/2.5 MHz transducer). The following 3099 echocardiograms were performed with another commercially available system (Acuson Cypress, with a 3V2c transducer). A standardized protocol was used, including two-dimensional scanning in the parasternal long axis view, parasternal short axis view, apical view and subcostal view, M-mode scanning in the parasternal long axis view, and pulsed wave Doppler scanning in the apical four chamber view.[20] Echocardiograms were recorded onto VHS tape and assessed at the reading center, which was located at Erasmus Medical Center.

Several structural parameters were assessed.[21] Left atrium diameter, left ventricular end systolic dimension (LVES), left ventricular end diastolic dimension (LVED), end diastolic interventricular septum thickness and end-diastolic left ventricular posterior wall thickness were measured in the parasternal long axis view using M-mode with two-dimensional guidance. With regard to systolic parameters, fractional shortening at the endocardium was calculated as (LVED-LVES)/LVED*100%.[21] Global left

ventricular systolic function was qualitatively assessed, without quantitative measurement, from the two-dimensional echocardiogram and classified as normal, fair, moderate or poor. Furthermore, diastolic parameters were measured.[22] Pulsed Doppler recordings of transmitral filling velocity were performed in the apical 4-chamber view, with the sample volume placed in the mitral valve orifice near the tips of the leaflets. Doppler peak E and peak A velocities were averaged over 3 cycles. E/A ratio was computed by dividing Doppler peak E velocity by Doppler peak A velocity. Early mitral valve velocity deceleration time was measured as the time between the peak E wave and the upper deceleration slope extrapolated to the zero baseline.

Echocardiograms were made and read by 4 trained echocardiographers. To assess intra-reader and inter-reader agreement, 32 participants were examined in duplicate. For continuous variables, overall median percent intra-reader and inter-reader measurement variabilities were calculated as the absolute measurement difference divided by the average of the two measurements, multiplied by 100. Overall median intra-reader and inter-reader variabilities for left ventricular end-diastolic dimension were 3% and 4%, respectively. For left ventricular end-systolic dimension both variabilities were 6%, and for fractional shortening both were 8%. Overall median intra-reader and inter-reader variabilities for E/A ratio were 15% and 18%, respectively, and for deceleration time both were 16%. Finally, the percentage agreement for categorization of left ventricular systolic function as normal or other than normal was 82% within readers and 86% between readers.

Statistical analysis

We calculated means, medians and proportions of cardiovascular risk factors and echocardiographic characteristics in men and women. Differences between the sexes were tested with t-tests, chi square tests and Mann-Whitney tests where appropriate.

Subsequently, we investigated the association between cardiovascular risk factors and echocardiographic parameters. We performed linear regressions with age, BMI, systolic blood pressure, diastolic blood pressure, total cholesterol, HDL-cholesterol, diabetes mellitus and smoking as independent variables and structural echocardiographic parameters (diameter of left atrium, left ventricular end-systolic dimension, left ventricular end-diastolic dimension, interventricular septum thickness, left ventricular posterior wall thickness), systolic parameters (fractional shortening) and diastolic parameters (mitral valve inflow peak E velocity, mitral valve inflow peak A velocity, E/A ratio and mitral valve inflow deceleration time) as dependent variables. Continuous independent variables were examined per standard deviation increase. For some of the dependent variables, the distribution of the residuals was skewed. After log-transformation of these variables, the residuals were normally distributed with a constant variance. To make the regression coefficients more informative, units of dependent variables were multiplied by 10^{-2}. The analysis was performed separately in men and women. We adjusted for age and type of ultrasonography system used, and subsequently, we also adjusted the analysis of each risk factor for all other risk factors and also for use of diuretics, beta-blockers, ACE inhibitors, lipid lowering drugs and heart rate.

We used analysis of covariance to compare risk factors according to categories of qualitatively assessed left ventricular systolic function and categories of E/A ratio and diastolic function. We categorized E/A ratio and deceleration time according to cut points used in previous reports.[3,23] Normal diastolic function was defined as E/A ratio between 0.75 and 1.50 and deceleration time between 150

ms and 240 ms. Impaired relaxation was defined as E/A ratio < 0.75 and deceleration time > 240 ms. Restrictive diastolic dysfunction was defined as E/A ratio > 1.50 and deceleration time < 150 ms. Participants were required to have both Doppler criteria consistent with impaired relaxation or restrictive dysfunction to be classified. Participants with one abnormal criterion were classified as indeterminate rather than as normal.

All analyses were performed using SPSS 11.0. Values for cardiovascular covariates were missing in less than 3% of participants, except for BMI, which was missing in 5.5%. Missing values were handled by single imputation using the expectation-maximization algorithm in SPSS 11.0. All tests were two-sided.

Results

Table 1 shows the distribution of cardiovascular risk factors in men and women. Women were significantly older than men, although this was a clinically modest difference of 1.1 years. In table 2, the distribution of echocardiographic characteristics is displayed in men and women. All characteristics showed significant differences across the sexes; in general, structural parameters were larger in men, systolic function was worse in men, and with regard to diastolic function, E/A ratio was lower in women.

For reasons of conciseness, the multivariable results are presented in the tables. Table 3 demonstrates the multivariable-adjusted associations between cardiovascular risk factors and structural parameters assessed by echocardiography. Overall, age, BMI, systolic blood pressure and diastolic blood

Table 1. Population characteristics.

Variable	Men (n=1736)	Women (n=2689)	P-value
Age (years)	70.8 ± 7.0	71.9 ± 7.5	< 0.001
Body mass index (kg/m^2)	27.2 ± 3.4	27.7 ± 4.5	< 0.001
Systolic blood pressure (mmHg)	148 ± 20	151 ± 22	< 0.001
Diastolic blood pressure (mmHg)	81 ± 11	79 ± 11	< 0.001
Total cholesterol (mmol/l)	5.4 ± 0.9	5.9 ± 0.9	< 0.001
HDL-cholesterol (mmol/l)	1.3 ± 0.3	1.6 ± 0.4	< 0.001
Diabetes mellitus (%)	13.4	12.7	0.49
Smoking			< 0.001
- Never (%)	13.4	43.1	
- Former (%)	68.2	42.8	
- Current (%)	18.4	14.0	
Use of diuretics (%)	5.4	11.8	< 0.001
Use of beta blockers (%)	11.9	15.6	< 0.001
Use of ACE-inhibitors (%)	11.2	11.0	0.79
Use of lipid-lowering drugs (%)	11.2	11.9	0.52

Categorical variables are expressed as percentage. Continuous variables are expressed as mean ± standard deviation. T-tests and chi square tests were used where appropriate.

Table 2. Echocardiographic characteristics.

Variable	Men (n=1736)	Women (n=2689)	P-value
Structural parameters			
Left atrium diameter (mm)	42 ± 5	39 ± 5	< 0.001
Left ventricular end systolic dimension (mm)	32 (29-36)*	29 (27-32)*	< 0.001
Left ventricular end diastolic dimension (mm)	53 ± 5	49 ± 5	< 0.001
Interventricular septum thickness (mm)	8 (8-10)*	8 (7-9)*	< 0.001
Left ventricular posterior wall thickness (mm)	8 (7-8)*	7 (7-8)*	< 0.001
Systolic parameters			
Fractional shortening	38 ± 7	40 ± 6	< 0.001
Left ventricular systolic function			< 0.001
- Normal (%)	55.0	64.9	
- Fair (%)	41.0	33.1	
- Moderate (%)	2.8	1.7	
- Poor (%)	1.1	0.4	
Diastolic parameters			
Mitral valve inflow peak E (m/s)	0.63 ± 0.15	0.66 ± 0.16	< 0.001
Mitral valve inflow peak A (m/s)	0.72 ± 0.16	0.80 ± 0.17	< 0.001
Mitral valve inflow deceleration time (ms)	219 ± 47	211 ± 45	< 0.001
E/A ratio	0.86 (0.71-1.00)*	0.81 (0.70-1.00)*	< 0.001

*Median and inter-quartile range because of skewed distribution.

Categorical variables are expressed as percentage. Continuous variables are expressed as mean ± standard deviation. T-tests, chi square tests and Mann-Whitney tests were used where appropriate.

pressure were significantly associated with most structural parameters in men and women. Age, BMI and systolic blood pressure generally showed positive associations, with the exception of an inverse association of age with left ventricular end diastolic dimension in women. Diastolic blood pressure showed inverse associations with left atrium diameter in men and women. Furthermore, total cholesterol showed associations with selected parameters. The age- and ultrasonography system adjusted results demonstrated additional associations between HDL-cholesterol and diabetes mellitus and most structural parameters (inverse and positive associations, respectively), which disappeared after further adjustment (data not shown).

In table 4, associations with fractional shortening are displayed. Age, BMI and diastolic blood pressure were inversely and independently associated with fractional shortening in men and women. Systolic blood pressure showed an independent, positive association in women; in men, the regression coefficient was comparable but did not reach statistical significance. The age- and ultrasonography system adjusted results were similar (data not shown). Table 5 shows associations of risk factors with left ventricular systolic function. Overall, the results were in accordance with table 4. Age was higher in men and women with fair and moderate function than in those with normal function. BMI was also significantly higher in men and women with fair function. Systolic blood pressure was lower in men with fair function than in men with normal function. In women, systolic blood pressure decreased and diastolic blood pressure increased according to systolic function categories.

Table 3. Multivariable adjusted regression coefficients for risk factors, describing the increase in structural parameters (with their appropriate units) per (sex-specific) standard deviation increase of the risk factors.

Variable	Diameter of left atrium ($mm*10^{-2}$)	Left ventricular end-systolic dimension ($mm*10^{-2}$), log transformed	Left ventricular end-diastolic dimension ($mm*10^{-2}$)	Interventricular septum thickness ($mm*10^{-2}$), log transformed	Left ventricular posterior wall thickness ($mm*10^{-2}$), log transformed
Men					
Age (years)	39.9 (12.0, 67.9)†	1.54 (0.64, 2.43)‡	-27.5 (-55.5, 4.54)	2.68 (1.54, 3.82)‡	1.67 (0.67, 2.67)†
Body mass index (kg/m^2)	203 (177, 229)‡	3.06 (2.23, 3.89)‡	126 (99.7, 151)‡	3.03 (2.02, 4.04)‡	3.10 (2.23, 3.98)‡
Systolic blood pressure (mmHg)	50.2 (21.0, 79.5)‡	0.54 (-3.89, 1.48)	57.9 (28.7, 87.1)‡	1.79 (0.60, 2.97)†	2.13 (1.10, 3.17)‡
Diastolic blood pressure (mmHg)	-18.8 (-48.4, 10.8)	-0.05 (-1.00, 0.89)	-44.7 (-74.1, -15.3)†	2.20 (1.00, 3.39)‡	0.33 (-0.71, 1.38)
Total cholesterol (mmol/l)	-11.6 (-36.5, 13.4)	-0.16 (-0.96, 0.64)	-1.65 (-26.5, 23.2)	1.06 (-2.07, -0.05)*	-0.99 (-1.88, -0.11)*
HDL-cholesterol (mmol/l)	4.06 (-21.1, 29.2)	-0.07 (-0.87, 0.74)	17.6 (-7.57, 42.7)	-0.44 (-1.46, 5.80)	-0.14 (-1.03, 0.75)
Diabetes mellitus	-3.21 (-75.1, 68.7)	1.04 (-1.25, 3.34)	34.0 (-37.7, 106)	2.74 (-0.17, 5.65)	-1.06 (-3.61, 1.49)
Current smoking	-39.5 (-100.6, 21.6)	0.87 (-1.08, 2.28)	-10.3 (-50.8, 71.4)	2.28 (-0.20, 4.76)	1.89 (-0.28, 4.05)
Women					
Age (years)	47.9 (26.0, 69.8)‡	0.50 (-0.17, 1.17)	-37.4 (-58.1, -16.7)‡	4.06 (3.26, 4.86)‡	2.18 (1.44, 2.91)‡
Body mass index (kg/m^2)	147 (126, 167)‡	2.86 (2.24, 3.49)‡	116 (96.4, 135)‡	4.00 (3.25, 4.74)‡	2.92 (2.24, 3.60)‡
Systolic blood pressure (mmHg)	34.4 (10.1, 58.6)†	0.03 (-0.71, 0.78)	32.9 (10.1, 55.7)†	1.40 (0.51, 2.28)†	0.68 (-0.13, 1.49)
Diastolic blood pressure (mmHg)	-27.9 (-51.2, -4.50)†	1.00 (0.29-1.72)†	6.77 (-15.3, 28.8)	0.97 (0.11, 1.83)*	0.78 (-229, 1.57)
Total cholesterol (mmol/l)	-2.19 (-21.5, 17.2)	-0.70 (-1.29, -0.11)†	-27.2 (-45.4, -9.00)†	0.74 (0.03, 1.44)*	-0.28 (-0.93, 0.37)
HDL-cholesterol (mmol/l)	-12.0 (-31.6, 7.56)	-0.03 (-0.63, 0.56)	4.51 (-13.9, 22.9)	-0.93 (-1.64, -0.21)*	0.05 (-0.60, 0.70)
Diabetes mellitus	13.9 (-43.7, 71.5)	1.08 (-0.67, 2.83)	62.5 (8.76, 116)*	0.96 (-1.14, 3.06)	2.17 (0.25, 4.10)*
Current smoking	-87.8 (-141, -34.7)†	0.73 (-0.87, 2.34)	19.1 (-30.3, 68.5)	1.32 (-0.60, 3.25)	2.32 (0.56, 4.08)*

* $0.01< p <0.05$, † $0.001< p <0.01$, ‡ $P<0.001$.

All models are adjusted (where appropriate) for ultrasonography system, age, BMI, systolic and diastolic blood pressure, total and HDL cholesterol, diabetes mellitus, smoking, use of diuretics, beta-blockers, ACE inhibitors, lipid lowering drugs and heart rate.

Table 4. Multivariable adjusted regression coefficients for risk factors, describing the increase in fractional shortening (% *10^{-2}) per (sex-specific) standard deviation increase of the risk factors.

	Fractional shortening (% *10^{-2})	
Variable	**Men**	**Women**
Age (years)	-125 (-163, -87.1)‡	-77.5 (-106, -49.6)‡
Body mass index (kg/m^2)	-39.4 (-74.4, -4.34)*	-26.1 (-52.0, -0.13)*
Systolic blood pressure (mmHg)	34.3 (-4.94, 73.6)	36.4 (5.52, 67.3)*
Diastolic blood pressure (mmHg)	-54.3 (-93.9, -14.6)†	-47.6 (-77.3, -17.8)†
Total cholesterol (mmol/l)	6.11 (-27.7, 39.9)	10.3 (-14.3, 34.9)
HDL-cholesterol (mmol/l)	20.0 (-14.0, 53.9)	3.59 (-21.2, 28.3)
Diabetes mellitus	-51.8 (-148.6, 45.0)	9.65 (-63.1, 82.4)
Current smoking	-41.8 (-124, 40.6)	-32.9 (-99.5, 33.7)

* 0.01< p <0.05, † 0.001< p <0.01, ‡ P<0.001.

All models are adjusted (where appropriate) for ultrasonography system, age, BMI, systolic and diastolic blood pressure, total and HDL cholesterol, diabetes mellitus, smoking, use of diuretics, beta-blockers, ACE inhibitors, lipid lowering drugs and heart rate.

Table 5. Population characteristics according to left ventricular systolic function, multivariable adjusted.

	Left ventricular systolic function			
Variable	**Normal**	**Fair**	**Moderate**	**Poor**
Men	n=938	n=699	n=48	n=19
Age (years)	69.7	72.0‡	73.6‡	72.0
Body mass index (kg/m^2)	26.9	27.5‡	26.7	27.5
Systolic blood pressure (mmHg)	149	147*	148	143
Diastolic blood pressure (mmHg)	81	82	82	83
Total cholesterol (mmol/l)	5.4	5.4	5.5	5.2
HDL-cholesterol (mmol/l)	1.33	1.31	1.30	1.39
Diabetes mellitus (%)	13.0	12.3	21.4	20.7
Current smoking (%)	16.6	21.1*	13.9	19.4
Women	n=1725	n=879	n=44	n=10
Age (years)	70.8	73.5‡	76.5‡	71.4
Body mass index (kg/m^2)	27.5	28.1‡	28.2	25.8
Systolic blood pressure (mmHg)	152	150†	144†	140*
Diastolic blood pressure (mmHg)	79	80‡	81*	81
Total cholesterol (mmol/l)	5.9	5.9	5.6*	5.4
HDL-cholesterol (mmol/l)	1.58	1.57	1.52	1.49
Diabetes mellitus (%)	11.9	14.0	6.5	11.8
Current smoking (%)	13.8	14.8	20.1	20.0

* 0.01< p <0.05, † 0.001< p <0.01, ‡ P<0.001, compared to normal left ventricular systolic function.

All models are adjusted (where appropriate) for ultrasonography system, age, BMI, systolic and diastolic blood pressure, total and HDL cholesterol, diabetes mellitus, smoking, use of diuretics, beta-blockers, ACE inhibitors, lipid lowering drugs and heart rate.

Table 6 demonstrates the multivariable-adjusted associations between cardiovascular risk factors and diastolic echocardiographic parameters. Age and diastolic blood pressure were inversely and significantly associated with E/A ratio in men and women, whereas systolic blood pressure showed no association. BMI was positively and significantly associated with E/A ratio in women; in men, the regression coefficient was of similar magnitude but did not reach significance. Age- and ultrasonography system adjusted analyses also showed inverse associations between BMI and systolic blood pressure and E/A ratio in both sexes; however, these did not persist after additional adjustment (data nor shown). Categorization of E/A ratio confirmed the associations of lower E/A ratio with higher age and diastolic blood pressure in both sexes (table 7). Of note is that it also showed significantly lower systolic blood pressure in women with E/A ratio < 0.75. In table 8 characteristics are shown according to categories of left ventricular diastolic function. In accordance with table 6 and 7, higher age was asso-

Table 6. Multivariable adjusted regression coefficients for risk factors, describing the increase in diastolic parameters (with their appropriate units) per (sex-specific) standard deviation increase of the risk factors.

Variable	Mitral valve inflow peak E ($m/s*10^{-2}$), log transformed	Mitral valve inflow peak A ($m/s*10^{-2}$)	Mitral valve inflow deceleration time ($ms*10^{-2}$)	E/A ratio ($*10^{-2}$), log transformed
Men				
Age (years)	-3.06 (-3.88, -2.23)‡	3.29 (2.43, 4.15)‡	1315 (1047, 1583)‡	-10.1 (-11.4, -8.70)‡
Body mass index (kg/m^2)	1.45 (0.67, 2.22)‡	2.46 (1.66, 3.26)‡	6.71 (-241, 255)	-1.16 (-2.42, 0.11)
Systolic blood pressure (mmHg)	4.02 (3.16, 4.88)‡	4.05 (3.15, 4.95)‡	-162 (-442, 117)	0.48 (-0.94, 1.90)
Diastolic blood pressure (mmHg)	-3.80 (-4.68, -2.93)‡	-2.03 (-2.95, -1.12)‡	259 (-26.7, 544)	-3.43 (-4.88, -1.98)‡
Total cholesterol (mmol/l)	-0.26 (-1.00, 0.47)	0.33 (-4.31, 1.09)	290 (55.0, 525)*	-0.79 (-2.01, 0.43)
HDL-cholesterol (mmol/l)	0.01 (-0.73, 0.76)	0.08 (-0.69, 0.86)	-136 (-375, 104)	0.04 (-1.17, 1.25)
Diabetes mellitus	2.58 (0.45, 4.70)*	3.98 (1.77, 6.20)‡	306 (-382, 995)	-2.66 (-6.16, 0.85)
Current smoking	-0.47 (-2.26, 1.32)	1.27 (-0.61, 3.14)	316 (-267, 890)	-2.73 (-5.71, 0.24)
Women				
Age (years)	-2.24 (-2.94, -1.54)‡	4.09 (3.37, 4.81)‡	1049 (842, 1256)‡	-9.90 (-11.0, -8.82)‡
Body mass index (kg/m^2)	0.43 (-0.22, 1.08)	1.90 (1.23, 2.56)‡	38.8 (-154, 232)	-1.59 (-2.59, -0.60)†
Systolic blood pressure (mmHg)	3.43 (2.65, 4.20)‡	3.73 (2.94, 4.53)‡	-423 (-650, -195)‡	0.95 (-0.24, 2.14)
Diastolic blood pressure (mmHg)	-3.18 (-3.93, -2.43)‡	-1.52 (-2.29, -0.76)‡	184 (-35.8, 403)	-3.61 (-4.76, -2.46)‡
Total cholesterol (mmol/l)	-0.48 (-1.10, 0.14)	-0.04 (-0.67, 0.59)	146 (-34.4, 327)	-0.61 (-1.56, 0.33)
HDL-cholesterol (mmol/l)	0.06 (-0.57, 0.68)	-0.55 (-1.19, 0.08)	-106 (-289, 76.9)	1.28 (0.32, 2.24)†
Diabetes mellitus	2.04 (0.19, 3.90)*	2.28 (0.39, 4.18)*	469 (-75.5, 1013)	0.78 (-2.05, 3.61)
Current smoking	-1.54 (-3.25, 0.16)	-0.89 (-2.61, 0.84)	447 (-50.7, 945)	-0.84 (-3.41, 1.73)

* $0.01 < p < 0.05$, † $0.001 < p < 0.01$, ‡ $P < 0.001$.

All models are adjusted (where appropriate) for ultrasonography system, age, BMI, systolic and diastolic blood pressure, total and HDL cholesterol, diabetes mellitus, smoking, use of diuretics, beta-blockers, ACE inhibitors, lipid lowering drugs and heart rate.

Table 7. Population characteristics according to E/A ratio, multivariable adjusted.

Variable	E/A ratio		
	E/A < 0.75	**0.75≤ E/A≤ 1.50**	**E/A > 1.50**
Men	n=457	n=1183	n=29
Age (years)	73.5‡	69.7	69.2
Body mass index (kg/m^2)	27.2	27.2	27.2
Systolic blood pressure (mmHg)	147	148	149
Diastolic blood pressure (mmHg)	82†	81	80
Total cholesterol (mmol/l)	5.4	5.4	5.2
HDL-cholesterol (mmol/l)	1.31	1.32	1.33
Diabetes mellitus (%)	14.7	12.1	16.0
Current smoking (%)	29.9	18.0	11.3
Women	n=824	n=1730	n=36
Age (years)	74.6‡	70.4	69.7
Body mass index (kg/m^2)	27.9	27.6	26.9
Systolic blood pressure (mmHg)	149†	151	152
Diastolic blood pressure (mmHg)	81‡	79	77
Total cholesterol (mmol/l)	6.0	5.9	5.7
HDL-cholesterol (mmol/l)	1.55*	1.59	1.59
Diabetes mellitus (%)	11.6	12.6	21.9
Current smoking (%)	14.6	14.4	13.0

* $0.01< p <0.05$, † $0.001< p <0.01$, ‡ $P<0.001$, compared to $0.75\leq E/A\leq 1.50$.

All models are adjusted (where appropriate) for ultrasonography system, age, BMI, systolic and diastolic blood pressure, total and HDL cholesterol, diabetes mellitus, smoking, use of diuretics, beta-blockers, ACE inhibitors, lipid lowering drugs and heart rate.

ciated with impaired relaxation in men and women. Diastolic blood pressure was significantly higher in women with impaired relaxation compared to women with normal diastolic function. In men with impaired relaxation, diastolic blood pressure, although higher, did not reach statistical significance. Finally, systolic blood pressure was significantly lower in women with impaired relaxation.

Discussion

Summarizing, in this study, we have examined structural, systolic and diastolic echocardiographic parameters and their associations with cardiovascular risk factors in a population-based cohort. Structural parameters were generally larger in men, and larger values were most consistently associated with higher values of age, BMI and systolic blood pressure in both sexes. Although we had excluded participants with prevalent heart failure, myocardial infarction, and atrial fibrillation and flutter, moderate or poor left ventricular systolic function was found to be present in the participants, albeit with a modest prevalence (a total of 3.9% of men and 2.1% of women). Higher age, higher BMI, lower systolic and higher diastolic blood pressure were most consistently associated with worse systolic function.

Table 8. Population characteristics according to left ventricular diastolic function, multivariable adjusted.

	Left ventricular diastolic function			
Variable	**Normal**, 0.75≤E/A≤1.50 150≤DT≤240	**Impaired relaxation**, E/A<0.75 DT>240	**Restrictive,** E/A>1.50 DT<150	**Indeterminate** (Other combination of E/A and DT)
Men	n=882	n=196	n=5	n=564
Age (years)	69.3	74.1‡	69.4	71.5‡
Body mass index (kg/m^2)	27.3	27.2	27.0	27.1
Systolic blood pressure (mmHg)	148	147	143	147
Diastolic blood pressure (mmHg)	81	82	84	82*
Total cholesterol (mmol/l)	5.4	5.4	6.0	5.4
HDL-cholesterol (mmol/l)	1.32	1.28	1.36	1.32
Diabetes mellitus (%)	12.0	13.5	29.6	13.5
Current smoking (%)	17.0	18.5	0.0	20.0
Women	n=1373	n=275	n=8	n=905
Age (years)	70.2	76.0‡	71.0	72.6‡
Body mass index (kg/m^2)	27.6	27.7	24.3*	27.8
Systolic blood pressure (mmHg)	151	148†	159	151
Diastolic blood pressure (mmHg)	79	81‡	76	79*
Total cholesterol (mmol/l)	5.9	6.0	5.6	5.9
HDL-cholesterol (mmol/l)	1.59	1.55	1.60	1.57
Diabetes mellitus (%)	12.56	15.4	50.4‡	11.0
Current smoking (%)	13.8	16.0	0.0	15.0

* 0.01< p <0.05, † 0.001< p <0.01, ‡ P<0.001, compared to normal left ventricular diastolic function.

E/A = E/A ratio. DT = deceleration time. All models are adjusted (where appropriate) for ultrasonography system, age, BMI, systolic and diastolic blood pressure, total and HDL cholesterol, diabetes mellitus, smoking, use of diuretics, beta-blockers, ACE inhibitors, lipid lowering drugs and heart rate.

With regard to diastolic parameters, we found that Doppler peak E and peak A velocities were larger in women than in men, but that E/A ratio was lower in women than in men. Higher age and higher diastolic blood pressure were most consistently associated with lower E/A ratio in both sexes.

Strengths of the present study include its population-based nature, its large size, and its standardized assessment of risk factors and echocardiographic characteristics. Nevertheless, several issues warrant consideration. First, we used global left ventricular function assessed qualitatively from two-dimensional images, without quantitative measurement of the ejection fraction. This approach is easily obtainable and reflects the routine procedure in echocardiography laboratories. Prior studies have reported that the reproducibility and accuracy of visual assessments are as good as those of quantitative methods,[24,25] and similar visual assessments have also been used in other studies such as the Framingham Study.[13] Furthermore, we have also measured fractional shortening which is a quantitative measure and also an indicator of systolic function. Associations of risk factors with fractional shortening showed a pattern similar to associations with qualitatively assessed left ventricular func-

tion. This suggests that assessment of left ventricular systolic function was performed appropriately in our study.

Second, we categorized diastolic function solely according to E/A ratio and deceleration time, while additional measures such as pulmonary venous flow or Doppler tissue imaging are needed to distinguish the pseudonormal pattern from normal diastolic function.[26] We were not able to assess such additional measures because of constraints in examination time available per participant, inherent to large-scale population-based research. Furthermore, several different recommendations have been issued for categorization of diastolic function based on E/A ratio and deceleration time.[27,28] We based our categorization on recent reports.[3,23] The use of only two criteria resulted in a substantive group of participants with "indeterminate" diastolic function (33%). Furthermore, only 0.3% of the population fell into the restrictive category, which made this group inadequately small for drawing statistically powered conclusions. When we made the criteria more stringent (E/A ratio < 0.50 or > 1.50 and deceleration time < 150 or > 280), percentages of the study population in the impaired relaxation, restrictive, and indeterminate categories were 0.3%, 0.3%, and 13.1%, respectively. From the above, it is clear that the choice of cut-points complicates the estimation of the prevalence of diastolic dysfunction. However, the categorization may still be a useful addition when examining the associations of risk factors with diastolic parameters.

Finally, we should bear in mind that some of the echocardiographic measurements displayed considerable variability. Inter-reader and intra-reader measurement variabilities for E/A ratio and deceleration time reflect the practical difficulties involved in estimating Doppler peak E velocity and Doppler peak A velocity. In contrast, intra-reader and inter-reader variability of structural and systolic parameters was in a much lower range.

Previously, fractional shortening has been measured in a subset of 2267 participants of the Rotterdam Study.[29] In accordance with the present study, fractional shortening was found to be somewhat higher in women (40±7%) than in men (38±8%). However, no significant association was found with age.

The distribution of echocardiographic parameters and their associations with cardiovascular risk factors have previously been examined in several population-based studies. The Cardiovascular Health Study has reported a prevalence of left ventricular ejection fraction abnormalities of 6.3% in men and 1.8% in women. Sex, age, hypertension and clinical coronary heart disease were the only independent predictors of left ventricular ejection fraction abnormalities.[8] In the same study, Doppler early and late diastolic left ventricular filling velocities were importantly related to sex, age, blood pressure and also to height, weight and heart rate.[30] In accordance with our study, women had higher early and late diastolic velocities than men, and diastolic blood pressure was inversely related and systolic blood pressure was positively related to Doppler early and late velocities. The Olmsted County study has reported a prevalence of 6.0% of systolic dysfunction, defined as an ejection fraction of ≤50%.[3] Furthermore, the prevalence of mild, moderate and severe diastolic dysfunction was 20.8%, 6.6% and 0.7%, respectively (definitions of mild and severe diastolic dysfunction roughly corresponding to our impaired relaxation and restrictive categories, respectively). The prevalence of diastolic dysfunction increased with age, was more common in participants with cardiovascular disease, diabetes, or systolic dysfunction, and was equally common in men and women. The Framingham study has reported a prevalence of 5.1% of abnormally low fractional shortening (≤30%) measured by M-mode echocar-

diography in 1493 men.[12] Later, they reported a prevalence of visually estimated ejection fraction ≤ 50% without having a history of heart failure of 6% in men and 0.8% in women.[13] Furthermore, this study has reported that E/A ratio is associated with age, sex, systolic blood pressure, heart rate, PR interval and left ventricular systolic function in a subset of 127 randomly selected, normal subjects.[5] The Strong Heart study has reported that left ventricular systolic dysfunction, defined as ejection fraction ≤54%, was present in 14% of the subjects and that is was independently associated with male sex, hypertension, overweight, overt heart failure and coronary heart disease, and less consistently with older age and diabetes.[6] With regard to diastolic parameters, 16% of the participants had E/A ratio < 0.6 and 3% had E/A ratio >1.5. Sex, age, BMI, systolic and diastolic blood pressure, heart rate, diabetes, baseline coronary heart disease and heart failure showed associations with abnormal E/A ratio (< 0.6 or > 1.5). The above underlines that prevalence of systolic and diastolic dysfunction varies across studies, probably in part due to differences in population characteristics and definitions of ventricular dysfunction. Nevertheless, associations of age, sex, and blood pressure with ventricular dysfunction appear to be rather consistent across studies.

In conclusion, we have found that moderate and poor left ventricular systolic function was present in asymptomatic men and women participating in the Rotterdam Study. With regard to diastolic parameters, we found that E/A ratio was on average lower in women than in men. Higher age, BMI and diastolic blood pressure and lower systolic blood pressure were most consistently associated with worse systolic function in both sexes. Higher age and diastolic blood pressure were most consistently associated with lower E/A ratio in both sexes. Further research is warranted to investigate the value of echocardiography in identification of individuals with preclinical ventricular dysfunction.

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Chapter 5.2

Echocardiographic parameters and mortality

Abstract

Background. Even when heart failure has not yet become clinically manifest, preclinical ventricular dysfunction may be present, and therapeutic interventions introduced at this time may reduce morbidity and mortality. However, data on the predictive value of echocardiographic characteristics in the general population remain relatively scarce.

Methods. The Rotterdam Study is a population-based cohort study in men and women aged ≥ 55 years. Participants with prevalent heart failure, myocardial infarction and atrial fibrillation and flutter at the time of echocardiography were excluded. Structural, systolic and diastolic parameters were assessed using two-dimensional, M-mode and Doppler echocardiography. Echocardiograms were available in 4425 participants.

Results. During a mean follow-up of 3.0 years, 226 participants died. Increased left ventricular mass was an independent risk factor for all-cause mortality, particularly in men (hazard ratio per standard deviation of natural log transformed left ventricular mass, 1.20 (95% CI, 1.01-1.43)). Fractional shortening and left ventricular systolic function did not show a clear association with mortality. E/A ratio < 0.75 was an independent risk factor in men (age-adjusted hazard ratio 1.82 (95% CI 1.23-2.69)). This was further reflected by diastolic function: impaired relaxation was a risk factor in men, but not in women.

Conclusions. Structural and diastolic echocardiographic parameters are associated with all-cause mortality in an asymptomatic population. However, the evidence is still inadequate to support the usefulness of echocardiography for screening to identify asymptomatic individuals with preclinical ventricular dysfunction.

Introduction

Heart failure has been shown to entail an increased risk of mortality.[1] This issue is gaining importance because the burden of heart failure is steadily increasing, in part as a result of better treatment of cardiovascular disease and of the aging of the population.[2] Even when heart failure has not yet become clinically manifest, preclinical ventricular dysfunction may be present.[3] It has been recognized that therapeutic interventions introduced even before the appearance of left ventricular dysfunction or symptoms may reduce morbidity and mortality.[2]

The above underlines the value of early recognition of preclinical abnormalities. Echocardiography may be useful for this purpose, and may also be useful to gain more insight into which of these preclinical features predispose to earlier mortality. Important advantages of echocardiography include its noninvasive and comprehensive nature. As a consequence, the predictive value of echocardiographic measures for occurrence of heart failure, cardiovascular disease and for mortality has been investigated within several population-based studies, such as the Framingham Study, Cardiovascular Health Study, The Strong Heart Study and the Olmsted County study.[3-13] Nonetheless, data on the predictive value of echocardiographic characteristics in the general population still remain relatively scarce.

To expand the current knowledge on echocardiographic parameters that may increase risk of death in the general population, we have performed a comprehensive investigation of structural, systolic as well as diastolic echocardiographic parameters in relation to all-cause mortality within a population-based cohort study in men and women aged 55 years and over.

Methods

Study population

The study was performed within the framework of the Rotterdam Study, a population-based cohort study aimed at assessing the occurrence of and risk factors for chronic diseases in the elderly.[14] Baseline visits of the Rotterdam Study took place in 1990-1993. All inhabitants of a suburb of Rotterdam aged 55 years and over were invited and 7983 agreed to participate (response 78%). Follow-up visits took place in 1993 to 1994 and 1997 to 1999. In 2000-2001, the cohort was extended with 3011 participants from the same suburb (response 67%), also aged 55 years and over. For the present study, data collection took place from 2002-2005. Within this period, the participants from the original cohort completed their fourth center visit (n=3550), and the participants of the extended cohort completed their second center visit (n=2389). Of these, 3052 and 2235, respectively, underwent cardiac echocardiography. Missing echocardiograms were largely caused by the absence of echocardiographers. Participants with prevalent heart failure (n=304), myocardial infarction, (n=561), or atrial fibrillation (n=124) or atrial flutter (n=13) at the time of echocardiography were excluded from the analysis; this led to the exclusion of a total of 862 persons and consequently resulted in a total of 4425 participants available for analysis. The medical ethics committee of Erasmus Medical Center, Rotterdam, approved the study. Participants gave written informed consent and permission to retrieve information from treating physicians.

Echocardiography

For each participant, an echocardiogram was obtained. The first 2188 echocardiograms were performed with a commercially available ultrasonography system (AU3 Partner, Esaote Biomedica, with a 3.5/2.5 MHz transducer). The following 3099 echocardiograms were performed with another commercially available system (Acuson Cypress, with a 3V2c transducer). A standardized protocol was used, including two-dimensional scanning in the parasternal long axis view, parasternal short axis view, apical view and subcostal view, M-mode scanning in the parasternal long axis view, and pulsed wave Doppler scanning in the apical four chamber view.[15] Echocardiograms were recorded onto VHS tape and assessed at the reading center, which was located at Erasmus Medical Center.

Several structural parameters were assessed.[16] Left atrium diameter, left ventricular end systolic dimension (LVES), left ventricular end diastolic dimension (LVED), end diastolic interventricular septum thickness (IVST) and end-diastolic left ventricular posterior wall thickness (PWT) were measured in the parasternal long axis view using M-mode with two-dimensional guidance. Left ventricular mass (grams) was calculated as $0.80(1.04*((IVST+LVED+PWT)^3-LVED^3))+0.6$, according to Devereux et al.[17] With regard to systolic parameters, fractional shortening at the endocardium was calculated as (LVED-LVES)/LVED*100%.[16] Global left ventricular systolic function was qualitatively assessed, without quantitative measurement, from the two-dimensional echocardiogram and classified as normal, fair, moderate or poor. Furthermore, diastolic parameters were measured.[18] Pulsed Doppler recordings of transmitral filling velocity were performed in the apical 4-chamber view, with the sample volume placed in the mitral valve orifice near the tips of the leaflets. Doppler peak E and peak A velocities were averaged over 3 cycles. E/A ratio was computed by dividing Doppler peak E velocity by Doppler peak A velocity. Early mitral valve velocity deceleration time was measured as the time between the peak E wave and the upper deceleration slope extrapolated to the zero baseline.

Echocardiograms were made and read by 4 trained echocardiographers. To assess intra-reader and inter-reader agreement, 32 participants were examined in duplicate. For continuous variables, overall median percent intra-reader and inter-reader measurement variabilities were calculated as the absolute measurement difference divided by the average of the two measurements, multiplied by 100. Overall median intra-reader and inter-reader variabilities for left ventricular end-diastolic dimension were 3% and 4%, respectively. For left ventricular end-systolic dimension both variabilities were 6%, and for fractional shortening both were 8%. Overall median intra-reader and inter-reader variabilities for E/A ratio were 15% and 18%, respectively, and for deceleration time both were 16%. Finally, the percentage agreement for categorization of left ventricular systolic function as normal or other than normal was 82% within readers and 86% between readers.

Assessment of cardiovascular risk factors and prevalent disease

A trained interviewer visited all participants at home and collected information on current health status, medical history, drug use, and smoking, using a computerized questionnaire. Clinical measures were obtained during a visit at the study center using standard procedures.[19] Presence of symptomatic heart failure at the time of echocardiography was assessed by using a validated score similar to the definition of heart failure of the European Society of Cardiology, as described previously,[20] and the information needed was obtained by screening of all medical records of general practitioners (GPs) in retrospect for the occurrence of heart failure and obtaining letters and discharge reports from

medical specialists when a case was found. Prevalent myocardial infarction was considered present when myocardial infarction was detected by screening GP medical records and reviewing letters and discharge reports from medical specialists, or when an ECG characteristic of prior myocardial infarction was detected during a follow-up visit. Presence of atrial fibrillation or atrial flutter at the time of echocardiography was assessed with electrocardiography by applying the Modular ECG Analysis System (MEANS), which has been extensively evaluated,[21,22] and is characterized by a high sensitivity (96.6%) and a high specificity (99.5%) in coding arrhythmias.[23] For a small sample of participants, ECGs were not available at the time this report was written because of logistic problems and were approximated by using ECGs from the previous examination (year 2000-2001).

Follow-up procedure

Information on vital status of the Rotterdam Study participants was obtained from general practitioner medical records and from municipal records. Follow-up for all-cause mortality started at the echocardiographic examination and for the present study was complete until February 21, 2007.

Statistical analysis

We calculated means, medians and proportions of cardiovascular risk factors and echocardiographic characteristics in men and women. To investigate the association between echocardiographic parameters and all-cause mortality, we used Cox proportional hazards models. The proportional hazards assumption was tested by drawing log minus log plots of the survival function or by adding interaction terms involving time to the models where appropriate. Diameter of left atrium, left ventricular end-systolic dimension, left ventricular end-diastolic dimension, interventricular septum thickness, left ventricular posterior wall thickness, left ventricular mass, fractional shortening, mitral valve inflow E-peak, mitral valve inflow A-peak, E/A ratio and mitral valve inflow deceleration time were entered as independent variables. Variables were natural log transformed when their distributions were skewed. Hazard ratios were computed per standard deviation increase in the independent variables. E/A ratio was also entered as a categorical variable with cut-points 0.75 and 1.50.[3,24] In model 1, we adjusted for age, sex and type of ultrasonography system, and in model 2, we adjusted for age, sex, type of ultrasonography system, body mass index, systolic blood pressure, diastolic blood pressure, total cholesterol, HDL-cholesterol, diabetes mellitus, smoking, use of diuretics, beta-blockers, ACE inhibitors and lipid lowering drugs, and heart rate. The analysis was repeated in men and women separately.

Left ventricular systolic and diastolic function were entered into the Cox proportional hazards model as categorical variables. We categorized diastolic function according to cut points used in previous reports.[3,24] Normal diastolic function was defined as E/A ratio between 0.75 and 1.50 and deceleration time between 150 ms and 280 ms. Impaired relaxation was defined as E/A ratio < 0.75 and deceleration time > 280 ms. Restrictive diastolic dysfunction was defined as E/A ratio > 1.50 and deceleration time < 150 ms. Participants were required to have both Doppler criteria consistent with impaired relaxation or restrictive dysfunction to be classified. Participants with one abnormal criterion were classified as indeterminate rather than as normal.

All analyses were performed using SPSS 11.0. Values for cardiovascular covariates were missing in less than 3% of participants, except for BMI, which was missing in 5.5%. Missing values were handled by single imputation using the expectation-maximization algorithm in SPSS 11.0. All tests were two-sided.

Results

Table 1 shows baseline characteristics in men and women. All echocardiographic characteristics showed significant differences across the sexes; in general, structural parameters were larger in men, systolic function was worse in men, and with regard to diastolic function, E/A ratio was lower in women.

During a mean follow-up time of 3.0 (standard deviation, 1.0) years, death occurred in 226 persons. In table 2, hazard ratios of all-cause mortality, adjusted for age, sex and type of ultrasonography system, are shown. The structural parameters interventricular septum thickness and left ventricular posterior wall thickness were significantly and positively associated with all-cause mortality. After stratifying on sex, the hazard ratio for interventricular septum thickness remained of similar magnitude in both sexes but lost significance in women, whereas left ventricular posterior wall thickness no longer showed an association in women. Left ventricular mass was positively associated with all-cause mortality in the total population and in men, but not in women. The estimates did not materially change after multivariable adjustment.

With regard to systolic parameters, fractional shortening did not show a significant association with all-cause mortality (table 2). As for qualitatively assessed left ventricular systolic function, risk of all-cause mortality was increased in participants with poor systolic function, but the risk estimates did not reach statistical significance (table 3). Overall survival at the end of follow-up in persons with normal, fair, moderate and poor systolic function was 96.6%, 92.4%, 92.4% and 89.7%, respectively.

As to diastolic parameters, E/A ratio showed a significant and inverse association with all-cause mortality in the total population (table 2). After stratification on sex, this effect only remained present in men. Accordingly, after categorization of E/A ratio, E/A ratio < 0.75 was associated with an increased risk of mortality in men. In women, E/A ratio > 1.50 resulted in an increased but non-significant risk (table 4). Furthermore, impaired relaxation was also associated with an increased risk in men, while a restrictive pattern was associated with an increased risk in both men and women (table 4). Overall survival at the end of follow-up in persons with normal, impaired and restrictive diastolic function was 97%, 91.0% and 76.9%, respectively. Results remained essentially the same after multivariable adjustment.

Table 1. Baseline characteristics.

Variable	All (n=4425)	Men (39%)	Women (61%)	P-value
Age (years)	71.4 ± 7.3	70.8 ± 7.0	71.9 ± 7.5	< 0.001
Body mass index (kg/m^2)	27.5 ± 4.1	27.2 ± 3.4	27.7 ± 4.5	< 0.001
Systolic blood pressure (mmHg)	150 ± 21	148 ± 20	151 ± 22	< 0.001
Diastolic blood pressure (mmHg)	80 ± 11	81 ± 11	79 ± 11	< 0.001
Total cholesterol (mmol/l)	5.7 ± 1.0	5.4 ± 0.9	5.9 ± 0.9	< 0.001
HDL-cholesterol (mmol/l)	1.5 ± 0.4	1.3 ± 0.3	1.6 ± 0.4	< 0.001
Diabetes mellitus (%)	12.9	13.4	12.7	0.49
Smoking				< 0.001
- Never (%)	31.5	13.4	43.1	
- Former (%)	52.7	68.2	42.8	
- Current (%)	15.8	18.4	14.0	
Use of diuretics (%)	9.3	5.4	11.8	< 0.001
Use of beta blockers (%)	14.1	11.9	15.6	< 0.001
Use of ACE-inhibitors (%)	11.1	11.2	11.0	0.79
Use of lipid-lowering drugs (%)	11.6	11.2	11.9	0.52
Left atrium diameter (mm)	40 ± 5	42 ± 5	39 ± 5	< 0.001
Left ventricular end systolic dimension (mm)	30 (28-34)*	32 (29-36)*	29 (27-32)*	< 0.001
Left ventricular end diastolic dimension (mm)	51 ±5	53 ± 5	49 ± 5	< 0.001
Interventricular septum thickness (mm)	8 (7-9)*	8 (8-10)*	8 (7-9)*	< 0.001
Left ventricular posterior wall thickness (mm)	7 (7-8)*	8 (7-8)*	7 (7-8)*	< 0.001
Left ventricular mass (g)	140 (117-167)*	157 (135-187)*	130 (110-151)*	< 0.001
Fractional shortening (%)	39 ± 7	38 ± 7	40 ± 6	< 0.001
Left ventricular systolic function				< 0.001
- Normal (%)	61.0	55.0	64.9	
- Fair (%)	36.2	41.0	33.1	
- Moderate (%)	2.1	2.8	1.7	
- Poor (%)	0.7	1.1	0.4	
Mitral valve inflow peak E (m/s)	0.65 ± 0.16	0.63 ± 0.15	0.66 ± 0.16	< 0.001
Mitral valve inflow peak A (m/s)	0.77 ± 0.17	0.72 ± 0.16	0.80 ± 0.17	< 0.001
Mitral valve inflow deceleration time (ms)	214 ± 46	219 ± 47	211 ± 45	< 0.001
E/A ratio	0.83 (0.71-1.00)*	0.86 (0.71-1.00)*	0.81 (0.70-1.00)*	< 0.001

*Median and inter-quartile range because of skewed distribution.

Categorical variables are expressed as percentage. Continuous variables are expressed as mean ± standard deviation. P-values are for differences between men and women. T-tests, chi square tests and Mann-Whitney tests were used where appropriate.

Table 2. Hazard ratios for all-cause mortality per standard deviation of echocardiographic parameters (natural log transformed when appropriate).

Variable	Hazard ratio per standard deviation (95% confidence interval)					
	Model 1			Model 2		
	All	Men	Women	All	Men	Women
Left atrium diameter (mm)	0.94 (0.82-1.08)	0.94 (0.78-1.12)	0.96 (0.80-1.15)	0.96 (0.83-1.11)	0.99 (0.81-1.21)	0.96 (0.78-1.17)
Left ventricular end systolic dimension (mm)‡	1.02 (0.89-1.17)	1.03 (0.85-1.23)	1.01 (0.85-1.21)	1.03 (0.89-1.18)	1.04 (0.86-1.27)	1.00 (0.83-1.20)
Left ventricular end diastolic dimension (mm)	0.98 (0.86-1.13)	1.01 (0.85-1.21)	0.96 (0.80-1.15)	0.99 (0.86-1.14)	1.04 (0.86-1.26)	0.96 (0.80-1.16)
Interventricular septum thickness (mm)‡	1.21 (1.06-1.38)†	1.25 (1.03-1.51)*	1.20 (0.99-1.46)	1.21 (1.05-1.39)†	1.26 (1.02-1.54)*	1.20 (0.97-1.47)
Left ventricular posterior wall thickness (mm)‡	1.13 (1.00-1.29)*	1.21 (1.02-1.44)*	1.07 (0.88-1.28)	1.12 (0.98-1.27)	1.19 (0.99-1.43)	1.05 (0.87-1.28)
Left ventricular mass (g)‡	1.15 (1.01-1.31)*	1.20 (1.01-1.43)*	1.09 (0.91-1.31)	1.16 (1.01-1.34)*	1.24 (1.02-1.51)*	1.10 (0.91-1.34)
Fractional shortening (%)	0.94 (0.83-1.08)	0.95 (0.78-1.15)	0.95 (0.79-1.13)	0.95 (0.83-1.08)	0.94 (0.77-1.15)	0.97 (0.81-1.16)
Mitral valve inflow peak E (m/s)	1.01 (0.89-1.16)	0.94 (0.78-1.13)	1.08 (0.91-1.29)	0.98 (0.86-1.12)	0.89 (0.73-1.08)	1.06 (0.89-1.27)
Mitral valve inflow peak A (m/s)	1.11 (0.98-1.26)	1.19 (1.03-1.37)*	1.00 (0.83-1.21)	1.10 (0.97-1.25)	1.16 (0.99-1.36)	1.02 (0.84-1.23)
Mitral valve inflow deceleration time (ms)	0.98 (0.86-1.12)	1.07 (0.89-1.29)	0.89 (0.74-1.07)	0.99 (0.87-1.12)	1.07 (0.89-1.28)	0.92 (0.76-1.10)
E/A ratio ‡	0.87 (0.75-1.00)*	0.75 (0.61-0.91)†	1.02 (0.84-1.23)	0.85 (0.74-0.98)*	0.75 (0.62-0.92)†	0.98 (0.80-1.19)

Model 1. Adjusted for age and type of ultrasonography system (and sex where appropriate).

Model 2. Adjusted for age, type of ultrasonography system, sex (where appropriate), body mass index, systolic blood pressure, diastolic blood pressure, total cholesterol, HDL-cholesterol, diabetes mellitus, smoking, heart rate, use of diuretics, beta-blockers, ACE inhibitors and lipid lowering drugs and heart rate.

* $0.01 < p < 0.05$; † $p < 0.01$; ‡ Natural log transformed because of skewed distribution.

Table 3. Hazard ratios for all-cause mortality per category of left ventricular systolic function.

	All		Men		Women	
Systolic function	Cases/ subjects	HR (95% CI)	Cases/ subjects	HR (95% CI)	Cases/ subjects	HR (95% CI)
Model 1						
Normal	91/ 2662	1.00 (ref.)	36/ 938	1.00 (ref.)	55/ 1724	1.00 (ref.)
Fair	119/ 1578	1.21 (0.91-1.61)	67/ 699	1.34 (0.88-2.03)	52/ 879	1.14 (0.76-1.69)
Moderate	7/ 92	0.90 (0.42-1.97)	5/ 48	1.05 (0.40-2.70)	2/ 44	0.69 (0.17-2.84)
Poor	3/ 29	1.74 (0.55-5.52)	2/ 19	1.48 (0.35-6.15)	1/ 10	3.16 (0.44-23.0)
Model 2						
Normal	91/ 2662	1.00 (ref.)	36/ 938	1.00 (ref.)	55/ 1724	1.00 (ref.)
Fair	119/ 1578	1.22 (0.92-1.62)	67/ 699	1.46 (0.96-2.22)	52/ 879	1.08 (0.73-1.61)
Moderate	7/ 92	0.95 (0.44-2.08)	5/ 48	1.22 (0.47-3.17)	2/ 44	0.70 (0.17-2.91)
Poor	3/ 29	1.85 (0.58-5.93)	2/ 19	1.68 (0.40-7.14)	1/ 10	3.94 (0.54-29.0)

HR= Hazard ratio, CI= Confidence interval

Model 1. Adjusted for age and type of ultrasonography system (and sex where appropriate).

Model 2. Adjusted for age, type of ultrasonography system, sex (where appropriate), body mass index, systolic blood pressure, diastolic blood pressure, total cholesterol, HDL-cholesterol, diabetes mellitus, smoking, heart rate, use of diuretics, beta-blockers, ACE inhibitors and lipid lowering drugs and heart rate.

Discussion

In summary, in this population-based study, we have examined structural, systolic and diastolic echocardiographic parameters and their associations with all-cause mortality. Increased left ventricular mass was an independent risk factor, particularly in men. Fractional shortening and left ventricular systolic function did not show a clear association. Low E/A ratio was an independent risk factor in men. This was further reflected by diastolic function: impaired relaxation was a risk factor in men, but not in women.

Strengths of the present study include its population-based nature, its large size, and its standardized assessment of risk factors and echocardiographic characteristics. Nevertheless, several issues warrant consideration. First, we used global left ventricular function assessed qualitatively from two-dimensional images, without quantitative measurement of the ejection fraction. This approach is easily obtainable and reflects the routine procedure in echocardiography laboratories. Prior studies have reported that the reproducibility and accuracy of visual assessments are as good as those of quantitative methods,[25,26] and similar visual assessments have also been used in other studies such as the Framingham Study.[13] Furthermore, we also measured fractional shortening which is a quantitative indicator of systolic function. Second, we categorized diastolic function solely according to E/A ratio and deceleration time, while additional measures such as pulmonary venous flow or Doppler tissue imaging are needed to distinguish the pseudonormal pattern from normal diastolic function.[27] Also, several different recommendations have been issued for categorization of diastolic function based on E/A ratio and deceleration time.[28,29] We based our criteria for diastolic dysfunction on recent reports.[3,24]

Table 4. Hazard ratios for all-cause mortality per category of E/A ratio and left ventricular diastolic function.

	All		Men		Women	
Variable	Cases/ subjects	HR (95% CI)	Cases/ subjects	HR (95% CI)	Cases/ subjects	HR (95% CI)
E/A ratio						
Model 1						
0.75-1.50	105/ 2912	1.00 (ref.)	53/ 1183	1.00 (ref.)	52/ 1729	1.00 (ref.)
< 0.75	103/ 1281	1.47 (1.11-1.95)*	56/ 457	1.82 (1.23-2.69)†	47/ 824	1.18 (0.78-1.78)
> 1.50	5/ 65	1.81 (0.74-4.44)	1/29	0.87 (0.12-6.31)	4/ 36	2.61 (0.94-7.25)
Model 2						
0.75-1.50	105/ 2912	1.00 (ref.)	53/ 1183	1.00 (ref.)	52/ 1729	1.00 (ref.)
< 0.75	103/ 1281	1.49 (1.12-1.98)†	56/ 457	1.79 (1.20-2.65)†	47/ 824	1.26 (0.83-1.93)
> 1.50	5/ 65	1.55 (0.62-3.88)	1/ 29	0.71 (0.09-5.28)	4/ 36	2.29 (0.79-6.62)
Diastolic function						
Model 1						
Normal	67/ 2254	1.00 (ref.)	33/ 882	1.00 (ref.)	34/ 1372	1.00 (ref.)
Impaired relaxation	42/ 471	1.55 (1.03-2.33)*	28/ 196	2.09 (1.23-3.57)†	14/ 275	1.01 (0.53-1.93)
Restrictive pattern	3/ 13	6.89 (2.15-22.1)†	1/ 5	17.0 (2.26-127)†	2/ 8	5.64 (1.34-23.8)*
Indeterminate	92/ 1469	1.49 (1.08-2.05)*	43/ 564	1.43 (0.90-2.27)	49/ 905	1.55 (0.99-2.41)
Model 2						
Normal	67/ 2254	1.00 (ref.)	33/ 882	1.00 (ref.)	34/ 1372	1.00 (ref.)
Impaired relaxation	42/ 471	1.55 (1.04-2.33)*	28/ 196	2.03 (1.19-3.46)*	14/ 275	1.08 (0.56-2.09)
Restrictive pattern	3/ 13	7.23 (2.16-24.2)†	1/ 5	22.6 (2.74-187)†	2/ 8	4.89 (1.10-21.8)*
Indeterminate	92/ 1469	1.49 (1.08-2.05)*	43/ 564	1.36 (0.85-2.16)	49/ 905	1.57 (1.01-2.46)*

HR= Hazard ratio, CI= Confidence interval

Model 1. Adjusted for age and type of ultrasonography system (and sex where appropriate).

Model 2. Adjusted for age, type of ultrasonography system, sex (where appropriate), body mass index, systolic blood pressure, diastolic blood pressure, total cholesterol, HDL-cholesterol, diabetes mellitus, smoking, heart rate, use of diuretics, beta-blockers, ACE inhibitors and lipid lowering drugs.

* $0.01 < p < 0.05$; † $p < 0.01$

The use of only two criteria complicated classification of diastolic dysfunction. It resulted in a substantial group of participants with "indeterminate" diastolic function, and results pertaining to the "restrictive pattern" category were based on very small numbers of participants. However, E/A ratio can by itself be used as an approximation of diastolic function, permitting classification of all participants. Finally, lack of power prevented us from examining incident cardiovascular disease and incident heart failure as endpoints at this point in time.

The association of echocardiographic measures with mortality has been investigated in several other population-based studies. Structural parameters have been examined in the Cardiovascular Health Study.[9] In accordance with this study, we did not find an association of left atrium diameter

with mortality in our study. Our finding of a positive association of end diastolic interventricular septum thickness and end diastolic left ventricular posterior wall thickness with mortality confirms the associations found in the Cardiovascular Health Study. These associations may reflect the association between left ventricular mass and mortality, found in the Cardiovascular Health Study, as well as the Framingham Study[4] and the present study. In contrast with the Cardiovascular Health Study, we did not find a significant association of left ventricular end systolic dimension with all-cause mortality.

In the present study, we did not find a significant association of fractional shortening and left ventricular systolic function with all-cause mortality. Investigation of left ventricular systolic function was complicated by a modest prevalence of moderate and poor function and consequently low numbers of events in these categories. The Framingham Study, Cardiovascular Health Study, the Strong Heart Study and the Olmsted County study have examined the impact of systolic parameters on all-cause mortality. The Framingham Study reported an independent association between asymptomatic left ventricular dysfunction, ascertained by visual assessment of echocardiograms, and all-cause mortality. [13] The discrepancy with our study may in part have been caused by selection bias in our study; the Framingham Study had up to 12 years of follow-up (mean 5 years), whereas in our study mean follow-up was 3.0 years, and participants who came to the research center may have been less likely to die in the first years of follow-up, because they were still relatively healthy. This may have attenuated some associations. The Cardiovascular Health Study also reported an independent association between ejection fraction assessed by echocardiography and all-cause mortality.[7] The fact that the participants of the Cardiovascular Health Study were on average ten years older than the Rotterdam Study participants may have contributed to this discrepancy.[30] The Strong Heart Study also reported an independent association of ejection fraction, measured from echocardiographic left ventricular linear dimensions, with all-cause mortality.[12] The difference with our study may at least in part have been caused by differences in study population, since the Strong Heart Study comprises American Indians with high prevalence of diabetes and obesity. Within residents of Olmsted County, low ejection fraction was found to be associated with increased all-cause mortality.[3] However, this analysis was only adjusted for age and sex.

With regard to diastolic measures, Bella et al demonstrated within the Strong Heart Study that E/A ratio >1.5 was associated with an increase in all-cause mortality, as was E/A ratio <0.6, the latter not independent of covariates.[11] In the Olmstedt County study, diastolic dysfunction, detected using Doppler examination of mitral inflow and pulmonary venous inflow and Doppler tissue imaging of the mitral annulus, was predictive of all-cause mortality, independent of age, sex and ejection fraction.[3] In the present study, in the entire cohort, E/A ratio <0.75 was independently associated with all-cause mortality. After stratification by gender, this association remained present in men but attenuated in women. This may reflect gender differences in the occurrence of heart failure. Categorization of diastolic function accordingly revealed increased risk for impaired relaxation in men. Furthermore, it revealed an increased risk for a restrictive pattern in both men and women, although the confidence intervals were wide because of low power in this category.

In conclusion, our study supports the associations of selected structural and diastolic echocardiographic parameters with all-cause mortality in an asymptomatic population. However, in view of the low prevalence of ventricular dysfunction in our study population, the evidence is still inadequate to support the usefulness of echocardiography for screening to identify asymptomatic individuals with preclinical ventricular dysfunction who may benefit from early treatment.

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Chapter 5.3

C-reactive protein level and heart failure

Abstract

Background. Experimental studies have shown that the known biological effects of proinflammatory cytokines could explain many aspects of the syndrome of heart failure. The inflammatory marker that presently seems most suitable to assess inflammation is C-reactive protein (CRP). This study was designed to investigate the association between serum CRP levels, as determined by high-sensitivity assay, and the occurrence of heart failure.

Methods. Serum CRP levels were available from 6437 men and women without heart failure, aged ≥55 years, from the prospective population-based Rotterdam Study. Cox proportional hazards analysis was used to determine risk of heart failure for sex-specific quartiles of CRP.

Results. CRP levels in the highest versus the lowest quartile showed increased hazard ratios of incident heart failure. The age-and sex-adjusted hazard ratio was 2.64 (95% confidence interval 2.04-3.43) for all participants. For men, the age adjusted hazard ratio was 4.37 (2.87-6.66), and for women, 1.86 (1.32-2.62). The interaction term of CRP with sex was highly significant. After additional adjustment for established cardiovascular risk factors, the association attenuated slightly in men and substantially in women, becoming 3.73 (2.40-5.78) and 1.42 (0.99-2.03), respectively. Excluding participants with prevalent coronary heart disease and accounting for incident coronary heart disease resulted in a further attenuation of the hazard ratios, which was proportionately larger in men than in women.

Conclusions. CRP is strongly and independently associated with occurrence of heart failure in men. In women, the association is weaker and does not persist after accounting for established cardiovascular risk factors.

Introduction

Recently, inflammatory markers have been implicated as predictors of heart failure. Experimental studies have shown that the known biological effects of proinflammatory cytokines could explain many aspects of the syndrome of heart failure, such as left ventricular dysfunction, pulmonary edema and the process of left ventricular remodeling, including myocyte hypertrophy and progressive myocyte loss through apoptosis.[1]

The inflammatory marker that presently seems most suitable to assess inflammation is C-reactive protein (CRP).[2] Elevated CRP levels have been associated with an adverse prognosis in patients with heart failure,[3-5] and elevated CRP levels have shown to be predictive of the development of heart failure in high-risk participants.[6,7]

Few population-based studies have examined the association between CRP and heart failure. In the Cardiovascular Health Study, increased CRP was an independent predictor of heart failure, the association persisting after adjustment for clinically prevalent as well as subclinical atherosclerotic disease.[8] The Framingham Heart Study demonstrated that participants with CRP serum levels of ≥5 mg/l experienced a significantly increased risk of heart failure, even after adjustment for prevalent cardiovascular disease and the occurrence of myocardial infarction during follow-up.[9] This study was limited by the use of a low-sensitivity CRP assay. Finally, in the Health ABC study, high levels of CRP independently predicted the incidence of events of heart failure.[10] In this study, incident heart failure was defined as any overnight hospitalization with this diagnosis, which does not account for all heart failure cases in a population.

These studies suggest that elevated levels of CRP precede heart failure. We sought to expand the evidence by examining the relation between CRP levels, as determined by high-sensitivity assay, and the occurrence of heart failure, in the Rotterdam Study, a prospective population-based cohort study in men and women aged ≥55 years.

Methods

Study population and baseline data collection

The present study is part of the Rotterdam Study, a population-based cohort study aimed at assessing the occurrence of and risk factors for chronic diseases in the elderly. Objectives and methods of the Rotterdam Study have been described in detail elsewhere.[11] The Rotterdam Study cohort includes 7983 men and women aged ≥55 years (78% of the eligible population), living in a well-defined suburb of the city of Rotterdam, the Netherlands. Baseline data were collected from 1990 until 1993. The obtained information included current health status, medical history, drug use and smoking.

In addition, in 7129 participants, established cardiovascular risk factors were determined at the research center according to standard procedures, as described previously.[12] These included body mass index, blood pressure, total cholesterol, high-density lipoprotein (HDL) cholesterol, diabetes mellitus, and presence of angina pectoris according to the Rose questionnaire.[13] Electrocardiograms (ECGs) were recorded and processed by the Modular ECG Analysis System[14] to obtain ECG measurements and interpretations. Myocardial infarction found on ECG was based on a set of criteria partly derived

from the Minnesota code.[15] A history of myocardial infarction was considered present in case of a self-report of myocardial infarction confirmed by ECG or additional clinical information, or the presence of an ECG characteristic of prior myocardial infarction.

The medical ethics committee of Erasmus Medical Center, Rotterdam, approved the study. Participants gave written informed consent and permission to retrieve information from treating physicians.

Population for analysis

The Rotterdam Study comprises 7983 participants. Two hundred fifty-four participants were excluded because of prevalent heart failure at baseline, five because of unclear heart failure status, and four because of missing heart failure status, leaving 7720 participants. Within this group, CRP levels were available for 6437 participants, which were used for analysis. CRP measurements were lacking for participants who did not visit the research center and for participants of whom no blood was available due to logistic reasons. Values for cardiovascular covariates were missing in <14% of participants, and these missing values were handled by single imputation using an expectation-maximization algorithm.

In further analyses, participants with coronary heart disease at baseline, defined as a history of myocardial infarction, percutaneous transluminal coronary angioplasty or coronary artery bypass grafting, were additionally excluded, yielding a group of 6881, in which CRP levels were available for 5691 participants.

Measurement of CRP

At baseline, a venipuncture was performed by application of minimal stasis with a 21-gauge Butterfly needle with tube (Surflo winged infusion set, Terumo, Tokyo, Japan). Non-fasting blood was collected, and all tubes were stored on ice before and after blood sampling. High-sensitivity CRP was determined in serum, which was stored at -20°C until performance of the CRP measurements in 2003 to 2004. CRP was measured using Rate Near Infrared Particle Immunoassay (Immage Immunochemistry System, Beckman Coulter, Fullerton, CA). This system measures concentrations from 0.2 to 1440 mg/l, with a within-run precision < 5.0%, a total precision < 7.5% and a reliability coefficient of 0.995.

Heart failure assessment

Assessment of prevalent heart failure at baseline in the Rotterdam Study has been described in detail elsewhere.[16,17] Briefly, a validated score was used, which was similar to the definition of heart failure of the European Society of Cardiology.[18] Furthermore, databases containing hospital discharge diagnoses from all hospitals in the Netherlands as of January 1, 1991, and all medical records from general practitioners in the area were screened in retrospect. Using these three methods, information on the presence of heart failure at baseline was available for all participants.

Follow-up started at the baseline examination and for the present study ended on January 1, 2000. Cases of incident heart failure were obtained by continuously monitoring participants of the Rotterdam Study for the occurrence of heart failure during follow-up through automated linkage with files from general practitioners. All available data on these events, including hospital discharge letters, were copied from the medical records. Furthermore, verified hospital discharge diagnoses were used

for case finding, gathered from all hospitals in the Netherlands as described above. The date of incident heart failure was defined as the day of the first occurrence of symptoms suggestive of heart failure, obtained from the medical records, or the day of receipt of a first prescription for a loop diuretic or an angiotensin-converting enzyme inhibitor, whichever came first.

The diagnosis of heart failure was classified as definite, probable, possible or unlikely. Only definite and probable cases were included in the analysis. In accordance with the criteria of the European Society of Cardiology,[18] definite heart failure was defined as a combination of heart failure diagnosed by a medical specialist and the presence of typical symptoms of heart failure, such as breathlessness at rest or during exertion, ankle edema, and pulmonary crepitations, confirmed by objective evidence of cardiac dysfunction (chest x-ray, echocardiography). Probable heart failure was defined as heart failure diagnosed by a general practitioner, with at least 2 typical symptoms suggestive of heart failure, and at least 1 of the following: history of cardiovascular disease, response to treatment for heart failure, or objective evidence of cardiac dysfunction, whereas symptoms could not be attributed to another underlying disease. Two research physicians independently classified all information on potential heart failure events. If there was disagreement, a consensus was reached in a separate session. Finally, a cardiologist verified all probable and possible cases, and all cases in which the 2 physicians could not reach consensus. If the cardiologist disagreed with the research physicians, the cardiologist's judgment was considered decisive.

Statistical analysis

Firstly, age-adjusted means or proportions for baseline characteristics were computed by analysis of covariance for men and women with and without incident heart failure. For this purpose, CRP was log-transformed because of its skewed distribution. Secondly, to allow for the demonstration of a possibly non-linear association, CRP was categorized into quartiles with cut-points 0.9, 1.9, and 3.6 mg/l and Cox proportional hazards analysis was performed to determine the hazard ratio of heart failure. Participants in the lowest quartile of CRP served as the reference group. P for trend was obtained by entering CRP into the Cox proportional hazards models as a continuous variable. Participants were censored at the time of occurrence of heart failure, death or the end of the study period. The proportional hazards assumption was tested by drawing log minus log plots of the survival function, which confirmed that the assumption was met. In model 1, we adjusted for age and sex. In model 2, we additionally adjusted for body mass index, hypertension, total cholesterol, HDL-cholesterol, smoking, diabetes mellitus and angina pectoris. Analyses were performed in all participants, and furthermore separate analyses were performed in men and women using sex-specific quartiles of CRP. While exploring the data in this way, we noted that remarkable differences were present in the hazard ratios between men and women. Thus, we tested interaction between CRP and gender using a Cox model containing sex, CRP and the product term of sex and CRP, entering CRP as a continuous variable. This interaction was similarly tested with additional adjustment for age, body mass index, hypertension, total cholesterol, HDL-cholesterol, smoking, diabetes mellitus and angina pectoris.

Finally, we excluded participants with presence of coronary heart disease at baseline. We performed Cox proportional hazards analysis, censoring participants at the time of occurrence of heart failure, death or the end of the study period, or occurrence of coronary heart disease (myocardial infarction, percutaneous transluminal coronary angioplasty, coronary artery bypass grafting) if this took

place before the occurrence of heart failure. Once more, we divided CRP into quartiles, the cut-points now being 0.9, 1.8 and 3.5 mg/L. In model 1, we adjusted for age and sex. In model 2, we adjusted for age, sex, body mass index, hypertension, total cholesterol, HDL-cholesterol, smoking, diabetes mellitus and angina pectoris. Similarly, analyses were first performed in all participants, and subsequently separate analyses were performed in men and women using sex-specific CRP quartiles. Analyses were performed with SPSS 11.0 for Windows (SPSS, Inc, Chicago, IL).

Results

Baseline characteristics and follow-up

Table 1 shows baseline characteristics of the total study population (n=6437) and for cases and noncases in men and women. The mean age of the total study population was 69.3 years and 40% were men. In both sexes, age, body mass index, systolic blood pressure, hypertension, smoking, history of myocardial infarction and C-reactive protein were significantly different in cases and noncases. Total cholesterol and diabetes mellitus were significantly different in cases and noncases in men but not in women, whereas diastolic blood pressure and HDL-cholesterol were significantly different in women but not in men.

Table 1. Baseline characteristics of the study population, crude for total population and age-adjusted for men and women.

		Men (n=2601)			Women (n=3836)		
Variable	Total population (n=6437)	Cases (n=262)	Non-cases (n=2339)	P-value	Cases (n=289)	Non-cases (n=3547)	P-value
Age (years)	69.3±9.1	73.2	67.7	<0.001	77.8	69.5	<0.001
Body mass index (kg/m^2)	26.2±3.7	26.3	25.6	<0.001	27.6	26.6	<0.001
Systolic blood pressure (mm Hg)	139±22	143	138	0.003	144	140	0.003
Diastolic blood pressure (mm Hg)	74±12	75	75	0.296	75	73	0.036
Hypertension (%)	33.9	39.1	28.7	0.001	47.0	35.8	<0.001
Total cholesterol (mmol/l)	6.6±1.2	6.5	6.3	0.004	6.8	6.8	0.981
HDL-cholesterol (mmol/l)	1.4±0.4	1.2	1.2	0.237	1.4	1.4	0.009
Smokers (%)							
- Never	34.2	4.8	8.5	0.042	48.2	54.3	0.049
- Current	22.4	33.8	29.6	0.174	25.0	17.7	0.003
- Former	40.6	61.4	61.9	0.891	26.9	28.0	0.682
Diabetes mellitus (%)	10	19.8	8.9	<0.001	13.3	10.0	0.074
C-reactive protein (mg/l)*	1.9 (0.9-3.6)	3.0	1.9	<0.001	2.2	1.8	0.001
History of myocardial infarction (%)	10.4	33.2	14.6	<0.001	14.5	7.0	<0.001

Categorical variables are expressed as percentage. Values of continuous variables are expressed as mean ± standard deviation.

*Median and interquartile range for total because of skewed distribution.

The mean follow-up time until occurrence of heart failure, death or study end was 6.5 years (standard deviation 2.4 years). During follow-up 551 incident cases of heart failure occurred.

C-reactive protein and risk of heart failure

After adjustment for age and sex, the risk of developing heart failure increased with increasing quartiles of CRP, with a hazard ratio of 2.64 (95% confidence interval (CI) 2.04-3.43) for the highest compared to the lowest quartile in the total population (table 2). The interaction between sex and CRP was highly significant (p=0.02, and p=0.01 after adjustment for established cardiovascular risk factors). In men, a hazard ratio of 4.37 (95% CI 2.87-6.66) was reached in the highest compared to the lowest quartile. In women, the hazard ratios were lower; they did not reach statistical significance in the second and third quartiles and became 1.86 (95% CI 1.32-2.62) in the highest quartile. After additional adjustment for established cardiovascular risk factors, the estimates declined moderately, providing a hazard ratio of 2.08 (95% CI 1.58-2.74) for the highest compared to the lowest quartile in the general population. Adjustment for established cardiovascular risk factors resulted in a larger proportionate decline of the risk estimates in women than in men, the multivariable-adjusted hazard ratio for the highest compared to the lowest quartile of CRP falling from 1.86 (95% CI 1.32-2.62) to 1.42 (95% CI 0.99-2.03) in the former and from 4.37 (95% CI 2.87-6.66) to 3.73 (95% CI 2.40-5.78) in the latter.

Table 2. Hazard ratios with 95% confidence intervals for developing heart failure in quartiles of C-reactive protein levels.

		Model 1	Model 2
	Events/ participants	**Hazard Ratio (95% CI)**	**Hazard Ratio (95% CI)**
Total			
Quartile 1	76/ 1610	1.00 (reference)	1.00 (reference)
Quartile 2	117/ 1612	1.41 (1.05-1.88)	1.28 (0.95-1.71)
Quartile 3	135/ 1607	1.55 (1.17-2.05)	1.32 (0.99-1.76)
Quartile 4	223/ 1608	2.64 (2.04-3.43)	2.08 (1.58-2.74)
P for trend		<0.001	<0.001
Men			
Quartile 1	27/ 650	1.00 (reference)	1.00 (reference)
Quartile 2	53/ 652	1.77 (1.11-2.81)	1.61 (1.01-2.58)
Quartile 3	65/ 649	2.21 (1.41-3.46)	1.84 (1.16-2.92)
Quartile 4	117/ 650	4.37 (2.87-6.66)	3.73 (2.40-5.78)
P for trend		<0.001	<0.001
Women			
Quartile 1	48/ 959	1.00 (reference)	1.00 (reference)
Quartile 2	65/ 967	1.23 (0.85-1.79)	1.12 (0.77-1.64)
Quartile 3	69/ 952	1.20 (0.83-1.74)	1.01 (0.69-1.47)
Quartile 4	107/ 958	1.86 (1.32-2.62)	1.42 (0.99-2.03)
P for trend		<0.001	0.010

Model 1 was adjusted for age and sex (when appropriate). Model 2 was adjusted for age, sex (when appropriate), body mass index, hypertension, total cholesterol, HDL-cholesterol, smoking, diabetes mellitus and angina pectoris.

After exclusion of participants with coronary heart disease at baseline and additionally censoring participants at the occurrence of coronary heart disease before heart failure during follow-up, the hazard ratios attenuated compared to the hazard ratios found for the total cohort (table 3). The multivariable-adjusted hazard ratio for the highest compared to the lowest quartile of CRP in men declined from 3.73 (95% CI 2.40-5.78) to 2.64 (95% CI 1.54-4.53), whereas in women, the proportionate decline was smaller, ranging from 1.42 (95% CI 0.99-2.03) to 1.39 (95% CI 0.92-2.12).

Additional adjustment for cardiac medication and antithrombotic and serum lipid-reducing agents did not materially change the results.

Table 3. Hazard ratios with 95% confidence intervals for developing heart failure in quartiles of C-reactive protein levels in participants without prevalent coronary heart disease.

		Model 1	Model 2
	Events/ participants	**Hazard Ratio (95% CI)**	**Hazard Ratio (95% CI)**
Total			
Quartile 1	59/ 1423	1.00 (reference)	1.00 (reference)
Quartile 2	94/ 1423	1.24 (0.87-1.75)	1.14 (0.80-1.62)
Quartile 3	106/ 1426	1.36 (0.97-1.90)	1.17 (0.82-1.65)
Quartile 4	157/ 1419	2.16 (1.58-2.96)	1.71 (1.23-2.38)
P for trend		<0.001	<0.001
Men			
Quartile 1	22/ 537	1.00 (reference)	1.00 (reference)
Quartile 2	40/540	1.32 (0.75-2.34)	1.30 (0.72-2.32)
Quartile 3	41/ 534	1.45 (0.83-2.54)	1.29 (0.72-2.29)
Quartile 4	69/ 536	3.02 (1.82-5.00)	2.64 (1.54-4.53)
P for trend		<0.001	<0.001
Women			
Quartile 1	37/ 887	1.00 (reference)	1.00 (reference)
Quartile 2	54/ 888	1.20 (0.78-1.87)	1.10 (0.71-1.72)
Quartile 3	64/ 883	1.24 (0.81-1.91)	1.05 (0.68-1.62)
Quartile 4	89/ 886	1.81 (1.21-2.70)	1.39 (0.92-2.12)
P for trend		<0.005	0.042

Model 1 was adjusted for age and sex (when appropriate). Model 2 was adjusted for age, sex (when appropriate), body mass index, hypertension, total cholesterol, HDL-cholesterol, smoking, diabetes mellitus and angina pectoris.

Additional analyses

To determine which covariates were responsible for the decline of the risk estimates after adjustment for established cardiovascular risk factors in women, we added body mass index, hypertension, total cholesterol, HDL-cholesterol, smoking, diabetes mellitus and angina pectoris to model 1 (CRP with age) one at a time, excluding participants with prevalent coronary heart disease. Adding hypertension yielded hazard ratios of 1.2 (95% CI 0.8-1.8), 1.2 (95% CI 0.8-1.8) and 1.7 (95% CI 1.1-2.5) for the second, third and fourth quartile of CRP, respectively; adding body mass index resulted in hazard

ratios of 1.1 (95% CI 0.7-1.7), 1.1 (95% CI 0.7-1.7) and 1.6 (95% CI 1.0-2.4), respectively; and adding HDL-cholesterol resulted in hazard ratios of 1.2 (0.8-1.8), 1.2 (0.8-1.8) and 1.7 (1.2-2.6), respectively. Adding the remaining risk factors did not materially change the risk estimates.

Furthermore, both for men and women, age, body mass index, hypertension, total cholesterol, HDL-cholesterol, smoking, diabetes mellitus and angina pectoris were entered into the Cox proportional hazards model by forward selection. Doing so, in women, age ($p<0.001$), hypertension ($p=<0.001$), body mass index ($p=0.01$) and current smoking ($p=0.01$) remained in the model, whereas in men, age ($p<0.001$), diabetes mellitus ($p<0.001$), and angina pectoris ($p<0.001$) remained in the model.

Discussion

In the present population-based study, CRP was a strong predictor of heart failure. The association persisted after exclusion of participants with coronary heart disease at baseline and after accounting for incident coronary heart disease in the follow-up period. The interaction between CRP and sex was highly significant. In men, the association was stronger than in women. After adjustment for established cardiovascular risk factors, in men, the association proved to be independent, whereas in women, the association strongly attenuated and lost statistical significance.

The strengths of our study include its population-based nature and the availability of >500 incident heart failure cases. Furthermore, a high-sensitivity CRP assay was used, giving us the possibility to examine steady-state CRP levels in the low reference range. Standardized assessment of various cardiovascular risk factors at baseline enabled us to account for possible confounding. Finally, we were able to account for incident coronary heart disease in our analysis.

There are several reasons for examining the association of inflammatory markers with heart failure.[1] Firstly, the biological role of inflammatory markers in the development of heart failure has been studied extensively, and the results suggest that many aspects of the syndrome of heart failure could be explained by the known biological effects of proinflammatory cytokines. These aspects include left ventricular dysfunction, pulmonary edema, left ventricular remodeling, myocyte hypertrophy, progressive myocyte loss through apoptosis and endothelial dysfunction. Furthermore, expression of cytokines is in direct relation to worsening New York Heart Association functional classification, which is very similar to the expression of the classic neurohormones (e.g., angiotensin II and norepinephrine) that are believed to play an important role in disease progression in heart failure. Moreover, there is growing evidence that critical interactions are present between inflammatory mediators and the mediators of the classic neurohormonal systems, and that many of the conventional therapies for heart failure may work, at least in part, through the modulation of proinflammatory cytokines.

Various analytes have been used to examine the association between inflammation and cardiovascular disease. However, only some of them are currently employable in clinical settings after consideration of the stability of the analyte, the commercial availability of assays, the standardization of those assays to allow comparison of results and the precision of the assays as measured by the coefficient of variation. Presently, comparison of the various inflammatory markers with respect to the above-mentioned characteristics favors CRP from the clinical chemistry perspective.[2]

Recent research has implied that CRP is associated with heart failure. Elevated CRP levels have been shown to result in an adverse prognosis in heart failure patients,[3-5] and elevated CRP levels seem to be predictive of the development of heart failure in high-risk participants.[6,7]

Until now, few population-based studies have examined the association between CRP and heart failure. Within the Cardiovascular Health Study cohort, which is based on Medicare eligibility lists at four locations, increased CRP was an independent predictor of heart failure in participants without prevalent heart failure.[8] The association persisted after adjustment for clinically prevalent as well as subclinical atherosclerotic disease, and for incident coronary heart disease. The Framingham Heart Study demonstrated that participants with CRP serum levels ≥5 mg/L experienced a significantly increased risk of heart failure, even after adjustment for prevalent cardiovascular disease and the occurrence of myocardial infarction on follow-up.[9] However, this study was limited by the use of a low-sensitivity CRP assay, and therefore could not discriminate between CRP levels in the low reference range. Finally, in the Health ABC study, high levels of CRP independently predicted the incidence of events of heart failure.[10] However, in this study, incident heart failure was defined as any overnight hospitalization for this diagnosis, which does not account for all heart failure cases in a population. Furthermore, although participants with cardiovascular disease at baseline were excluded, there was no adjustment for incident cardiovascular disease.

In all of the above-mentioned studies, sex-specific relative risks of developing heart failure were not provided. However, there is evidence that there are gender differences in heart failure. Women are more likely to have preserved left ventricular systolic function; systolic dysfunction is more common in men and diastolic dysfunction tends to occur more often in women. Heart failure that occurs after myocardial infarction is characterized by profound ventricular dilation, wall thinning, increased heart size, eccentric hypertrophy and systolic dysfunction. Heart failure that occurs due to long-standing hypertension is characterized by (concentric) left ventricular hypertrophy, increased myocardial mass and diastolic dysfunction, especially in the early stages. Therefore, men have coronary artery disease as an underlying factor for heart failure more frequently than women. Women are more likely to have hypertension as an underlying factor.[19,20] In the present study, gender differences in the association between CRP and risk of heart failure were found. In women, the risk estimates were lower, and furthermore, adjustment for established cardiovascular risk factors resulted in a larger proportionate decline of the risk estimates. This suggests that some of the established risk factors for which we adjusted may play a larger part in the development of heart failure in women than in men. Separate evaluation of the risk factors in women and application of a forward selection model implicated hypertension and body mass index. This concurs with the higher prevalence of diastolic dysfunction generally found in women. Contrastingly, applying forward selection in men resulted in a model that included angina pectoris and diabetes mellitus, suggesting that coronary heart disease, and thus systolic dysfunction, may be important in the development of heart failure in men. Moreover, although men and women were found to have equal risks of coronary heart disease for increasing CRP levels (data not shown), the hazard ratios for developing heart failure in men declined after accounting for prevalent and incident coronary heart disease, whereas in women, the proportionate decline was much smaller. Once again this suggests that in men, coronary heart disease may play a more important part in the development of heart failure than in women. In summary, these results may be a reflection of gender differences in the etiology of heart failure.

Some aspects of this study warrant further consideration. Firstly, serum samples had been stored for approximately ten years at -20ºC before CRP measurements were carried out. We compared these CRP measurements with CRP measurements in serum that had been stored at -80ºC in a random sample of 29 participants. Although the median CRP serum level was somewhat lower in -20ºC serum, Spearman's correlation coefficient for the association between CRP serum level in -20ºC samples and -80ºC samples was highly significant (correlation coefficient 0.99; $p<0.001$). Therefore, associations should be unaffected. Secondly, CRP measurements were lacking for 1283 out of 7720 participants at risk for heart failure. These participants were mainly persons who did not come to the research center at baseline. In these participants, 176 cases of heart failure occurred (13.7%), versus 551 cases in 6437 participants with CRP measurements available (8.6%). These 1283 participants were older, consisted for a higher percentage of women, had a lower percentage of current smokers, and had a higher prevalence of myocardial infarction. We believe that the higher heart failure incidence in these participants may be attributable to their higher age and worse health status. However, we consider it unlikely that this could have affected the difference we found between the sexes in the association of CRP with heart failure.

In conclusion, CRP is strongly and independently associated with occurrence of heart failure in men. In women, the association does not persist after accounting for established cardiovascular risk factors. The results of this study provide further support for the hypothesis that CRP may participate in the development and progression of heart failure, and suggest that the association between CRP and heart failure is different in men and women, possibly reflecting gender differences in the etiology of heart failure.

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Chapter 5.4

Lipoprotein-associated phospholipase A2 activity and heart failure

Abstract

Aims. Evidence is accumulating that inflammation plays a role in the pathophysiology of heart failure. Lipoprotein-associated phospholipase A2 (Lp-PLA2) has pro-inflammatory properties. We investigated whether Lp-PLA2 activity is associated with heart failure.

Methods and results. Lp-PLA2 activity was determined in a random sample of 1820 subjects from the Rotterdam Study, a population-based cohort study among persons aged 55 years and over. During a mean follow up of 6.7 years, 94 heart failure cases occurred. We excluded participants with heart failure or coronary heart disease at baseline and we accounted for incident coronary heart disease during follow-up. We used Cox proportional hazards models to compute hazard ratios adjusted for age, sex, non-HDL cholesterol, HDL cholesterol, body mass index, systolic blood pressure, diastolic blood pressure, hypertension, diabetes mellitus, smoking and C-reactive protein. The hazard ratio per unit increase of Lp-PLA2 activity was 1.03 (95% confidence interval, 1.01 to 1.05), *P* for trend = 0.011. Hazard ratios for the second, third and fourth quartiles were 1.06 (0.55 to 2.04), 1.43 (0.73 to 2.81) and 2.33 (1.21 to 4.49), respectively, using the lowest quartile of Lp-PLA2 activity as the reference category.

Conclusion. This study suggests that Lp-PLA2 activity is independently associated with incident heart failure.

Introduction

During the last fifteen years, an interest has developed for the potential role of inflammatory mediators in the pathofysiology of heart failure. Associations have been found between elevated inflammatory markers, such as interleukin-6[1], tumor necrosis factor-α[2] and C-reactive protein (CRP)[3] and congestive heart failure. It has been shown that inflammatory mediators may influence left ventricular remodeling, left ventricular function and pulmonary edema.[4,5] Furthermore, a correlation has been found between high blood levels of these inflammatory markers and worsening functional NYHA class, increased hospitalization rates and poorer survival of heart failure patients.[6]

Recently, several studies have found an independent association between the inflammatory marker lipoprotein-associated phospholipase A2 (Lp-PLA2) and risk of coronary heart disease.[7-11] Lp-PLA2 is an enzyme that circulates in the blood bound to low density lipoprotein (LDL) cholesterol. The enzyme has pro-inflammatory properties because of its capacity to hydrolyze oxidized phospholipids.[12] However, it is also suggested to have anti-inflammatory properties because of its ability to hydrolyze platelet-activating factor.[13,14] The relationship found between Lp-PLA2 and coronary heart disease suggests that the pro-inflammatory properties of Lp-PLA2 outweigh its anti-inflammatory properties.

To our knowledge no studies have yet been conducted on Lp-PLA2 as a predictor of heart failure. Therefore, we investigated the association between Lp-PLA2 and risk of heart failure in the Rotterdam Study, a population-based cohort study among men and women aged 55 years and over.

Methods

Rotterdam Study

The Rotterdam Study is a population-based cohort study comprising 7983 men and women aged 55 years and over. Its overall aim is to assess the occurrence of and risk factors for chronic diseases in the elderly. A detailed description of the objectives and methods of the Rotterdam Study has been given elsewhere.[15] All residents of a Rotterdam suburb aged 55 and over were invited to participate in the study and 78% participated. Baseline measurements started in 1990 and were completed in 1993.

The Medical Ethics Committee of Erasmus Medical Center, Rotterdam, approved the study. All participants gave written informed consent. This study complies with the Declaration of Helsinki.

Study Population

Lp-PLA2 activity was determined in a random subcohort of 1820 subjects. Prevalent heart failure cases at baseline (n = 47) were excluded for the current analysis. In addition, 183 subjects were excluded because they had a history of myocardial infarction, a history of coronary artery bypass grafting (CABG) or a history of percutaneous transluminal coronary angioplasty (PTCA) at baseline, leaving 1590 subjects, who were used for analysis.

Measurement of Lp-PLA2 activity

Plasma aliquots prepared from non-fasting blood samples were collected at baseline and stored at -80°C. Lp-PLA2 activity was measured with a high throughput radiometric activity assay, as described in detail previously.[11] Lp-PLA2 activity was expressed as nanomoles of platelet-activating factor hydrolyzed per minute per millilitre of plasma samples.

Prior to analysis of plasma samples from the Rotterdam Study, a pre-study validation was conducted to determine the reliability of the LpPLA2 activity assay. Six plasma samples were tested in triplicate, and the coefficient of variation (CV) for intra-assay precision ranged from 3.51-8.96%. To assess inter-assay precision, six plasma samples were tested on three occasions, and CV ranged from 8.48 -15.08%. Three cycles of freeze-thaw of frozen plasma did not result in appreciable loss of activity. The assay was therefore considered suitable for analysis of the Rotterdam Study samples, which were tested in duplicate. Samples were re-tested if the replicate CV was > 25%. The range of detection was 8-150 nmol/min per mL.

Measurement of covariates at baseline

At baseline, a trained interviewer visited all participants at home using a computerized questionnaire. The information obtained included current health status, medical history, drug use and smoking behavior. Additionally, established cardiovascular risk factors were measured at the research center. Height and weight were measured and the body mass index was calculated (weight (kg)/height^2 (m)). Blood pressure was measured at the right brachial artery with a random-zero sphygmomanometer with the participant seated. We defined hypertension as a systolic blood pressure ≥160 mmHg or a diastolic blood pressure ≥100 mmHg or the use of blood pressure lowering medication with an indication for hypertension.

Non-fasting blood samples were drawn and total cholesterol and high-density lipoprotein (HDL) cholesterol were measured within two weeks, as described previously.[16] Non-HDL cholesterol was computed by subtracting HDL cholesterol from total cholesterol. LDL cholesterol was determined in fasting blood samples in 120 randomly selected subjects by use of an enzymatic method (Roche). We calculated Pearson's correlation coefficient to compute the correlation of non-HDL cholesterol with LDL cholesterol, $r = 0.97$, $P < 0.001$.We defined diabetes mellitus as a random or post load glucose level ≥11.1 mmol/l or the use of blood glucose lowering medication.[17] Using a nephelometric method (Immage, Beckman Coulter), we measured CRP in blood samples kept frozen at –20°C.

A 12-lead resting electrocardiogram (ECG) was recorded with an ACTA electrocardiograph (ESAOTE, Florence, Italy) at a sampling frequency of 500Hz, and stored digitally. All ECGs were processed by the Modular ECG Analysis System (MEANS) to obtain ECG measurements and interpretations.[18] Myocardial infarction found on ECG was based on a comprehensive set of criteria that partly derive from the Minnesota code.[19] A history of myocardial infarction was considered present in case of a self-report of myocardial infarction confirmed by electrocardiography (ECG) or additional clinical information, or the presence of an ECG characteristic of prior myocardial infarction. In identifying incident myocardial infarctions (ICD-10 code I21), all available information, which included ECG, cardiac enzyme levels, and the clinical judgment of the treating specialist, was used. Revascularization procedures were identified by review of hospital discharge letters from the medical specialist.

Ascertainment of heart failure cases

Assessment of prevalent heart failure at the baseline examination in the Rotterdam Study has been described in detail earlier.[20,21] Briefly, a validated score was used, similar to the definition of heart failure of the European Society of Cardiology.[22] This score was based on the presence of at least two symptoms suggestive of heart failure (shortness of breath, ankle swelling and pulmonary crepitations) or use of medication for the indication of heart failure, in combination with objective evidence of cardiovascular disease.

Information on the presence of heart failure at baseline was obtained for all participants using either of the following three methods: interview questions on indication of cardiovascular medication and breathlessness, linkage of the Rotterdam Study database to a database containing hospital discharge diagnoses from all hospitals in the Rotterdam area as of 1 January 1991, and screening of all medical records in retrospect for the occurrence of heart failure in the majority of participants of the Rotterdam Study.

For the present study, follow-up started at the baseline examination, from 1990 till 1993 and was complete until 1 January 2000. Follow-up has been described in detail previously.[21] Briefly, cases of incident heart failure were obtained by continuously monitoring participants of the Rotterdam Study for the occurrence of heart failure during follow-up through automated linkage with files from general practitioners. Each participant's medical record was fully screened for incident heart failure. All available data on these events, such as hospital discharge letters and notes from general practitioners, were copied from the medical records. Apart from this systematic follow-up procedure, we used verified hospital discharge diagnoses for case finding, gathered from all hospitals in the Rotterdam area as described above. The diagnosis of heart failure was classified as definite, probable, possible or unlikely.[21] Two research physicians independently classified all information on potential heart failure events. If there was disagreement, a consensus was reached in a separate session. Finally, a cardiologist verified all probable and possible cases, and all cases in which the two physicians could not reach consensus. If the cardiologist disagreed with the research physicians, the cardiologist's judgement was considered decisive. The research physicians and the cardiologist based their decisions on the same data. Only definite and probable cases were included in the analyses.

Statistical analysis

To compare the baseline characteristics of the random sub-cohort to the remainder of the Rotterdam Study we used a χ^2 test for dichotomous variables, a t-test for continuous variables and a Mann-Whitney test for CRP, because its distribution was skewed. We used ANCOVA to display age- and sex adjusted baseline characteristics of the participants in different Lp-PLA2 activity quartiles. We log-transformed CRP because of its skewed distribution and we computed the geometric mean. We computed the standard deviation and interquartile range from the standard error. To compute P-value for trend for the baseline characteristics, we used logistic regression for dichotomous variables and linear regression for continuous variables. In both cases, continuous plasma values of Lp-PLA2 activity were used as the independent variable.

We used Cox proportional hazards models to evaluate the association of Lp-PLA2 activity with risk of heart failure. Subjects were censored at the time of occurrence of either heart failure, death or at the end of the study period. Furthermore, we censored subjects at the time of occurrence of myocar-

dial infarction, PTCA or CABG if these took place before the occurrence of heart failure, to account for coronary heart disease. The proportional hazards assumption was tested by drawing log minus log plots of the survival function, which confirmed that the assumption was met. In model 1 we adjusted for age and sex. Lp-PLA2 is tightly associated with lipoproteins; in humans it is predominantly located on LDL, and to a smaller extent on HDL. Because these factors were most likely to be confounders, in model 2, we additionally adjusted for non-HDL cholesterol and HDL cholesterol. In model 3 we additionally adjusted for body mass index, systolic blood pressure, diastolic blood pressure, hypertension, smoking status, diabetes mellitus and C-reactive protein. First, we entered the continuous plasma values of Lp-PLA2 activity into the models, to obtain the hazard ratio for heart failure per unit increase in Lp-PLA2 activity. By this means we also obtained the *P* for trend. Second, to allow for the demonstration of a possibly non-linear association, we made quartiles of Lp-PLA2 activity, with cut-points 35.9, 42.9 and 50.8 nmol/min per mL plasma and used the lowest quartile as the reference category.

To compare survival time until the occurrence of heart failure in the quartiles of Lp-PLA2 activity, C-reactive protein and non-HDL cholesterol we made event free survival curves adjusted for age and sex.

We conducted a subgroup analysis to compare the association between Lp-PLA2 activity and heart failure in subjects with a non-HDL cholesterol level below and above the median (cutpoint 5.20 mmol/L). Lp-PLA2 was dichotomized in this analysis using the median as cutoff point (42.9 nmol/min per mL plasma). We adjusted for age, sex, HDL cholesterol, body mass index, systolic blood pressure, diastolic blood pressure, hypertension, smoking status, diabetes mellitus and CRP. We did a similar subgroup analysis in strata of C-reactive protein (cutpoint 1.79 mg/L), adjusting for non-HDL cholesterol instead of C-reactive protein in the Cox proportional hazards model.

To test for interaction between Lp-PLA2 activity and non-HDL cholesterol and CRP, we entered interaction terms into the model using continuous values of Lp-PLA2 activity instead of quartiles of Lp-PLA2 activity and using non-HDL cholesterol and CRP as continuous variables. In this analysis we adjusted for age and sex.

Values for covariates were missing in <3%, except for C-reactive protein (6% missing values). We used single imputation based on expectation maximization to handle missing values.

Results

The mean follow-up time until censoring was 6.7 years (standard deviation 2.3 years). During follow-up, 113 incident heart failure cases occurred. Of these cases, 19 were preceded by coronary heart disease. Therefore, 94 incident cases of heart failure were left for analysis.

Table 1 shows the baseline characteristics of the total random cohort and the remainder of the Rotterdam Study. The characteristics of the random cohort were similar to the remainder of the Rotterdam Study except for age, systolic blood pressure and hypertension. Subjects in the random cohort were slightly younger (69.1 versus 71.1 years), had a lower systolic blood pressure (138.2 mm Hg versus 139.9 mm Hg) and had a lower prevalence of hypertension (33.1% versus 37.1%). Table 2 shows the baseline characteristics of participants in different quartiles of Lp-PLA2 activity adjusted for age and sex (when appropriate) and the *P*-value for trend. In all linear regression models we used, the residuals were normally distributed with a constant variance. Quartiles with a higher Lp-PLA2 activity

Table 1. Baseline characteristics of the study population.

Variable	Random Cohort (n = 1820)	Remainder Rotterdam Study (n = 6163)	P-value
Age, years	69.1 ± 9.1	71.1 ± 9.9	<0.01
Men, %	38.3	39.1	0.55
Body mass index, kg/m^2	26.2 ± 3.7	26.3 ± 3.8	0.30
Systolic blood pressure, mm Hg	138.2 ± 22.3	139.9 ± 22.4	<0.01
Diastolic blood pressure, mm Hg	73.3 ± 11.2	73.8 ± 11.8	0.12
Hypertension, %	33.1	37.1	<0.01
Non-HDL cholesterol, mmol/L	5.30 ± 1.24	5.24 ± 1.23	0.10
HDL-cholesterol mmol/L	1.35 ± 0.38	1.34 ± 0.37	0.38
Diabetes mellitus, %	9.8	10.7	0.31
Smokers, %			
- Current	23.0	22.5	0.68
- Former	41.7	40.4	0.34
- Never	35.3	37.0	0.18
CRP, mg/L*	1.78 (0.90-3.59)	1.93 (0.92-3.71)	0.07

Continuous variables are expressed as mean ± standard deviation. Categorical variables are expressed as percentage.

* Median and interquartile range because of skewed distribution.

Table 2. Baseline characteristics according to quartiles of Lp-PLA2 activity.

Variables	Quartile 1	Quartile 2	Quartile 3	Quartile 4	P-value for trend
Age, years	68.6 ±9.1	69.7 ±9.0	68.8 ±9.0	69.5 ±9.1	0.29
Men, %	27.1	35.2	42.0	48.9	<0.01
Body Mass Index, kg/m^2	25.8 ±3.6	26.1 ±3.6	26.5 ±3.6	26.5 ±3.6	<0.01
Systolic blood pressure, mm Hg	135.9 ±21.5	138.4 ±21.4	137.7 ±21.4	140.8 ±21.5	<0.01
Diastolic blood pressure, mm Hg	72.8 ±11.2	73.8 ±11.2	73.4 ±11.2	73.3 ±11.2	0.84
Hypertension, %	29.0	30.7	33.3	39.5	<0.01
Non-HDL cholesterol, mmol/L	4.49 ±1.09	5.13 ±1.08	5.53 ±1.06	6.06 ±1.08	<0.01
HDL-cholesterol mmol/L	1.50 ±0.36	1.38 ±0.36	1.28 ±0.36	1.25 ±0.36	<0.01
CRP, mg/L*	1.67 (1.62-1.73)	1.69 (1.64-1.75)	1.83 (1.77-1.89)	1.97 (1.91-2.04)	<0.01
Diabetes, %	8.8	9.6	11.0	10.0	0.18
Smokers, %					
- Current	24.5	20.6	22.5	24.3	0.76
- Former	42.7	44.0	42.7	37.5	0.10
- Never	32.8	35.4	34.8	38.2	0.05

Continuous variables are expressed as mean ± standard deviation. Categorical variables are expressed as percentage. All (geometric) means and percentages are adjusted for age and sex, except for age (only adjusted for sex) and sex (only adjusted for age).

*Geometric mean and interquartile range because of skewed distribution.

contained a higher percentage of men and hypertensive participants. They had a significantly higher body mass index, systolic blood pressure, non-HDL cholesterol and CRP. HDL-cholesterol was lower in participants within higher quartiles of Lp-PLA2 activity.

Lp-PLA2 activity was associated with risk of heart failure (Table 3). After adjustment for age and sex, the hazard ratio for heart failure per unit increase in Lp-PLA2 activity was 1.02 (95% confidence interval (CI) 1.00-1.03), $P = 0.026$. After additional adjustment for non-HDL cholesterol and HDL cholesterol, this was 1.02 (95% CI 1.00-1.04), $P = 0.024$, and after additional adjustment for known cardiovascular risk factors, 1.03 (95% CI 1.01-1.05), $P = 0.011$. Participants in the second, third and fourth quartiles of Lp-PLA2 activity had a hazard ratio of 0.99 (95% CI 0.52-1.86), 1.27 (95% CI 0.68-2.40), 1.93 (95% CI 1.09-3.42), respectively, for heart failure, using the first quartile of Lp-PLA2 activity as the reference category and adjusting for age and sex. Using model 2, this was 1.01 (95% CI 0.53-0.92), 1.34 (0.69-2.60) and 2.16 (95% CI 1.13-4.11), respectively. Using model 3, this further increased to 1.06 (95% CI 0.55-2.04), 1.43 (95% CI 0.73-2.81) and 2.33 (95% CI 1.21-4.49), respectively.

The event-free survival curve according to quartiles of Lp-PLA2 activity shows that the survival

Table 3. Hazard ratios for heart failure according to quartiles of Lp-PLA2 activity

Lp-PLA2	Cases/	Hazard Ratio (95% confidence interval)		
(nmol/min per mL)	Subjects	Model 1	Model 2	Model 3
Unit increase	94/ 1590	1.02 (1.00-1.03)	1.02 (1.00-1.04)	1.03 (1.01-1.05)
P-value for trend		0.026	0.024	0.011
Quartile 1	18/ 397	1 (reference)	1 (reference)	1 (reference)
Quartile 2	20/ 398	0.99 (0.52-1.86)	1.01 (0.53-1.92)	1.06 (0.55-2.04)
Quartile 3	21/ 398	1.27 (0.68-2.40)	1.34 (0.69-2.60)	1.43 (0.73-2.81)
Quartile 4	35/ 397	1.93 (1.09-3.42)	2.16 (1.13-4.11)	2.33 (1.21-4.49)

Model 1 adjusted for age and sex; model 2 adjusted for age, sex, non-HDL cholesterol and HDL cholesterol; model 3 adjusted for age, sex, non-HDL cholesterol, HDL cholesterol, body mass index, systolic blood pressure, diastolic blood pressure, hypertension, diabetes mellitus, smoking status and C-reactive protein.

time until the occurrence of heart failure was higher in the lowest quartile than in the highest quartile (Figure 1). The curve also illustrates that the difference in risk between quartiles 1 and 4 is rather consistent over time. Figure 2 and 3 show the event free survival curve of C-reactive protein and non-HDL cholesterol, respectively. Although C-reactive protein was significantly related to the event free survival time, no clear association was found for non-HDL cholesterol.

Figure 4 shows the results of our subgroup analyses. The hazard ratios for heart failure associated with Lp-PLA2 activity for the subgroups below and above the median of non-HDL cholesterol level were 1.89 (95% CI 1.05-3.39) and 1.77 (95% CI 0.83-3.79), respectively. The hazard ratio was somewhat larger in subjects with a non-HDL cholesterol below the median, but no significant interaction was found between Lp-PLA2 activity and non-HDL cholesterol (*P* for interaction = 0.817) in relation to risk of heart failure. The hazard ratio for the subjects with a CRP below the median was 3.83 (95% CI 1.64-8.93), which was higher than the hazard ratio for the subjects with CRP above the median, namely 1.26 (95% CI 0.71-2.23). However, the interaction term for Lp-PLA2 activity and CRP was not significant (*P* for interaction = 0.364).

Figure 1. Survival time until the occurrence of heart failure according to quartiles of Lp-PLA2, adjusted for age and sex.

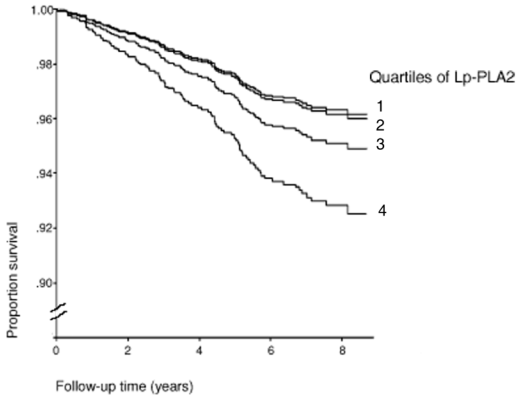


Figure 2. Survival time until the occurrence of heart failure according to quartiles of C-reactive protein (CRP), adjusted for age and sex.

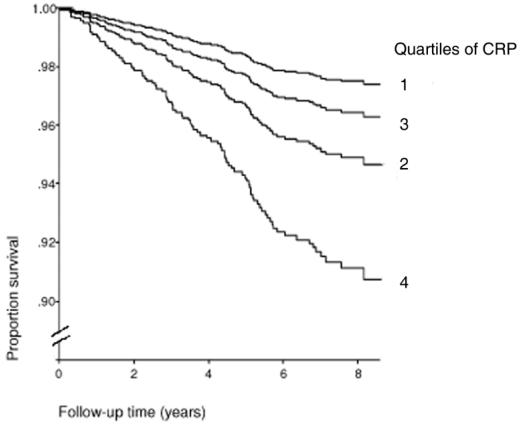


Figure 3. Survival time until the occurrence of heart failure according to quartiles of non-HDL cholesterol, adjusted for age and sex.

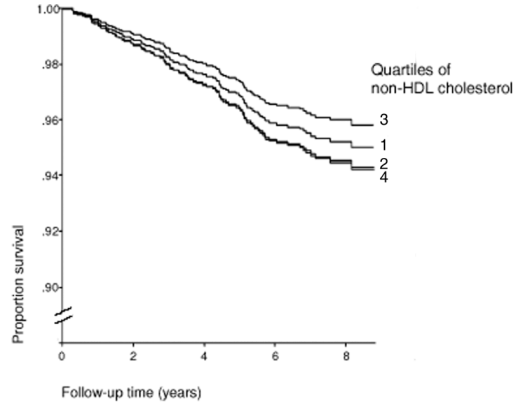
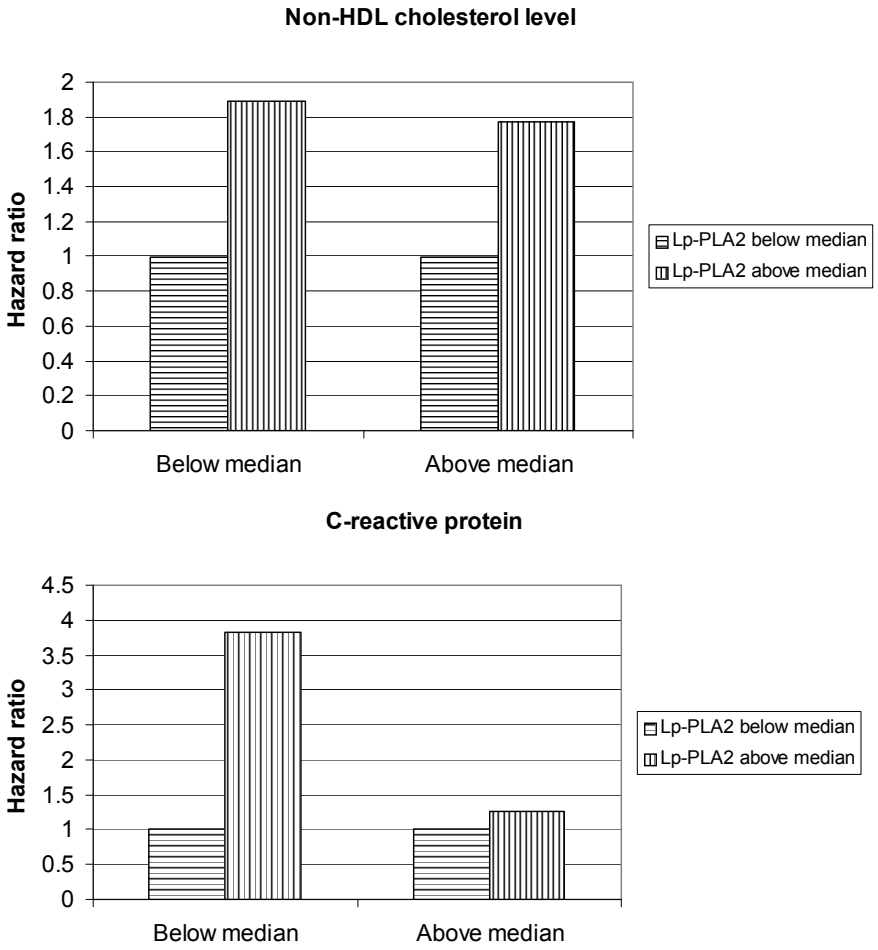


Figure 4. Hazard ratios for heart failure associated with Lp-PLA2 activity in strata of non-HDL cholesterol level and C-reactive protein.



Discussion

In the present population-based cohort study, Lp-PLA2 activity was an independent predictor of heart failure. The association persisted after we adjusted for known cardiovascular risk factors and C-reactive protein. A significant trend was seen, and subjects in the highest quartile had no less than a doubled risk of developing heart failure compared to subjects in the lowest quartile, even though we excluded subjects with prevalent coronary heart disease at baseline and censored subjects with incident coronary heart disease during follow-up. This suggests that the association found between Lp-PLA2 activity and heart failure is independent of coronary heart disease.

To our knowledge, this is the first study performed on the association between Lp-PLA2 and risk of heart failure. The present study is a population-based prospective cohort study, which guards our study from selection and recall bias. Strengths of our study include the ability to account for possible

confounding by incorporating established cardiovascular risk factors into the statistical models. Finally, we were able to account for prevalent and incident coronary heart disease in our analysis.

The pathophysiology of heart failure is complex. While heart failure was once merely considered to be a cardiocirculatory impairment, now it is known that the neuroendocrine system is involved. Evidence is accumulating that inflammation also plays a direct role in the pathophysiology of heart failure. In former studies several inflammatory markers, such as interleukin-6[1], tumor necrosis factor-α[2] and C-reactive protein[3], have been associated with incidence of heart failure. Inflammatory mediators have been found to affect left ventricular remodeling, left ventricular dysfunction, pulmonary edema, fetal gene expression and cardiomyopathy.[4,5] Finally, a correlation has been found between high blood levels of inflammatory markers and poorer prognosis in heart failure patients.[6]

In the Atherosclerosis Risk in Communities study, an association between Lp-PLA2 and incident coronary heart disease was present after adjustment for age, sex and race. After further adjustments for cardiovascular risk factors, the association was only present in subjects with a low LDL cholesterol.[9] Our subgroup analysis showed that the association found between Lp-PLA2 activity and heart failure is present in subjects with a non-HDL cholesterol level below the median as well as in subjects with a non-HDL cholesterol level above the median, although in the latter group the association lacked significance. The association between Lp-PLA2 and heart failure was much stronger in subjects with a C-reactive protein level below the median than in subjects with a C-reactive protein level above the median. We have no explanation for this difference in risk estimates. The interaction between Lp-PLA2 and C-reactive protein was not significant, so the difference may be due to chance.

Several studies have investigated the association between LDL cholesterol and heart failure.[23-25] Although the Framingham Study found a positive relation,[26] subsequent studies were not able to confirm this. In our study, we also failed to find a clear relation between non-HDL cholesterol and risk of heart failure. Lp-PLA2 is an enzyme bound to LDL cholesterol and therefore Lp-PLA2 activity is highly correlated with LDL cholesterol levels. In the present study, we found that the association of Lp-PLA2 with heart failure was independent of non-HDL cholesterol. We used non-HDL cholesterol for adjustment, since no measurements of LDL cholesterol were available. Because of the high correlation between LDL cholesterol and non-HDL cholesterol in a random sample of our cohort ($r = 0.97$, $P < 0.001$), we believe that residual confounding by LDL cholesterol cannot explain our results.[11]

In conclusion, our findings suggest that Lp-PLA2 activity is independently associated with risk of heart failure. Our study provides further evidence inflammation is involved in the etiology of heart failure.

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Part III

Prevalence of atherosclerosis

Chapter 6

Prevalence of atherosclerosis in men and women in the Rotterdam Study

Chapter 6.1

The female advantage in cardiovascular disease

Abstract

The female advantage in coronary heart disease occurrence is not completely understood. To characterize gender differences in cardiovascular disease by vascular site, the authors compared degrees of coronary, carotid, peripheral, and aortic atherosclerosis in men and women aged ≥55 years from the population-based Rotterdam Study (Rotterdam, the Netherlands). Data were collected between 1997 and 2000. A subset of 2,013 participants had data on both coronary calcification and one or more measures of extracoronary atherosclerosis, including intima-media thickness (IMT), carotid plaques, ankle-arm index (AAI), and aortic calcification. The multivariable adjusted male:female odds ratios for calcium score > 1,000 were 7.8 (95% confidence interval (CI): 3.2, 19.3), 5.4 (95% CI: 2.8, 10.2), and 3.0 (95% CI: 1.7, 5.2) in the lowest, middle, and highest age tertiles, respectively. For IMT > 1.0 mm, severe carotid plaques, AAI < 0.90, and severe aortic calcification, ratios did not decline with age. Overall multivariable-adjusted male:female odds ratios for these measures were 2.9 (95% CI: 2.0, 4.1), 2.0 (95% CI: 1.4, 2.8), 0.9 (95% CI: 0.7, 1.3), and 1.0 (95% CI: 0.8, 1.5), respectively. The authors conclude that the gender difference in atherosclerosis is larger in the coronary vessels than in other vascular beds. Remarkably, it is absent in the aorta and the lower-extremity vessels. Factors causing this site-specific gender difference require further investigation.

Introduction

After two decennia of rigorous research, the remarkable gender difference in coronary heart disease occurrence is still not completely understood. It cannot be fully explained by risk factors such as lifestyle, lipid profile, and blood pressure.[1] The hypothesis that estrogen carries a cardioprotective effect is still being debated and could not be proven in large randomized controlled trials on estrogen therapy.[2,3] Although the gender difference is also present to a much smaller extent for stroke and peripheral artery disease, it is most prominent for coronary heart disease.[4-9] This suggests that gender differences in cardiovascular disease may differ according to vascular sites. Autopsy studies support such a differential gender effect.[10-13] Although studies have been performed in living populations to examine gender differences in atherosclerosis at single vascular sites,[14-17] to our knowledge, no studies have been performed in living populations to compare gender differences in atherosclerosis at different sites of the vascular tree. To further characterize gender differences in cardiovascular disease according to various vascular sites, we compared degrees of coronary, carotid, aortic, and peripheral atherosclerosis between men and women in participants aged 55 years or more in a population-based cohort study.

Materials and Methods

Study population

This study was embedded in the Rotterdam Study, a population-based study aimed at addressing the occurrence of and risk factors for chronic diseases in the elderly. The rationale and design of the Rotterdam Study have been described elsewhere.[18] The study started with a baseline examination conducted between 1990 and 1993, which included 7,983 men and women aged 55 years or more (78 percent of the eligible population) living in a well-defined suburb of the city of Rotterdam, the Netherlands. Follow-up visits took place in 1993–1994 and 1997–1999. From 1997 onwards, participants through 85 years of age who completed the third phase of the Rotterdam Study were invited to participate in the Rotterdam Coronary Calcification Study and to undergo electron-beam computed tomography for assessment of coronary calcification in the epicardial coronary arteries.[19] The medical ethics committee of Erasmus Medical Center, Rotterdam, approved the study. Participants gave written informed consent and permission to retrieve information from treating physicians.

Coronary calcification

Imaging was performed with a C-150 Imatron scanner (Imatron, South San Francisco, California), and has been described in detail previously.[19] Briefly, quantification of coronary calcification was performed with AccuImage software (AccuImage Diagnostics Corporation, South San Francisco, California) displaying all pixels with a density of over 130 Hounsfield Units. A calcium score was obtained as proposed by Agatston et al.[20] We added the scores for individual calcifications, resulting in a calcium score for the entire epicardial coronary system. A calcium scores above 1,000 was considered the outcome measure. Interobserver reliability for calcium scoring has been found to be excellent, with

correlation coefficients for calcium scores obtained by different observers being greater than 0.99 and only small differences in absolute calcium scores being observed.[21]

Measures of extracoronary atherosclerosis

During the third phase of the Rotterdam Study (1997–1999), several noninvasive measurements of atherosclerosis were conducted. A detailed description of the procedures used has been given previously.[22] Ultrasonography of both carotid arteries was performed according to the protocol of the Rotterdam Study. Measurements of common carotid intima-media thickness (IMT) involved regions of the common carotid arteries proximal to the carotid bulb, starting at a distance of 1 cm from the bulb. IMT was determined as the average of mean near- and far-wall measurements, computing the average of left and right common carotid IMT. We considered carotid IMT above 1.0 mm to be high carotid IMT. In a reproducibility study of IMT measurements carried out among 80 participants of the Rotterdam Study, intraclass correlation coefficients between ultrasonographers, readers, and visits to the study center were 0.63, 0.88, and 0.74, respectively.[23]

The left and right common carotid arteries, bifurcations, and internal carotid arteries were evaluated for the presence of atherosclerotic lesions (plaques). A plaque was defined as a focal widening relative to adjacent segments, with protrusion into the lumen. The anterior and posterior walls were evaluated and the number of affected locations counted. This resulted in a plaque score between 0 and 6. Carotid plaque scores of 4 or greater were considered severe. A reproducibility study among 166 Rotterdam Study participants on the assessment of plaques in the carotid bifurcation revealed kappa values of 0.59 for the left carotid artery, 0.65 for the right carotid artery, and 0.60 for plaques in either side.[24] These findings were statistically significant ($p < 0.001$) and indicated moderate agreement.

Using a random-zero sphygmomanometer, sitting blood pressure was measured at the right upper arm, and systolic blood pressure at both ankles (posterior tibial artery) was measured in the supine position. We computed the ratio of systolic blood pressure at the ankles to systolic blood pressure at the arm to obtain the ankle-arm index (AAI). For the current analyses, we used the lowest measurement. Because of possible measurement artifacts reflecting the presence of rigid or calcified walls, participants with AAIs greater than 1.5 were excluded. AAIs smaller than 0.90 were considered anomalous.

Aortic calcification was diagnosed by radiographic detection of calcified deposits in the abdominal aorta. Lateral abdominal radiographs were made from a fixed distance with the participant seated. Calcifications in the abdominal aorta were classified as present when linear densities were seen in an area parallel and anterior to the lumbar spine. The extent of aortic calcification was scored according to the length of the involved area of the posterior wall, with scores 0–5 corresponding to 0, <1.0, 1.0–2.4, 2.5–4.9, 5.0–9.9, and ≥10.0 cm. Severe aortic calcification was considered present when the length of the area was 5 cm or more. A comparison study conducted in our department involving computed tomography among 56 subjects showed that abdominal calcification could be detected radiographically in 32 subjects; in all but one of these subjects, calcification was located in the aorta on the corresponding computed tomography images.[25]

Assessment of covariates

A trained interviewer visited all participants at home and collected information using a computerized questionnaire. Information obtained included current health status, medical history, smoking, and medication use, including use of hormone replacement therapy. Clinical measurements were obtained during a visit to the research center. Height and weight were measured, and body mass index (weight (kg)/height $(m)^2$) was calculated. Blood pressure was measured at the right brachial artery using a random-zero sphygmomanometer with the participant in a sitting position. Blood samples were obtained after overnight fasting. Serum total cholesterol level was determined by an enzymatic procedure, and high density lipoprotein (HDL) cholesterol was measured similarly after precipitation of the non-HDL fraction.[26] Glucose was determined enzymatically by the hexokinase method. Diabetes was defined as the use of antidiabetic medication and/or a fasting glucose level ≥7.0 mmol/liter and/or a nonfasting glucose level ≥11.1 mmol/liter.[27] Using a nephelometric method (Immage; Beckman Coulter, Inc., Fullerton, California), C-reactive protein was measured in serum samples, which were kept frozen at −80°C.

Population for analysis

Of the 3,371 participants who completed the third phase of the Rotterdam Study and were eligible for electron-beam computed tomography scanning, scans were obtained for 2,063 participants (a response of 61 percent). For several reasons (i.e., metal clips from cardiac surgery, severe artifacts, and registration errors (electrocardiogram, acquisition), image acquisition data could not be reconstructed or analyzed for 50 participants. Thus, calcium scores were available for 2,013 participants. The present study was conducted in these participants. In this group, IMT, carotid plaque scores, AAI, and aortic calcification measurements were available for 1,859, 1,736, 1,926, and 1,753 participants, respectively. The analysis was repeated excluding participants with prevalent cardiovascular disease; prevalent myocardial infarction, stroke, heart failure, and angina pectoris and claudicatio intermittens, both diagnosed by Rose questionnaire,[28] were present in 255, 71, 74, 138, and 64 of the 2,013 participants, respectively. Their exclusion resulted in exclusion of 468 participants, leaving 1,545 participants for whom calcium scores were available. In this group, IMT, carotid plaque scores, AAI, and aortic calcification measurements were available for 1,432, 1,341, 1,490, and 1,347 participants, respectively.

Statistical analysis

In the statistical analysis, we first tested for differences in baseline characteristics between men and women. For this purpose, we used *t* tests for continuous variables and chi-squared tests for categorical variables. A Mann-Whitney *U* test was used for C-reactive protein because of the skewed data distribution. Second, we examined the associations of baseline characteristics with measures of atherosclerosis in men and women using linear regression, adjusting for age.

Subsequently, participants were stratified according to gender and age. Age, ranging from 61 to 85 years, was divided into tertiles (61–67, 68–73, and 74–85 years). Mean values were computed for IMT and AAI, and median values were computed for coronary calcium score, carotid plaque score, and aortic calcification score because of their skewed distributions, for men and women in strata of age. Using logistic regression, male:female odds ratios were calculated for having coronary calcification above 1,000, IMT > 1.0 mm, severe carotid plaques, AAI < 0.90, or severe aortic calcification in different

age tertiles. In model 1, we adjusted for age. In model 2, we additionally adjusted for body mass index, systolic and diastolic blood pressure, total and HDL cholesterol, smoking, diabetes mellitus, C-reactive protein, hormone replacement therapy in women, and use of diuretics, beta-blockers, angiotensin-converting enzyme inhibitors, and lipid-lowering drugs (defined as those with World Health Organization Anatomical-Therapeutic-Chemical (ATC) code C10; www.whocc.no/atcddd). Analyses were repeated after excluding participants with prevalent myocardial infarction, stroke, heart failure, angina pectoris, and claudicatio intermittens.

Values for cardiovascular covariates were missing in less than 4 percent of participants, except for the presence of diabetes mellitus, for which 6.8 percent of values were missing. These missing values were handled by single imputation using an expectation-maximization algorithm. Analyses were performed using SPSS 11.0 for Windows (SPSS, Inc., Chicago, Illinois).

Results

Table 1 shows baseline characteristics of the study population. Mean age was 71.3 years, and the population consisted of 46 percent men and 54 percent women. Men had a significantly lower body mass index, significantly higher systolic and diastolic blood pressures, significantly lower total and HDL cholesterol levels, and significantly higher prevalences of smoking and diabetes mellitus than

Table 1. Baseline characteristics of the study population.

Variable	Total		Men		Women		p value
	No. or mean	%	No. or mean	%	No. or mean	%	
No. and % of subjects	2,013	100	933	46	1,080	54	
Mean age (years)	71.3 (5.7)*		71.2 (5.6)		71.3 (5.8)		0.77
Mean body mass index†	27.0 (3.9)		26.5 (3.2)		27.4 (4.4)		<0.001
Mean systolic blood pressure (mmHg)	143 (21)		144 (21)		142 (21)		0.04
Mean diastolic blood pressure (mmHg)	76 (11)		77 (11)		75 (11)		<0.001
Mean total cholesterol level (mmol/liter)	5.8 (1.0)		5.8 (0.9)		6.0 (0.9)		<0.001
Mean high density lipoprotein cholesterol level (mmol/liter)	1.4 (0.4)		1.2 (0.3)		1.5 (0.4)		<0.001
Smoking status (%)							
- Never smoker		30		10		47	
- Current smoker		16		18		15	<0.001
- Former smoker		54		72		39	
Diabetes mellitus (%)		14		16		12	0.01
Median‡ C-reactive protein level (mg/liter)	2.5 (1.3–4.5)		2.6 (1.3–4.6)		2.4 (1.3–4.4)		0.37
Use of hormone replacement therapy (%)						21	
Mean age (years) at last menstrual period					48.8 (5.2)		

* Numbers in parentheses, standard deviation. † Weight (kg)/height $(m)^2$. ‡ The median value and interquartile range are presented because of the skewed distribution of the data.

Table 2. Age-adjusted regression coefficients for risk factors, describing the increase in log calcium score, intima-media thickness and ankle arm index per standard deviation increase of the cardiovascular risk factors, adjusted for age.

Variable	Log coronary calcium score		Intima-media thickness		Ankle arm index	
	Men	Women	Men	Women	Men	Women
Body mass index	0.24 (0.11, 0.37)	0.19 (0.06, 0.32)	0.03 (0.02, 0.04)	0.01 (0.001, 0.02)	0.01 (-0.01, 0.02)	0.01 (0.00, 0.02)
Systolic blood pressure	0.19 (0.06, 0.33)	0.11 (-0.03, 0.25)	0.04 (0.03, 0.06)	0.04 (0.03, 0.04)	-0.05 (-0.07, -0.04)	-0.04 (-0.05, -0.04)
Diastolic blood pressure	0.02 (-0.11, 0.16)	0.00 (-0.14, 0.14)	0.01 (0.003, 0.03)	0.01 (0.001, 0.02)	0.00 (-0.01, 0.01)	-0.01 (-0.02, 0.00)
Total cholesterol	-0.05 (-0.19, 0.08)	0.07 (-0.06, 0.21)	0.01 (0.00, 0.03)	0.00 (-0.01, 0.01)	-0.01 (-0.02, 0.01)	0.00 (-0.01, 0.01)
HDL-cholesterol	-0.04 (-0.18, 0.09)	-0.23 (-0.37, -0.09)	-0.01 (-0.02, -0.00)	-0.01 (-0.02, -0.00)	0.01 (-0.001, 0.02)	0.01 (0.001, 0.02)
Smoking (ever)	0.55 (0.12, 0.99)	0.61 (0.35, 0.88)	0.03 (-0.004, 0.07)	0.01 (-0.01, 0.03)	-0.04 (-0.08, 0.01)	-0.03 (-0.05, -0.01)
Diabetes mellitus	0.56 (0.20, 0.93)	0.84 (0.42, 1.26)	0.05 (0.02, 0.08)	0.02 (0.00, 0.05)	-0.05 (-0.08, -0.01)	-0.01 (-0.05, 0.01)
C-reactive protein*	0.20 (0.07, 0.33)	0.16 (0.02, 0.29)	0.02 (0.01, 0.03)	0.01 (0.002, 0.02)	-0.03 (-0.04, -0.02)	0.00 (-0.01, 0.01)

* Log transformed because of skewed distribution.

did women. Age and C-reactive protein level were similar in men and women. Associations of baseline characteristics with coronary calcium score, IMT, and AAI showed similar patterns in men and women (table 2). For carotid plaques, current smoking and diabetes were significant risk factors in men, whereas in women, they did not reach statistical significance (table 3). For aortic calcification, systolic blood pressure was a significant risk factor in men but did not reach statistical significance in women (table 4).

Figure 1 shows median coronary calcium score, mean IMT, median carotid plaque score, mean AAI, and median aortic calcification score according to age and gender. Median coronary calcium score was higher in men than in women in all age categories. A similar, though less pronounced, pattern was seen for IMT and carotid plaque score. For AAI and aortic calcification score, the pattern was more heterogeneous, with less obvious differences between men and women.

Crude percentages of participants with atherosclerosis, according to gender and age, are shown in figure 2 for each of the vascular locations. For all four measures of atherosclerosis, percentages of participants with high degrees of atherosclerosis increased with increasing age. Percentages with severe coronary calcification, IMT > 1.0 mm, and severe carotid plaques were notably higher in men than in women.

Table 5 shows numbers of cases and participants in gender and age categories and male:female odds ratios as computed by logistic regression. Coronary calcification showed the highest male:female odds ratios as compared with the other measures of atherosclerosis. The age-adjusted male:female odds ratios for having a calcium score above 1,000 in the two lowest age tertiles were 6.9 (95 percent

Table 3. Association between baseline characteristics and presence of severe carotid plaques in men and women adjusted for age.

Variable	Men			Women		
	No severe carotid plaques	Severe carotid plaques	P for trend	No severe carotid plaques	Severe carotid plaques	P for trend
Body mass index (kg/m^2)	26.3	26.4	0.80	27.2	26.8	0.86
Systolic blood pressure (mm Hg)	142	150	< 0.001	142	143	0.002
Diastolic blood pressure (mm Hg)	77	77	0.49	75	73	0.65
Total cholesterol (mmol/l)	5.5	5.6	0.04	6.0	6.1	0.26
HDL-cholesterol (mmol/l)	1.3	1.3	0.40	1.5	1.4	0.68
Smoking						
- Former (%)	72.6	69.7	0.55	37.5	45.2	0.07
- Current (%)	15.9	26.2	<0.001	12.9	23.9	0.20
Diabetes mellitus (%)	13.4	20.8	0.001	10.6	14.8	0.13
C-reactive protein (mg/l)	2.2*	2.8*	<0.001	2.1*	2.7*	0.01

* Geometric mean because of skewed distribution.

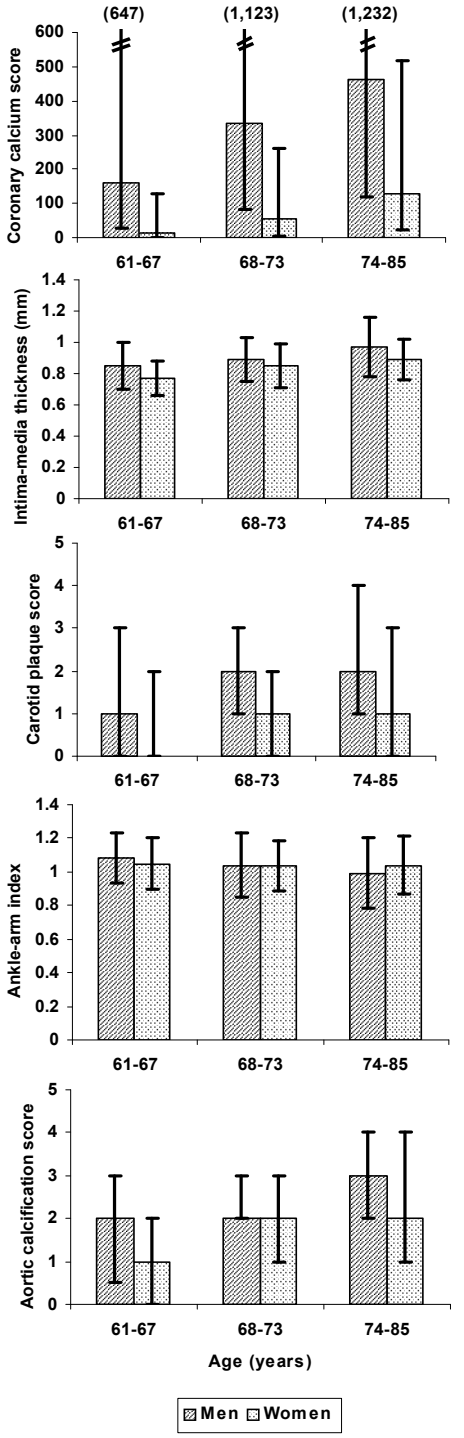
Table 4. Association between baseline characteristics and presence of severe aortic calcification in men and women, adjusted for age.

Variable	Men			Women		
	No severe aortic calcification	Severe aortic calcification	P for trend	No severe aortic calcification	Severe aortic calcification	P for trend
Body mass index (kg/m^2)	26.4	26.7	0.05	27.3	27.8	0.35
Systolic blood pressure (mm Hg)	143	146	0.001	142	143	0.13
Diastolic blood pressure (mm Hg)	77	76	0.997	75	73	0.48
Total cholesterol (mmol/l)	5.6	5.6	0.44	6.1	6.1	0.48
HDL-cholesterol (mmol/l)	1.3	1.3	0.04	1.5	1.4	<0.001
Smoking						
- Former (%)	71	76	0.86	38.1	44.0	0.56
- Current (%)	17.7	16.9	<0.001	12.7	20.1	<0.001
Diabetes mellitus (%)	14.6	23.5	0.03	10.7	18.9	0.03
C-reactive protein (mg/l)	2.2*	2.7*	0.01	2.2*	2.6*	<0.001

* Geometric mean because of skewed distribution.

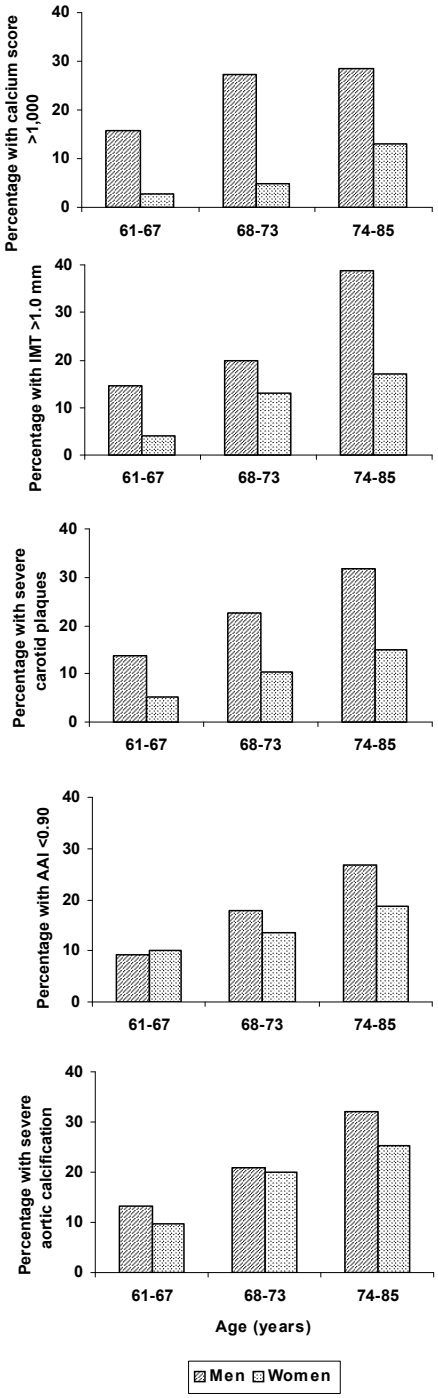
confidence interval (CI): 3.4, 13.9) and 7.4 (95 percent CI: 4.3, 12.7), showing that the men had a substantially higher degree of coronary calcification than the women. In the highest age tertile, the odds ratio declined to 2.7 (95 percent CI: 1.8, 4.0). The odds ratios for IMT, carotid plaques, AAI, and aortic

Figure 1. Measures of atherosclerosis according to gender (stripes, men; dots, women) and age tertile.



Mean values and standard deviations (bars) are displayed for ankle-arm index and intima-media thickness, and median values and interquartile ranges (bars) are displayed for coronary calcium score, carotid plaque score, and aortic calcification score, because of their skewed distributions. For coronary calcium score, the 75th percentiles are shown in parentheses.

Figure 2. Crude percentages of participants with severe atherosclerosis at various vascular locations, according to gender (stripes, men; dots, women) and age tertile.



IMT, intima-media thickness; AAI, ankle-arm index.

Table 5. Male:female odds ratios for atherosclerosis among all participants and by age tertile.

	Age tertile			Total
	1 (61–67 years)	2 (68–73 years)	3 (74–85 years)	
Coronary calcification > 1,000				
No. of men	49/308*	88/321	87/304	224/933
No. of women	10/364	17/349	48/367	75/1,080
Male:female OR† (model 1)‡	6.9 (3.4, 13.9)§	7.4 (4.3, 12.7)	2.7 (1.8, 4.0)	4.4 (3.3, 5.8)
Male:female OR (model 2)¶	7.8 (3.2, 19.3)	5.4 (2.8, 10.2)	3.0 (1.7, 5.2)	4.3 (3.0, 6.3)
Intima-media thickness > 1.0 mm				
No. of men	41/282	60/301	108/278	209/861
No. of women	13/334	42/322	58/342	113/998
Male:female OR (model 1)	4.2 (2.2, 8.0)	1.7 (1.1, 2.6)	3.1 (2.1, 4.5)	2.6 (2.0, 3.4)
Male:female OR (model 2)	4.7 (2.0, 11.2)	1.6 (0.9, 2.9)	4.1 (2.4, 7.1)	2.9 (2.0, 4.1)
Severe carotid plaques				
No. of men	37/268	62/274	84/263	183/805
No. of women	17/319	31/298	47/314	95/931
Male- to-female OR (model 1)	2.8 (1.5, 5.2)	2.6 (1.6, 4.1)	2.7 (1.8, 4.0)	2.7 (2.0, 3.5)
Male:female OR (model 2)	2.2 (1.0, 4.7)	2.4 (1.3, 4.3)	1.8 (1.1, 3.2)	2.0 (1.4, 2.8)
Ankle-arm index < 0.90				
No. of men	28/302	55/309	76/285	159/896
No. of women	36/353	45/336	64/341	145/1,030
Male:female OR (model 1)	0.9 (0.5, 1.5)	1.4 (0.9, 2.1)	1.6 (1.1, 2.3)	1.3 (1.0, 1.7)
Male:female OR (model 2)	0.9 (0.5, 1.7)	1.0 (0.6, 1.7)	1.0 (0.6, 1.8)	0.9 (0.7, 1.3)
Severe aortic calcification				
No. of men	36/273	58/277	86/269	180/819
No. of women	31/321	59/296	80/317	170/934
Male:female OR (model 1)	1.4 (0.8, 2.4)	1.1 (0.7, 1.6)	1.4 (1.0, 2.0)	1.3 (1.0, 1.6)
Male:female OR (model 2)	1.4 (0.7, 2.8)	0.8 (0.5, 1.4)	1.2 (0.7, 2.0)	1.0 (0.8, 1.5)

* Number of cases/total number of participants. † OR, odds ratio. ‡ Adjusted for age. § Numbers in parentheses, 95% confidence interval. ¶ Adjusted for age, body mass index, systolic and diastolic blood pressure, total and high density lipoprotein cholesterol, smoking, diabetes mellitus, C-reactive protein, hormone replacement therapy, and use of diuretics, beta-blockers, angiotensin-converting enzyme inhibitors, and lipid-lowering drugs.

calcification did not show an evident decline with increasing age, and the overall age-adjusted odds ratios for these measures were 2.6 (95 percent CI: 2.0, 3.4), 2.7 (95 percent CI: 2.0, 3.5), 1.3 (95 percent CI: 1.0, 1.7), and 1.3 (95 percent CI: 1.0, 1.6), respectively.

Adjustment for established cardiovascular disease risk factors, C-reactive protein level, hormone replacement therapy, and use of cardiac medication changed the estimates slightly. Multivariable adjusted odds ratios are displayed in figure 3. This figure emphasizes that the gender difference in severe coronary calcification is particularly present at lower age; at high age, the difference is less marked as compared with other measures of atherosclerosis.

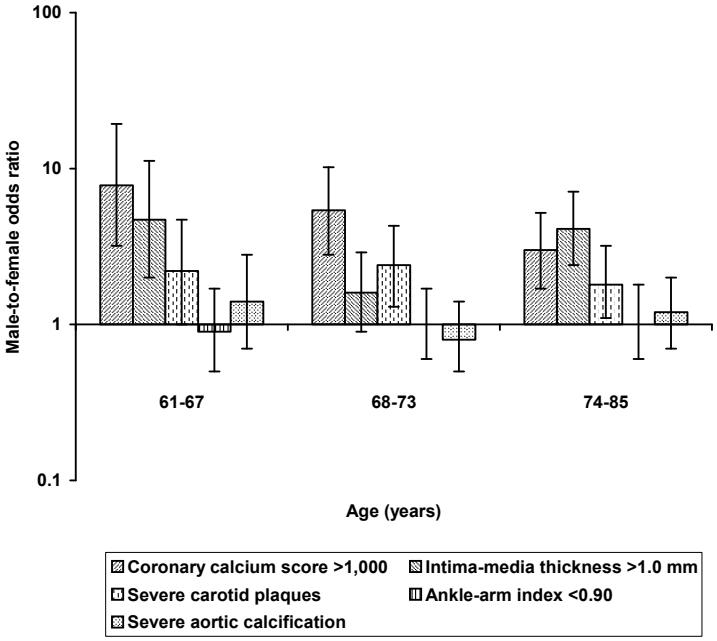
Table 6. Male:female odds ratios for atherosclerosis in participants without prevalent myocardial infarction, cerebrovascular accident, heart failure, angina pectoris,* or claudicatio intermittens,* among all participants and by age tertile.

	Age tertile			Total
	1 (61–67 years)	2 (68–73 years)	3 (74–85 years)	
Coronary calcification > 1,000				
No. of men	28/249†	44/218	30/184	102/651
No. of women	5/327	10/287	25/280	40/894
Male:female OR‡ (model 1)§	8.4 (3.2, 22.1)¶	7.0 (3.4, 14.3)	2.0 (1.1, 3.5)	4.1 (2.8, 6.1)
Male:female OR (model 2)#	11.3 (3.3, 38.9)	5.6 (2.4, 13.3)	2.7 (1.2, 6.1)	4.7 (2.9, 7.7)
Intima-media thickness > 1.0 mm				
No. of men	30/230	38/210	56/167	124/607
No. of women	8/299	38/264	33/262	79/825
Male:female OR (model 1)	5.5 (2.5, 12.2)	1.3 (0.8, 2.2)	3.5 (2.2, 5.7)	2.6 (1.9, 3.5)
Male:female OR (model 2)	6.8 (2.4, 19.4)	1.5 (0.8, 2.9)	6.1 (3.0, 12.5)	3.1 (2.1, 4.7)
Severe carotid plaques				
No. of men	28/220	35/189	40/159	103/568
No. of women	11/286	21/246	26/241	58/773
Male- to-female OR (model 1)	3.6 (1.8, 7.5)	2.5 (1.4, 4.4)	2.8 (1.6, 4.8)	2.9 (2.0, 4.0)
Male:female OR (model 2)	3.4 (1.3, 8.8)	2.5 (1.2, 5.3)	1.9 (0.9, 4.0)	2.2 (1.4, 3.4)
Ankle-arm index < 0.90				
No. of men	18/245	31/211	32/175	81/631
No. of women	30/319	36/278	47/262	113/859
Male:female OR (model 1)	0.8 (0.4, 1.4)	1.2 (0.7, 1.9)	1.0 (0.6, 1.7)	1.0 (0.7, 1.4)
Male:female OR (model 2)	0.8 (0.4, 1.8)	0.7 (0.4, 1.4)	0.7 (0.4, 1.5)	0.7 (0.5, 1.1)
Severe aortic calcification				
No. of men	24/224	37/188	42/161	103/573
No. of women	23/289	42/240	55/245	120/774
Male:female OR (model 1)	1.4 (0.8, 2.5)	1.1 (0.7, 1.9)	1.2 (0.8, 2.0)	1.2 (0.9, 1.7)
Male:female OR (model 2)	1.9 (0.8, 4.3)	0.8 (0.4, 1.6)	1.4 (0.7, 2.6)	1.2 (0.8, 1.7)

* As assessed by Rose questionnaire. † Number of cases/total number of participants. ‡ OR, odds ratio. § Adjusted for age. ¶ Numbers in parentheses, 95% confidence interval. # Adjusted for age, body mass index, systolic and diastolic blood pressure, total and high density lipoprotein cholesterol, smoking, diabetes mellitus, C-reactive protein, hormone replacement therapy, and use of diuretics, beta-blockers, angiotensin-converting enzyme inhibitors, and lipid-lowering drugs.

When we repeated the analysis after excluding participants with prevalent myocardial infarction, stroke, heart failure, angina pectoris, and claudicatio intermittens, the pattern of the risk estimates did not materially change (table 6).

Figure 3. Male:female odds ratios for different measures of atherosclerosis according to age tertile.



Odds ratios were adjusted for age, body mass index, systolic and diastolic blood pressure, total and high density lipoprotein cholesterol, smoking, diabetes mellitus, C-reactive protein, hormone replacement therapy, and use of diuretics, beta-blockers, angiotensin-converting enzyme inhibitors, and lipid-lowering drugs. Bars, 95% confidence interval.

Discussion

This study demonstrates that the gender difference in atherosclerosis in the coronary vessels is large, that it is particularly high in younger participants, and that it remains present at older ages. The gender difference in the coronary vessels is strikingly larger than in the other studied vascular beds. The gender difference in carotid atherosclerosis is also substantial, yet smaller and less consistent. Remarkably, differences in the aorta and the lower extremity vessels are virtually absent. The difference in gender ratio between sites is not explained by differences in cardiovascular disease risk factors.

Strengths of the present study include its population-based nature, its large size, the availability of several noninvasive measures of extracoronary atherosclerosis in combination with a noninvasive measure of coronary atherosclerosis, and standardized assessment of risk factors. Nevertheless, some issues warrant consideration.

First, in the assessment of coronary calcium, 61 percent of the invited population participated. In general, slight differences were present between responders and nonresponders, such as the younger age of the scanned population (mean age difference, 1.7 years) and the relatively higher proportion of men in the scanned population (46.3 percent vs. 37.8 percent).[19] Female participants in electron-beam computed tomography scanning more frequently were former smokers (38.9 percent vs. 33.4 percent) than nonparticipants. Male participants in electron-beam computed tomography scanning were also

more frequently former smokers (72.2 percent vs. 66.9 percent) than nonparticipants. Furthermore, they had a somewhat higher body mass index (26.5 vs. 26.1) and a somewhat higher diastolic blood pressure (77 mmHg vs. 76 mmHg). Additional missing values for the other measures of atherosclerosis in the scanned population were missing for logistic reasons, and therefore were considered to be missing at random. Any differences in cardiovascular disease risk factors between participants and nonparticipants in these measurements were also of very small magnitude. Although we think it is unlikely that selection bias occurred, we cannot fully exclude the possibility of a slight underestimation or overestimation of the studied associations.

Second, although interobserver reproducibility of coronary calcium scoring has been found to be excellent,[14] when interpreting our results, it must be noted that the measures of extracoronary atherosclerosis have lower reproducibility. Still, it has been shown that measurement of IMT is highly reproducible[23] and measurement of carotid plaques is moderately reproducible.[24] Radiographic detection of aortic calcification has been validated.[25] Reproducibility of AAI measurements is dependent on the standardization of the technique and the experience of the personnel;[29-30] both were optimized in the Rotterdam Study.

Finally, the definition of peripheral arterial disease is conventionally stated as an AAI < 0.90.[31-33] However, using the cutpoint of 0.90 in both men and women assumes equal sensitivity for arterial obstruction, whereas in truth it may differ between the genders. It was not possible for us to examine the association of AAI with true arterial obstruction in our population empirically. The disadvantage of choosing a gender-specific cutpoint in an attempt to approximate equal sensitivity in men and women is that this may potentially introduce a new bias into the gender estimates. Therefore, we used the cutpoint of 0.90 in the analysis. In line with this thinking, we applied the frequently used absolute cutpoint of 1.0 mm for IMT in the analyses.[34,35] Furthermore, the meaning of mean AAI (figure 1) in a population may be questioned; while the interpretation of a low AAI as indicative of arterial obstruction has been validated, the meaning of the mean AAI is less clear. However, AAI has been suggested to have an association with cardiovascular disease across its entire range,[36-38] which supports the possibility that AAI may be used as a continuous variable.

The differential gender effect for vascular sites was demonstrated in autopsy studies several decades ago.[10-13] Present studies demonstrate the prominence of the gender difference in coronary heart disease.[4] The gender difference in diseases which originate in other vascular beds, such as stroke and peripheral artery disease, is not nearly as large.[5-9] In the present study, we found that the male:female odds ratio for severe coronary calcification was almost 8 in the lowest age tertile and diminished with increasing age. The large male:female ratio is in accordance with prior data;[39] the incidence of hospitalization for a first myocardial infarction in the Netherlands results in a male-to female ratio of 4.2 in the age category 50–59 years, diminishing to 1.2 in persons aged 90 years or older. Furthermore, in the present study, the overall male:female odds ratio was somewhat lower for IMT > 1.0 mm and severe carotid plaques, with values of approximately 3 and 2, respectively. Prior data have demonstrated that the male:female ratio for cerebral infarction ranges from 1.6 in persons aged 55–64 years to 1.0 in persons aged 85 years or older.[40] Note that the contribution of atherothromboembolism to ischemic stroke is limited to 50 percent;[41] however, this should not influence the gender ratio, unless this percentage differs highly between men and women. With regard to peripheral arterial disease, research performed previously within the Rotterdam Study has demonstrated that the prevalences in 5-year

age categories are similar for men and women.[7] Gender differences in cardiovascular events such as myocardial infarction may be attributable to differences in exposure to risk factors, differences in the development of atherosclerosis, differences in "trigger" factors given equal severity of atherosclerosis, or a combination of these processes. The present findings underscore the role of the development of atherosclerosis in gender differences in the occurrence of coronary events.

To explain the differential gender effects across vascular sites, we should search for risk factors that have different effects on atherosclerosis in men and women but also have varying effects on atherosclerosis in different vascular beds. As demonstrated by our study, traditional cardiovascular disease risk factors do not seem to provide an explanation. Cardiovascular risk factors are considered to be generally the same for both genders, with the exception of diabetes mellitus, HDL cholesterol, and triglycerides, which have been found to have stronger effects among women.[42] Furthermore, low density lipoprotein particle size may be of particular importance for coronary heart disease in men.[43] In general, risk factors for atherosclerosis in different vascular beds are the same as those that predispose to disease in each individual vascular bed.[44-47] The impact of these risk factors may vary according to vascular site; for instance, smoking and diabetes have a larger impact on peripheral arterial disease than the other risk factors.[48] Although the risk factors are generally the same, differences in vascular anatomy, leading to regional disturbances of blood flow, and local changes in the arterial wall that affect interaction with blood components may still cause differences between the vascular sites. Not much is known about risk factors for atherosclerosis that simultaneously differ between men and women and between vascular sites. Finding these factors may provide clues as to why men have substantially more atherosclerosis than women in the coronary arteries but not in the aorta and peripheral vessels. This may help to elucidate the reason for the gender gap in coronary heart disease.

In conclusion, our study illustrates that the gender difference in coronary calcification is impressive, that it is particularly high in younger participants, and that it remains present at older ages. In younger participants, the multivariable-adjusted male:female odds ratio in the coronary arteries was close to 8. In the carotid arteries, the gender difference was also substantial, reaching 3 overall, but in the aorta and the peripheral vessels the male:female odds ratio remained close to the null value of 1. Presently known risk factors do not fully explain why the gender difference in cardiovascular disease varies in the different vascular beds. We believe that investigation of the causes of the site-specific gender differences may shed more light on the gender gap in coronary heart disease.

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Chapter 7

General discussion

The objective of this thesis was to expand the knowledge on inflammatory markers and inflammatory genes that may play a part in the pathophysiology of cardiovascular disease. Most studies described were conducted within the framework of the Rotterdam Study, a population-based cohort study among men and women aged 55 years and over. In the present chapter, methodological aspects will be addressed, the main findings of this thesis will be placed in the context of ongoing research in the field of inflammation and cardiovascular disease, and potential clinical implications and directions for future research will be discussed.

Methodological considerations

Methodological considerations pertaining to the separate studies have been described in the specific chapters. Here, general methodological considerations will be discussed with regard to observational studies and genetic association studies.

Observational studies[1]

Observational studies include cohort studies, in which subjects are classified according to their exposure status and followed over time to ascertain disease incidence; case-control studies, in which subjects are selected according to their disease status and further classified according to their exposure status; and cross-sectional studies, which include as subjects all persons in the population at the time of ascertainment or a representative sample of all such persons, and have an objective limited to describing the population at that time. The latter two designs may also be applied within the framework of a cohort study. In follow-up studies, the design may be retrospective or prospective. A retrospective design enables the researcher to identify large groups in the past and to follow them up over long periods of time until the present moment. However, the available data may not be of desired quality. Using a prospective design, the researcher has full control of the data collection. However, large groups need to be followed over many years to examine disease frequencies. The Rotterdam Study is an observational, prospective cohort study, and therefore has the advantages of careful and appropriate data collection. The designs of the studies described in this thesis were either prospective, with the ability to assess temporal relationships, or cross-sectional.

A disadvantage of observational studies is that they may be subject to bias. Types of bias include selection bias, information bias and confounding bias. Selection bias occurs when the relation between exposure and disease is different for those who participate and those who should be theoretically eligible for the study, including those who do not participate. Examples of studies that may be prone to selection bias in this thesis are the studies involving coronary calcification (chapters 2.2, 3.2, 4.1 and 6.1). A subgroup of the Rotterdam study was used for these studies, which was not completely random, and this resulted in slight differences in baseline characteristics between participants and non-participants. However, we have no reason to believe that this would have affected the relation between the exposure and the disease in participants or non-participants, and therefore deem distortion of the results unlikely.

Information bias occurs when misclassification of the outcome occurs that is related to the determinant, or vice versa. This has been termed differential misclassification. In the Rotterdam Study,

exposure data were collected before the disease onset, and missing values were mostly caused by periodic absence of research assistants or technical problems. Therefore, differential misclassification of the exposure is unlikely. Furthermore, disease outcomes were assessed by persons blinded to the exposure status, which makes differential misclassification of the outcome unlikely as well.

Confounding bias may occur when the apparent effect of the exposure of interest is distorted because the effect of an extraneous factor is mistaken for or mixed with the actual exposure effect. This implies that a confounder is associated with both the exposure and the exposure effect. When investigating the association of biomarkers such as C-reactive protein (CRP) with coronary heart disease (chapter 2.1), many confounders of the association are present. The analyses can be adjusted for known confounders. However, confounders unknown to the researcher may still lead to a distortion of the results. Such "residual confounding" is difficult to solve in observational studies. A way to avoid these problems is to perform a double-blind, randomized controlled trial, in which participants are randomized to therapeutic measures influencing the exposure of interest or placebo and followed for the occurrence of events. Clinical trials may lead to different conclusions than observational studies.

Another issue that may pose problems in observational studies is causal inference. To determine whether an observed association is causal, a criterion that can be used is that of temporality: if the putative cause did not precede the effect, that is indisputable evidence that the observed association is not causal. In some cases, it may not be straightforward to establish whether the cause preceded the effect. For example, it may not be clear whether CRP plays an etiologic part in the development of atherothrombotic disease or whether it merely reflects the inflammatory state, and in this case the severity of the atherosclerotic burden, already present in the individual at the time of CRP measurement (chapter 2.1). To solve this problem of reverse causation, a different study design should be chosen. Within the observational setting, a "Mendelian randomization" approach could be chosen. Alternatively, an interventional approach could be chosen to provide more insight into causality, such as performing a randomized controlled clinical trial aimed to assess whether CRP reduction per se lowers risk, preferentially with agents that do not have alternative known beneficial effects. The extent to which Mendelian randomization and randomized trials are able to prove causality are discussed in more detail below.

Genetic association studies

To perform genetic association studies, candidate genes can be selected, and the association between variation in these genes and disease occurrence can be investigated. Genes may be selected on the basis of their presumed roles in disease pathophysiology and variants in these genes, such as single nucleotide polymorphisms (SNPs), may be selected because of their functional implications.[2] On the other hand, genetic variants may be selected because they "tag" gene haplotypes.[3] A haplotype is a set of SNPs, on a single chromatid, that are statistically associated. It is thought that the identification of a few alleles of a haplotype block can unambiguously identify all other polymorphic sites in its region. In this thesis, a presumably functional genetic variant is investigated in chapter 2.3: the complement factor H (CFH) Tyr402His SNP may alter the CRP binding site of CFH. In chapter 4.1, tagging SNPs are determined and FGG-FGA haplotypes, which may influence fibrinogen structure and function, are investigated.

Genetic variants may also influence blood levels of biomarkers. Investigation of such variants may provide solutions for problems encountered with causal inference in observational studies, such as residual confounding and reverse causation. If the blood level of a biomarker truly increases risk of disease, then carriage of a genetic variant that exposes individuals to a long-term elevation of this biomarker should confer an increased risk of disease proportional to the difference in biomarker level attributable to the variant. This implies that if non-genetic observational studies were unbiased, the increase in risk estimated from these studies should be consistent with the increase in risk conferred by carriage of the genetic variant. This approach should circumvent the problem of residual confounding, because the inheritance of a genetic variant associated with different biomarker levels should be subject to the random assortment of maternal and paternal alleles at the time of gamete formation. It should also circumvent the problem of reverse causation, because the genetic variant of interest is present in an individual before the disease occurs.[4] An application of this study design can be found in chapter 2.1 of this thesis, where CRP haplotypes are investigated in relation to CRP serum levels and coronary heart disease.

The above approach, known as "Mendelian randomization", has gained interest lately. Although it may solve several problems, it is based on multiple assumptions and therefore entails some problems of its own.[5] The first assumption is that the genetic variant is only associated with the biomarker of interest and not with other factors that may play a part in the disease process. Another assumption is that the genetic variant is only associated with the quantity but not the quality of the biomarker of interest. Both assumptions may not always hold true. Furthermore, it is assumed that there is no "canalisation" or compensation for the altered gene expression, in this case different biomarker levels due to the genetic variant, during development. However, given the complexity of the pathways involved and the multifactorial nature of cardiovascular disease, such compensation may not be unlikely in some cases. These issues should be borne in mind and dealt with when performing Mendelian randomization studies.

Genetic association studies in general may have several other shortcomings.[6] An association found between a single genetic variant and a disease outcome may have been caused by linkage disequilibrium with another genetic variant. A more comprehensive approach is obtained by constructing gene haplotypes that capture the common variation across a gene, as described above. Furthermore, large sample sizes are needed to provide enough statistical power to detect the effect of a genetic variant on diseases with a multifactorial origin. Such diseases are thought to result from variations in a large spectrum of genes with small individual effects. For example, as demonstrated in chapter 2.1, for CRP haplotypes, we were able to detect a relative risk of coronary heart disease of 1.21 with a power of 80% in our study; the true effect may actually have been smaller and thus have gone undetected. Finally, reproducibility of the results of genetic association studies may pose problems; lack of reproducibility may result from effect modification or population stratification, or, again, from insufficient statistical power.

Main findings in the context of ongoing research

Inflammation and atherothrombotic disease

C-reactive protein

Although proof of a causal role of CRP in cardiovascular disease has to be awaited, several pathophysiological mechanisms involving CRP have been suggested. CRP is involved in endothelial dysfunction, inducing the expression of adhesion molecules such as intercellular cell adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule (VCAM) and E-selectin. Also, it appears to be involved in recruitment of monocytes, infiltration of monocytes into the vessel wall and subsequent development into foam cells. Foam cell formation might be caused in part by uptake of CRP-opsonized native LDL. CRP exposure increases vascular smooth muscle cell proliferation and migration. Finally, CRP is able to activate the classical route of complement activation, which leads to the production of a variety of pro-inflammatory molecules.

Numerous epidemiologic studies have reported that CRP is an independent risk factor for cardiovascular disease, and a meta-analysis using more than 7000 cases has demonstrated that the multivariable-adjusted odds ratio for coronary heart disease in the highest versus the lowest tertile of CRP is 1.45 (95% CI 1.25-1.68).[7] Our results, presented in chapter 2.1 and showing that CRP serum level is an independent risk factor for coronary heart disease, are in agreement with these studies. To explore the potential causality of the CRP-coronary heart disease association, in chapter 2.1, we determined CRP gene haplotypes and investigated their associations with CRP serum level and coronary heart disease. Although haplotypes were associated with CRP serum level, they were not associated with coronary events. A possible explanation for this is that lack of power prevented us from demonstrating an association that may have been of small magnitude given the multifactorial origin of cardiovascular disease. Several other studies have attempted to replicate these findings. Some have found significant associations between CRP haplotypes and cardiovascular disease, while others have failed to do so.[8-10] Again, lack of power may underlie these inconsistent results.

With regard to atherosclerosis, epidemiologic studies support independent relations between CRP and carotid plaques[11-13] and low ankle-arm index.[14-16] In general, associations between CRP and coronary calcification and CRP and intima-media thickness have been less consistent.[11,12,14,17-22] This population-based study shows graded associations of CRP with extent and progression of atherosclerosis. However, the strength of the associations depends on the applied measure of atherosclerosis. We found an independent, graded association between CRP and extent and progression of carotid plaques and AAI. Furthermore, CRP was independently related to the highest level of carotid IMT, while the association with change in IMT was not significant. Although there was an independent, graded relation between CRP and aortic calcification, no independent association was found with progression of aortic calcification, nor with the amount of coronary calcification. A possible explanation for the less consistent findings with progression is, that more random variation is present in measures of progression of atherosclerosis than in measures of burden of atherosclerosis. This results from the fact that progression is derived from at least two measurements, which both have their own random variation, while measures of burden of atherosclerosis consist of only one measurement. Consequently, associations of determinants with these measures of progression may be more difficult

to demonstrate; a phenomenon that has previously been demonstrated for progression of intima-media thickness. In general, little is known about determinants of progression of calcification, but a similar problem may be present here. Our findings are generally supported by previous studies on the relation between CRP and quantity of atherosclerosis. The inconsistency in the literature on the relation between CRP and atherosclerosis may, at least partly, be explained by differences in applied measures of atherosclerosis and lack of quantification.

Complement factor H

Linkage studies for age-related macular degeneration have mapped a gene at chromosome 1q32, and through both fine-mapping and a whole genome association study, the Y402H variant of CFH gene was identified as the dominant susceptibility factor for age-related macula degeneration with ORs ranging from 2.5 to 7.4.[23-25] The pathophysiological processes pertaining to CFH are the following. The complement system contains several plasma and membrane-associated proteins that are organized in three activation pathways: the classical, the lectin and the alternative pathway. CFH is a plasma protein that plays an important part in the inhibition of the alternative pathway; it restricts the action of complement to activating surfaces by binding to C3b, accelerating the decay of the alternative pathway C3-convertase (C3bBb) and acting as a cofactor for the factor I-mediated proteolytic inactivation of C3b. This mechanism allows for activation of the early complement cascade by opsonization and may thereby play a protective role, however, complement activation is limited to the C3 level, and does not lead to full complement activation with cell lysis and ensuing inflammation. As such, CFH may play a part in the pathophysiology of atherosclerosis. The complement system contributes to inflammation in the arterial intima, and thus may exert unfavourable effects on atherosclerosis. Research in coronary artery specimens suggests that interaction of CFH with proteoglycans may be the mechanism by which complement activation in the superficial layer of the coronary intima is controlled.[26] Within the Rotterdam Study cohort, we demonstrated a 1.8-fold odds ratio for myocardial infarction among those individuals harboring the His allele of Y402H (chapter 2.3). There have been a few studies that have attempted to replicate these findings; Pai et al. demonstrated a protective effect of the His allele in women, but not in men,[27] while other studies failed to demonstrate significant associations.[28-30] Underlying reasons for these discrepancies may include smaller sample size, leading to low power and leaving room for a modest association, and use of atherosclerosis as the outcome instead of hard events. Furthermore, we cannot exclude the possibility that the association between Y402H and coronary heart disease is a false positive finding.

Binding of CRP to CFH has been suggested to augment the ability of CFH to down-regulate the effect of complement in atherosclerotic lesions. Furthermore, the CFH Tyr402His polymorphism has been suggested to influence the ability of CFH to bind CRP.[31] In chapter 2.4, we have demonstrated that the combined presence of unfavorable CRP and CFH genetic profiles is associated with risk of myocardial infarction. Unfortunately, we did not have an independent replication cohort at our disposal to consolidate the findings. Our paper may stimulate other research groups to investigate this association.

Lipoprotein-associated phospholipase A2

Lipoprotein-associated phospholipase A2 (Lp-PLA2) is an emerging biomarker that has gained attention recently because it may play a part in the pathophysiology of cardiovascular disease. Lp-PLA2 is an enzyme that circulates in the blood, and, in humans, is bound predominantly to LDL. Oxidative modification to the phospholipid component of LDL provides the substrate for the enzyme; following LDL oxidation, Lp-PLA2 acts rapidly to hydrolyze one of the fatty moieties of the phospholipid to produce two inflammatory molecules- oxidized fatty acids and lysophosphatidylcholine. Given that the majority of oxidized LDL resides within the intima, the products of Lp-PLA2 are generated within the vessel wall where the inflammatory processes of atherosclerosis occur. Both oxidized fatty acids and lysophosphatidylcholine are highly soluble, diffuse throughout atheroma, and affect the various cell types involved in atherosclerosis.

Overall, epidemiologic studies suggest that Lp-PLA2 is an independent risk factor for cardiovascular disease.[32-35] Research performed within the Rotterdam Study has led to the same conclusion.[36] Studies on Lp-PLA2 and coronary atherosclerosis seem to lean into the same direction.[37-42] However, in the Rotterdam Study, the association between Lp-PLA2 and coronary calcification was not independent of cholesterol (chapter 3.2). Furthermore, results from the Rotterdam Study have also suggested that the association of Lp-PLA2 with extracoronary atherosclerosis is not independent of cholesterol (chapter 3.3). Extracoronary atherosclerosis has been studied less extensively in the literature.[43,44]

As such, an issue of interest is whether the effect of Lp-PLA2 on cardiovascular events is truly independent of LDL-cholesterol, to which it is bound. Until now, findings generally seem to support an independent role for Lp-PLA2 in cardiovascular risk prediction. Another issue of interest is that Lp-PLA2 has been suggested to have both pro-atherogenic and anti-atherogenic properties. In humans, given that high levels of Lp-PLA2 are associated with cardiovascular events, evidence is building up that the pro-atherogenic properties of Lp-PLA2 outweigh the anti-atherogenic properties. However, research on genetic variation in the Lp-PLA2 gene appears to yield inconclusive results with regard to this matter.[45-50]

Fibrinogen

Apart from being an inflammatory marker, fibrinogen is an important coagulation factor, acting as an adhesive protein essential for platelet aggregation as well as forming insoluble fibrin fibers in the final stage of the blood coagulation cascade. Several prospective epidemiological studies have demonstrated an independent association between fibrinogen level and cardiovascular disease. This has recently been confirmed in a large, individual participant meta-analysis.[51] Changes in the structure of the fibrin network may also represent a pathophysiological mechanism contributing to the progression of atherosclerosis.[52,53] A correlation has been demonstrated between fibrin structure and coronary disease, and a possible underlying mechanism is the relationship between fibrin structure and hypofibrinolysis. Fibrin structure is in part determined by genetic influences. Therefore, genetic variants altering fibrinogen structure and function and consequently fibrin structure may provide an opportunity to further investigate the involvement of fibrinogen in atherogenesis. As such,we have demonstrated that FGG and FGA fibrinogen haplotypes were not associated with coronary events, coronary calcification or extracoronary atherosclerosis (chapter 4.1).

With regard to replication of these findings, until now, we are only aware of two other studies on fibrinogen haplotypes and coronary events. Uitte de Willige et al. found no association between FGG

gene haplotypes and myocardial infarction in the "Study of Myocardial Infarctions Leiden"(SMILE), which is in agreement with our study.[54] Mannila et al. studied the association between fibrinogen gene haplotypes and myocardial infarction in the Stockholm Coronary Artery Risk Factor (SCARF) study, and found that FGG and FGA haplotypes were associated with risk of myocardial infarction.[55] The discrepancy with the Rotterdam Study may have arisen because of differences in study population and the fact that the SCARF study was designed to study determinants of premature myocardial infarction, whereas the Rotterdam Study was designed to examine myocardial infarction in a general population.

Heat shock protein 27

Heat shock proteins (HSPs) from the HSP60 and HSP70 families have been most widely investigated in relation to atherosclerosis.[56,57] Recently, cardiovascular attention has also focused on HSP27.[58] The pathophysiological role of HSP27 has been suggested to include chaperoning activity, inhibition of F-actin polymerization, protection against apoptosis and involvement in the presentation of oxidized proteins to the proteosome degradation machinery. Using atherosclerotic carotid endarterectomy samples and control endarteries, Martin-Ventura et al demonstrated that HSP27 secretion correlates negatively with atherosclerotic plaque complexity by comparing the complicated versus the non-complicated adjacent area from the same specimen and control endarteries. They also reported reduced HSP27 plasma levels in atherosclerotic patients compared with healthy subjects.[59] Park et al. used the same strategy but examined the tissue compartment, and also reported that HSP27 expression is increased in the normal-appearing vessel adjacent to atherosclerotic plaque compared to both the plaque core area and the reference arteries.[60] By contrast, however, they reported that HSP27 plasma level was increased in acute coronary syndrome patients compared to normal reference subjects.

In this thesis, we performed a prospective, nested case-control study within the Women's Health Study to examine whether baseline levels of HSP27 among initially healthy individuals are associated with future cardiovascular event rates. No association was found between baseline HSP27 plasma level and risk of cardiovascular events (chapter 4.2). Further, although HSP27 was inversely associated with age, it was not associated with other established cardiovascular risk factors. To explain these results, we have to consider the possibility that HSP27 plasma level might not closely reflect the secretion of HSP27 from atherosclerotic plaques. If we address the hypothesis that HSP27 may be degraded by culprit atherosclerotic plaque,[61] it is unlikely that variations in HSP27 plasma level could be detected until the disease is advanced, as demonstrated in patients with a mean age of nearly 70 years with carotid atherosclerosis.[59] Another possibility is that HSP27 plasma level rises in the acute phase of ischemic events, as shown for HSP70,[62] in which case raised plasma level would not precede cardiovascular disease. This would help to explain the positive results found by Park et al., who used acute coronary syndrome patients and drew blood within 24 hours from presentation to the emergency department.[60]

Inflammation and heart failure

While heart failure was once merely considered to be a cardiocirculatory impairment, now it is known that the neuroendocrine system is involved. Furthermore, multiple lines of evidence support the "cytokine hypothesis," which suggests that inflammation plays an important role in the development

and progression of heart failure. Pathophysiologically, many aspects of the syndrome of heart failure can be explained by the known biological effects of proinflammatory cytokines. When expressed at sufficiently high concentrations, such as those observed in heart failure, cytokines are sufficient to mimic some aspects of the heart failure phenotype, including progressive left ventricular dysfunction, pulmonary edema, left ventricular remodeling, and cardiomyopathy. Thus, heart failure may progress, at least in part, as a result of the toxic effects exerted by endogenous cytokine cascades on the heart and the peropheral circulation. Consequently, circulating markers of inflammation, such as tumor necrosis factor alpha, interleukin 6, and C-reactive protein, may be useful in establishing the diagnosis, gauging prognosis, and evaluating the response to therapy in patients with heart failure.[63]

In this thesis, first, we examined the associations of established cardiovascular risk factors with echocardiographic parameters in asymptomatic persons (chapter 5.1). Ventricular systolic and diastolic dysfunction was present in participants that had not been diagnosed with heart failure or myocardial infarction. Higher age, higher BMI, lower systolic and higher diastolic blood pressure were most consistently associated with worse systolic function. Higher age and higher diastolic blood pressure were most consistently associated with lower E/A ratio. This was generally in line with previous studies.[64-68] Furthermore, chapter 5.2 demonstrates an association of selected structural and diastolic echocardiographic parameters with all-cause mortality in our study population. This is also in line with previous studies.[69-73] The above results underline the importance of insight into the identification of individuals with preclinical ventricular dysfunction who may benefit from early treatment.

Subsequently, we explored the role of inflammation in heart failure. Chapter 5.3 describes the association of CRP serum levels with incident heart failure. Positive associations had already been found in the Cardiovascular Health Study, the Framingham Study and the Health ABC study.[74-76] Sex-specific relative risks of developing heart failure were not provided in these studies. We found that in men, the association of CRP with heart failure was partly explained by presence of coronary heart disease, whereas in women, it was partly explained by hypertension and body mass index. The reason for this may lie in the fact that men are known to have coronary artery disease as an underlying factor for heart failure more frequently than women, and women are more likely to have hypertension as an underlying factor, and both coronary heart disease and hypertension are associated with CRP. In chapter 5.4 we investigated the association of Lp-PLA2 and heart failure in a random subcohort of the Rotterdam Study. Our findings suggest that Lp-PLA2 activity is independently associated with risk of heart failure. To our knowledge, no other studies have examined this association. Altogether, our findings support the hypothesis that inflammation plays a role in the development of heart failure.

Prevalence of atherosclerosis in men and women in the Rotterdam Study

The gender difference in coronary heart disease occurrence is not completely understood, and cannot be fully explained by risk factors such as lifestyle, lipid profile and blood pressure. The hypothesis that estrogen carries a cardio protective effect is being debated and could not be proven in large randomized controlled trials on estrogen therapy. Although the gender difference is also present to a much smaller extent for stroke and peripheral artery disease, it is most prominent for coronary heart disease. This suggests that gender differences in cardiovascular disease differ according to vascular sites. Autopsy studies support such a differential gender effect. Although studies have been performed in living populations to examine gender differences in atherosclerosis at single vascular sites,[77-80] no

studies have been performed in living populations to compare gender differences in atherosclerosis at different sites of the vascular tree.

In chapter 6.1 of this thesis, we have demonstrated that the gender difference in atherosclerosis in the coronary vessels is large, that it is particularly high in younger participants, and that it remains present at older age. The gender difference in the coronary vessels is strikingly larger than in the other studied vascular beds. The gender difference in carotid atherosclerosis is also substantial, yet smaller, and less consistent. Remarkably, the difference in the aorta and the lower extremity vessels is virtually absent. The difference in gender ratio between sites is not explained by differences in cardiovascular risk factors.

Clinical implications and future research topics

In this thesis, we have investigated the roles of CRP and Lp-PLA2 in cardiovascular disease. In general, the role of CRP has been most widely investigated; CRP has been reported to be independently and consistently associated with cardiovascular events in men and women, in healthy persons and in cardiovascular disease patients, and in strata of Framingham risk.[81] Furthermore, several studies have reported that CRP has additional value to established cardiovascular risk factors in cardiovascular risk stratification.[82-85] As a result, the Centers for Disease Control and Prevention and the American Heart Association have issued a statement supporting the usefulness of CRP in cardiovascular risk stratification.[86] Nonetheless, the role of CRP in cardiovascular risk stratification remains controversial. Literature-based meta-analyses have demonstrated significant, independent associations of CRP with coronary heart disease.[7,87] In the future, an individual-based meta-analysis of CRP serum levels and risk of cardiovascular disease could provide more precise risk estimates, carefully adjusted for potential confounders, stratified on potential effect modifiers and adjusted for regression dilution. However, an independent association with cardiovascular disease does not prove usefulness for risk stratification. Furthermore, in contrast with the above, several papers have reported a lack of additional value of CRP to established risk factors in cardiovascular risk stratification.[88-90] This discrepancy may at least in part be due to the fact that no consensus has yet been reached as to the strategy that is most appropriate to investigate the added value of biomarkers to established cardiovascular risk factors for risk stratification. A strategy that is often used is comparison of the area under the ROC curve for models containing established cardiovascular risk factors with models additionally containing the biomarker of interest.[91] The ROC curve is a plot of sensitivity versus 1–specificity that offers a summary of sensitivity and specificity across a range of cut points for a continuous predictor. Perfect discrimination is achieved if the scores for all the cases are higher than those for all the non-cases, with no overlap. The appropriateness of this method for the above issue has been questioned.[92] A biomarker with an odds ratio of 3 may have little effect on the area under the ROC curve, yet an increased level could shift estimated 10-year cardiovascular risk for an individual considerably, which may lead to different treatment recommendations.[92]

Apart from usefulness in risk stratification, another issue that is still controversial and warrants further investigation is the potential etiologic role of CRP in cardiovascular disease. Although basic research suggests several pathophysiological mechanisms through which CRP may act as a causal

agent,[93] the possibility exists that CRP is merely a marker that reflects the degree of inflammation present. Of course, it should be kept in mind that CRP merely being a marker would not exclude the possibility that inflammation in itself is a causal agent. A randomized controlled clinical trial using an intervention that selectively reduces CRP could potentially provide more insight into the nature of the CRP-cardiovascular disease association. Unfortunately, no such selective intervention currently exists. Although statins are known to lower CRP, they also have major effects on lipid profile and glucose metabolism. Interestingly, patients with acute coronary syndromes who have low CRP levels after statin therapy have been shown to have better clinical outcomes than those with higher CRP levels, regardless of the resultant level of LDL cholesterol.[94] The JUPITER trial, presently ongoing, will evaluate the potential benefits and risks of statin therapy among those with normal LDL cholesterol but raised CRP.[95] However, the basis for the trial does not depend on a causal relationship of CRP with coronary heart disease, but only whether CRP is a useful indicator for people at increased risk not identified by traditional risk factors. Mendelian randomization may be a suitable alternative to gain more insight into the role of CRP in cardiovascular disease. Cohort studies applying this method may be pooled to obtain adequate statistical power. However, when applying the Mendelian randomization approach, the above-mentioned limitations of this method should be kept in mind.

Lp-PLA2 has generally been studied less extensively. Although the body of evidence is not as vast as for CRP, a large number of observational epidemiological studies have reported generally positive associations between circulating mass and activity levels of Lp-PLA2 and risk of cardiovascular disease. Few studies have been large enough to provide reliable estimates in different subgroups. More insight is needed into the precise nature of the association, including the influence of confounders such as cholesterol and effect modifiers such as gender, and this may be provided by an individual-based meta-analysis, which is on the way.[96] Also, adequate data on the additional value of Lp-PLA2 to established cardiovascular risk factors for cardiovascular risk stratification are needed. Should, in the end, Lp-PLA2 prove to be useful in risk stratification, then guidelines should be formulated stating which type of assay (mass or activity) is most appropriate, which Lp-PLA2 cut-off points should be used for risk stratification in clinical practice, and in which subject categories these cut-off points should be applied for risk stratification. On the other hand, in contrast to CRP, selective inhibitors of Lp-PLA2 have been developed and are currently under investigation.[97] Although this research is still in a relatively early phase, in the future, it may provide us with the opportunity to investigate the pathophysiological effects of Lp-PLA2 by means of a randomized clinical trial.

Finally, HSPs are plausible candidates for a role in atherogenesis. HSP60 and HSP70 families and antibodies against these HSPs have been most widely investigated in relation to atherosclerosis; several of these factors have been associated with carotid disease and ischemic stroke,[98-100] with presence and severity of coronary atherosclerosis,[101,102] and with restenosis after percutaneous transluminal coronary angioplasty.[103] They have also been associated with aortic disease and peripheral vascular disease.[104-106] The Women's Health Study is the first prospective study to examine the association of HSP27 with cardiovascular disease. Therefore, confirmation of the findings by other prospective studies is warranted. However, given the potential mechanisms of action, measuring HSP27 in persons in whom advanced cardiovascular disease is present may be a more appropriate method to examine the role of HSP27 in cardiovascular disease.

In this thesis, we have performed genetic association studies concerning CFH genotype and fibrinogen FGG/FGA haplotypes and risk of cardiovascular disease. Our findings need to be confirmed in other study populations. As described above, reproducibility of genetic association studies remains problematic.[6] The direction that is currently gaining attention in genetic research is the application of genome wide association studies in population-based cohorts. Such studies investigate direct gene associations with large numbers of markers. SNPs are known to occur with sufficient frequency in the human genome to allow testing on this scale and genotyping platforms are now available to analyze upwards of 500K SNPs. Improvements in the efficiency of the design can be made by careful SNP selection, taking advantage of the background patterns of linkage disequilibrium (LD) and focusing genotyping on a subset of "tag SNPs", acting as proxies for nearby correlated variants. The International Haplotype Map (HapMap) project offers a comprehensive view of the structure of LD throughout the genome in multiple populations.[107] This, coupled with recent improvements in the efficiency of high-throughput genotyping platforms, has paved the way for effective whole genome association experiments. Several issues need to be addressed when using this approach. The primary concern is one of statistical power to observe an effect of a specific size. A number of 1000 cases and 1000 controls may be a realistic standard for detection of genes of moderate effect sizes; this is relatively large compared with most genetic case-control studies.[108] Another issue in genome-wide experiments is the choice of source tissue. Although the amount of DNA required is relatively small, the experiments are exquisitely sensitive to DNA quality. Consequently, quality of the sample source material may be as important as concerns over study design and sample size.[108] Furthermore, as the density of GW-SNP genotyping platforms continues to increase, the data volume and consequently data storage poses a challenge. Also, available tools for data management and analysis of GW-SNP data are relatively scarce.[109] A few exist or are under development, such as PLINK and GenABEL. A large number of test statistics will be generated when performing the analysis, and a major problem is how to best evaluate results taking this large number of tests into account. Ways to deal with multiple testing include applying Bonferroni's correction or the slightly less conservative Sidak's correction; the false discovery rate (FDR) method, which calculates the proportion of null results among significant results and is appropriate for situations in which multiple "hits" are expected in large-scale experiments; or permutation testing.[109] As genome wide association studies will continue to gain popularity, the above-mentioned issues will need to be dealt with adequately.

In this thesis, we have examined the distribution of echocardiographic parameters in the Rotterdam Study population and the associations of these parameters with established cardiovascular risk factors and with mortality. Insight into echocardiographic parameters is important, because it has been recognized that preclinical ventricular dysfunction may be present even when heart failure has not yet become clinically manifest,[110] and preclinical ventricular dysfunction has several characteristics rendering it a suitable target for screening. It has a natural history that is known to be of progressive nature, eventually resulting in overt heart failure, and as such it is a recognizable early asymptomatic stage of this condition. Heart failure is an important health problem that is gaining further momentum with the aging of the population and increased survival of patients with coronary heart disease. Also, preclinical ventricular dysfunction is prone to therapeutic interventions that reduce morbidity and mortality from heart failure.[111,112] It should be noted here that the lack of a clear association of

asymptomatic systolic dysfunction with all-cause mortality in our study may in part have been caused by selection bias in our study; mean follow-up was 3.0 years, and participants who came to the research center may have been less likely to die in the first years of follow-up, because they were still relatively healthy. This may have attenuated the association.

When considering whether screening community-dwelling individuals for preclinical ventricular dysfunction is appropriate some issues still need attention.[113] We found that moderate left ventricular systolic function was present in 2.8% of men and 1.7% of women without prevalent heart failure, myocardial infarction or atrial fibrillation and flutter, while poor systolic function was present in 1.1% and 0.4% of men and women, respectively. Global systolic function was qualitatively assessed from two-dimensional images by trained echocardiographers in our study. This approach reflects the routine practice in echocardiography laboratories and its reproducibility and accuracy has been shown to be as good as those of quantitative methods;[114,115] as such, we believe the measurement of systolic function was performed appropriately in our study. This low prevalence of moderate and poor left ventricular function does not provide additional support for screening. However, it should be kept in mind that in general, prevalence of asymptomatic left ventricular dysfunction varies widely across studies, in part because of differences in study populations and in definitions of asymptomatic left ventricular dysfunction.

Furthermore, appropriate screening tools should be applied. Although echocardiography is a strategy that detects ventricular dysfunction accurately in asymptomatic patients, when considering screening its costs- relative to its benefits- need to be investigated. Options that are cheaper may also be investigated; these include electrocardiograms and natriuretic peptides such as brain natriuretic peptide (BNP). The drawback of electrocardiography is that it may have low specificity and a low positive predictive value,[116] while the opposite is essential when screening large populations of asymptomatic individuals with a low prevalence of disease. Although BNP is useful for ruling out heart failure as the cause of symptoms, the capacity of BNP level to identify persons with ventricular systolic dysfunction may be limited,[117] and low sensitivities and specificities have been reported using various cut-points.[113]

In this thesis, we have found associations of CRP and Lp-PLA2 with incident heart failure, and these findings support the hypothesis that inflammation plays a role in the development of heart failure. Inflammatory biomarkers that have been investigated most extensively in this regard are tumor necrosis factor and interleukin-6.[63] In addition to their potential as heart failure biomarkers, inflammatory cytokines have been investigated as targets of heart failure therapy. Results for therapies directed against specific cytokines (such as tumor necrosis factor alpha) have thus far been disappointing.[63] Although one interpretation of these findings is that inflammatory mediators are not viable targets in heart failure, the countervailing point of view is that we simply have not targeted proinflammatory mediators with agents that can be used safely in the context of heart failure or that targeting a single component of the inflammatory cascade is not sufficient in a disease as complex as heart failure. Despite the unfavorable beginning with targeted anti-inflammatory approaches, strategies that use agents that have a broad spectrum of anti-inflammatory properties and immunomodulatory strategies that activate antiinflammatory pathways are currently being evaluated. Well-designed clinical trials are needed to provide more insight into this matter.

Finally, in this thesis, we have demonstrated that the gender difference in atherosclerosis in the coronary vessels is strikingly larger than in the other studied vascular bed, and that the difference in gender ratio between sites is not explained by differences in cardiovascular risk factors. To explain the differential gender effect across vascular sites, we should search for risk factors that have different effects on atherosclerosis in men and women but also have varying effects on atherosclerosis in different vascular beds. Cardiovascular risk factors are considered to be generally the same for both genders, with the exception of diabetes mellitus, HDL cholesterol and triglycerides, which have been found to have stronger effects among women,[118] and LDL particle size, which may be of particular importance for coronary heart disease in men.[119] Furthermore, risk factors for atherosclerosis in different vascular beds are generally the same as those that predispose to disease in each individual vascular bed,[120-123] although the impact of these risk factors may vary according to vascular site; for instance, smoking and diabetes have a larger impact on peripheral arterial disease than the other risk factors.[124] As such, it seems that to explain the gender difference, we should search for risk factors other than the established cardiovascular risk factors. Besides risk factors, differences in vascular anatomy, leading to regional disturbances of blood flow, and local changes in the arterial wall that affect interaction with blood components may cause differences between the vascular sites.

Not much is known about factors that simultaneously differ between men and women and between vascular sites. Finding them may provide clues as to why men have substantially more atherosclerosis than women in the coronary arteries but not in the aorta and peripheral vessels. This may help to elucidate the reason for the gender gap in coronary heart disease.

Main conclusions

Summarizing, the main conclusions of this thesis with respect to inflammation and cardiovascular disease are as follows.

CRP serum level is clearly and independently associated with coronary events. However, the strength of its association with atherosclerosis depends on the applied measure of atherosclerosis. An association between CRP gene haplotypes and coronary events could not be demonstrated. However, a causal role for CRP in coronary heart disease could not be excluded because of insufficient statistical power.

The CFH Tyr402His polymorphism is associated with increased risk of myocardial infarction, as is the combined presence of unfavorable CRP and CFH genetic profiles, which points towards an interaction between CRP and the alternative complement system in coronary heart disease.

Lp-PLA2 activity is associated with coronary and extra-coronary atherosclerosis, but these associations are not independent of cholesterol.

Both CRP serum level and Lp-PLA2 activity are associated with increased risk of heart failure, which supports the hypothesis that inflammation plays a part in the development of heart failure.

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Chapter 8

Summary - Samenvatting

Summary

Cardiovascular disease remains the leading cause of death in the western world. Of note is that a considerable part of the cases occurs in individuals that have none of the established cardiovascular risk factors. This prompts a search for remaining, unknown risk factors. In the last ten years, it has been recognized that inflammation plays an important role in cardiovascular disease. Insight into the role of inflammatory markers and genes in atherosclerosis may therefore provide a further understanding of the pathophysiology and may aid in identifying individuals at high risk.

In this thesis, we examined the role of emerging inflammatory markers and genes in cardiovascular disease within a population-based cohort setting. Most studies were conducted within the Rotterdam Study, a study among 7983 men and women aged 55 years and over living in a well-defined suburb of Rotterdam, the Netherlands. Inflammatory markers and genetic variations were assessed in the participants and associations with coronary events, coronary calcification, extracoronary atherosclerosis and heart failure were investigated.

Part I focuses on inflammation, atherosclerosis and coronary events. First, the associations between C-reactive protein (CRP) serum level, CRP gene haplotypes and risk of coronary heart disease were examined (**chapter 2.1**). CRP serum level was associated with higher risk of coronary heart disease, and CRP gene haplotypes were associated with CRP serum level. However, CRP gene haplotypes were not associated with coronary heart disease. These findings do not provide evidence for a causal role for CRP in coronary heart disease. In **chapter 2.2** the association of CRP serum level with measures of atherosclerosis was evaluated. An independent, graded association of CRP with extent and progression of carotid plaques and ankle-arm index was found. CRP was independently related to the highest level of carotid intima-media thickness, while the association with change in intima-media thickness was not significant. Although there was an independent, graded relation between CRP and aortic calcification, no independent association was found with progression of aortic calcification, nor with the amount of coronary calcification. In short, graded associations of CRP with extent and progression of atherosclerosis were present, but the strength of the associations depended on the applied measure of atherosclerosis. **Chapter 2.3** addresses the role of complement factor H (CFH) in myocardial infarction. An association was demonstrated between the His allele of the Tyr402His single nucleotide polymorphism of the CFH gene and increased risk of myocardial infarction. This finding underlines the importance of the alternative complement pathway in coronary heart disease. Binding of CRP to CFH has been suggested to augment the ability of CFH to down-regulate the effect of complement in atherosclerotic lesions. In **chapter 2.4**, we demonstrated that the combined presence of unfavorable CRP and CFH genetic profiles is associated with risk of myocardial infarction.

Chapter 3 is a review on the emerging biomarker lipoprotein-associated phospholipase A2 (Lp-PLA2) and cardiovascular disease. It addresses cohort studies that have examined Lp-PLA2 in relation to incident cardiovascular events, studies on Lp-PLA2 and measures of atherosclerosis, and studies on the Lp-PLA2 gene and cardiovascular disease. Overall, these studies suggest an independent, pro-atherogenic role for Lp-PLA2 in cardiovascular disease. Still, several issues remain to be further addressed and these are also discussed. In the Rotterdam Study, an association between Lp-PLA2 activity and coronary calcification was found, but this association was not independent of cholesterol

(**chapter 3.2**). The same was true for the association of Lp-PLA2 activity with extracoronary atherosclerosis, also found in the Rotterdam Study (**chapter 3.3**).

In **chapter 4.1**, we addressed the role of genetic variants altering fibrinogen structure and function, and consequently fibrin structure, in atherogenesis. FGG and FGA fibrinogen haplotypes were not associated with coronary events, coronary calcification or extracoronary atherosclerosis. In **chapter 4.2**, we examined whether baseline levels of heat shock protein 27 (HSP27) are associated with future cardiovascular events among initially healthy individuals, by means of a nested case-control study within the Women's Health Study, a prospective study of initially healthy women. However, no such association could be demonstrated.

Part II focuses on inflammation and heart failure. First, the associations of established cardiovascular risk factors with echocardiographic parameters were examined in asymptomatic persons (**chapter 5.1**). Ventricular systolic and diastolic dysfunction was present in participants that had not been diagnosed with heart failure or myocardial infarction. Higher age, higher BMI, lower systolic and higher diastolic blood pressure were most consistently associated with worse systolic function. Higher age and higher diastolic blood pressure were most consistently associated with lower E/A ratio. In **chapter 5.2**, an association of selected structural and diastolic echocardiographic parameters with all-cause mortality was demonstrated.

Subsequently, the role of inflammation in heart failure was explored. **Chapter 5.3** describes the positive association of CRP serum level with incident heart failure. In men, the association was partly explained by presence of coronary heart disease, whereas in women, it was partly explained by hypertension and body mass index. The reason for this may lie in the fact that men are known to have coronary artery disease as an underlying factor for heart failure more frequently than women, and women are more likely to have hypertension as an underlying factor, and both coronary heart disease and hypertension are associated with CRP. In **chapter 5.4**, an independent association of Lp-PLA2 activity with heart failure was demonstrated. Altogether, these findings support the hypothesis that inflammation plays a role in the development of heart failure.

Finally, in **Part III**, differences in prevalence of atherosclerosis between the genders are illustrated within the Rotterdam Study. We demonstrated that the gender difference in atherosclerosis in the coronary vessels is large, that it is particularly high in younger participants, and that it remains present at older age (**chapter 6.1**). The gender difference in the coronary vessels was found to be strikingly larger than in the other studied vascular beds. The gender difference in carotid atherosclerosis was also substantial, yet smaller, and less consistent. Remarkably, the difference in the aorta and the lower extremity vessels was virtually absent. The difference in gender ratio between sites was not explained by differences in cardiovascular risk factors. These findings underline the need for investigation of the causes of the site-specific gender differences in atherosclerosis, which may shed more light on the gender gap in coronary heart disease.

In **chapter 7**, the general discussion, methodological considerations with regard to the studies in this thesis are described; this includes aspects of observational studies and genetic association studies. Also, the main findings are reviewed in the context of ongoing research. Clinical implications, such as

usefulness of biomarkers for risk stratification, are addressed. Furthermore, future research directions including clinical trials and genome-wide association studies are discussed.

Samenvatting

Hart- en vaatziekten vormen nog steeds de belangrijkste doodsoorzaak in de westerse wereld. Een aanzienlijk deel van de gevallen betreft individuen bij wie geen van de bekende cardiovasculaire risicofactoren aanwezig is. Dit vormt een aanleiding om op zoek te gaan naar nieuwe, onbekende risicofactoren. In de afgelopen tien jaar is onderkend dat ontsteking een belangrijke rol speelt in hart- en vaatziekten. Inzicht in de rol van inflammatoire markers en genen in atherosclerose zou kunnen resulteren in een beter begrip van de pathofysiologie en zou daardoor de identificatie van individuen met een verhoogd risico kunnen vergemakkelijken.

In dit proefschrift hebben we de rol van in opkomst zijnde inflammatoire markers en genen in hart- en vaatziekten bestudeerd in een populatiegebaseerde setting. De meeste studies zijn uitgevoerd binnen het Erasmus Rotterdam Gezondheid Onderzoek (ERGO), een studie in 7983 mannen en vrouwen van 55 jaar en ouder, wonend in de Rotterdamse wijk Ommoord. Inflammatoire markers en genetische varianten zijn bepaald in de deelnemers en hun verband met gevallen van coronaire hartziekte, coronaire calcificatie, extracoronaire atherosclerose en hartfalen is onderzocht.

Deel I is gericht op inflammatie, atherosclerose en coronaire hartziekte. Eerst is het verband tussen C-reactief proteïne (CRP) serum waarden, CRP gen haplotypes en het risico op coronaire hartziekte bestudeerd (**hoofdstuk 2.1**). CRP serum waarden waren geassocieerd met een hoger risico op coronaire hartziekten, en CRP gen haplotypes waren geassocieerd met CRP serum waarden. Echter, CRP gen haplotypes waren niet geassocieerd met coronaire hartziekten. Deze bevindingen ondersteunen een causale rol van CRP in het ontstaan van hart -en vaatziekten niet. In **hoofdstuk 2.2** is het verband tussen CRP serum waarden en maten van atherosclerose onderzocht. Een onafhankelijk en gegradeerd verband is gevonden tussen CRP en mate van ernst en progressie van zowel carotisplaques als enkel-arm index. CRP vertoonde een onafhankelijk verband met de grootste intima-media dikte. De associatie met verandering van intima-media dikte was echter niet significant. Hoewel er onafhankelijke, gegradeerde associatie was tussen CRP en aortacalcificatie, is er geen onafhankelijk verband gevonden met progressie van aortacalcificatie, noch met mate van coronaire calcificatie. Kortom, er waren gegradeerde associaties met mate en progressie van atherosclerose, doch de sterkte van de associaties hing af van de maat van atherosclerose die toegepast werd. **Hoofdstuk 2.3** richt zich op de rol van complement factor H (CFH) in het ontstaan van een myocardinfarct. Een verband tussen het His allel van het Tyr402His genetische polymorfisme van het CFH gen en een verhoogd risico op het optreden van een myocardinfarct werd aangetoond. Deze bevinding onderstreept het belang van de alternatieve complement route bij het optreden van coronaire hartziekte. Mogelijk versterkt binding van CRP aan CFH het vermogen van CFH om het effect van complement in atherosclerotische lesies te verzwakken. In **hoofdstuk 2.4** is aangetoond dat de gecombineerde aanwezigheid van zowel ongunstige genetische CRP varianten als de bovengenoemde ongunstige genetische CFH variant geassocieerd is met een verhoogd risico op het optreden van een myocardinfarct.

Hoofdstuk 3.1 is een overzicht van de literatuur over de in opkomst zijnde biomarker lipoproteïne-gebonden fosfolipase A2 (Lp-PLA2) in relatie tot hart - en vaatziekten. Cohort studies die Lp-PLA2 in relatie tot gevallen van hart - en vaatziekte hebben bestudeerd, worden besproken, net als studies over Lp-PLA2 en maten van atherosclerose, en studies over het Lp-PLA2 gen en cardiovasculaire aan-

doeningen. In het algemeen suggereren deze studies dat Lp-PLA2 een onafhankelijke, pro-atherogene rol speelt in het ontstaan van hart-en vaatziekten. Toch zijn er enkele zaken die verdere aandacht behoeven; deze worden tevens besproken. In het ERGO onderzoek werd een verband gevonden tussen Lp-PLA2 activiteit en coronaire calcificatie (**hoofdstuk 3.2**). Dit verband was echter niet onafhankelijk van cholesterol. Dit gold ook voor het verband tussen Lp-PLA2 activiteit en extracoronaire atherosclerose, tevens aangetoond in het ERGO onderzoek (**hoofdstuk 3.3**).

Varianten in het fibrinogeen gen zijn van invloed op de structuur en functie van fibrinogeen, en derhalve ook op de structuur van fibrine. In **hoofdstuk 4.1** werd onderzocht of deze varianten een rol spelen bij de atherogenese. De resultaten lieten zien dat FGG en FGA fibrinogeen haplotypes niet geassocieerd waren met het optreden van gevallen van coronaire hartziekte, noch met coronaire calcificatie, noch met extracoronaire atherosclerose. In **hoofdstuk 4.2** werd onderzocht of baseline waarden van 'heat shock' eiwit 27 (HSP27) geassocieerd zijn met het toekomstige optreden van gevallen van cardiovasculaire aandoeningen. Dit werd gedaan binnen de 'Women's Health Study', een prospectieve studie in aanvankelijk gezonde vrouwen. Een dergelijk verband kon echter niet worden aangetoond

Deel II is gericht op inflammatie en hartfalen. Eerst werden de verbanden bestudeerd tussen cardiovasculaire risicofactoren en structurele, systolische en diastolische echocardiografische parameters in asymptomatische personen (**hoofdstuk 5.1**). Ventriculaire systolische en diastolische dysfunctie was aanwezig in deelnemers zonder hartfalen of myocardinfarct in hun voorgeschiedenis. Hogere leeftijd, hogere BMI, lagere systolische en hogere diastolische bloeddruk waren meest consistent geassocieerd met een slechtere systolische functie. Hogere leeftijd en hogere diastolische bloeddruk waren meest consistent geassocieerd met een lagere E/A ratio. In **hoofdstuk 5.2** werd een verband aangetoond tussen bepaalde structurele en diastolische echocardiografische parameters en algehele mortaliteit.

Hierna werd de rol van inflammatie in het ontstaan van hartfalen onderzocht. In **hoofdstuk 5.3** werd een positief verband aangetoond tussen CRP serum waarden en incident hartfalen. In mannen werd het verband gedeeltelijk verklaard door de aanwezigheid van coronaire hartziekten, terwijl het in vrouwen gedeeltelijk werd verklaard door aanwezigheid van hypertensie en hogere body mass index. Een mogelijke verklaring hiervoor is dat in mannen, zoals bekend, coronaire hartziekten vaker de onderliggende oorzaak zijn voor hartfalen dan in vrouwen, terwijl in vrouwen, hypertensie vaker de onderliggende oorzaak is, en zowel coronaire hartziekten als hypertensie zijn geassocieerd met hogere CRP waarden. In **hoofdstuk 5.4** werd een onafhankelijk verband tussen Lp-PLA2 activiteit en hartfalen aangetoond. Deze bevindingen ondersteunen de hypothese dat inflammatie een rol speelt bij het ontstaan van hartfalen.

Tenslotte worden in **deel III** verschillen in prevalentie van atherosclerose tussen mannen en vrouwen in het ERGO onderzoek geïllustreerd. We toonden aan dat het geslachtsverschil in de mate van atherosclerose in de coronairvaten groot is, dat het met name indrukwekkend is in jongere deelnemers, en dat het blijft bestaan op oudere leeftijd (**hoofdstuk 6.1**). Het geslachtsverschil was opmerkelijk groter in de coronairvaten dan in de andere vaatbedden. In de carotisvaten was het verschil tevens substantieel, echter duidelijk kleiner en minder consistent. Opmerkelijk was, dat het verschil in de aorta en

de vaten van de onderste extremiteit vrijwel afwezig bleek. Het verschil in de geslachtsratio tussen de locaties werd niet verklaard door verschillen in cardiovasculaire risicofactoren. Deze bevindingen onderstrepen het belang van onderzoek naar de oorzaken van de locatiegebonden geslachtsverschillen in atherosclerose. Deze oorzaken zouden meer inzicht kunnen verschaffen in het geslachtsverschil in het optreden van coronaire hartziekten.

In **hoofdstuk 7**, de algemene discussie, worden methodologische overwegingen met betrekking tot de studies in dit proefschrift beschreven, zowel wat betreft observationele studies als genetische associatie studies. Ook worden de belangrijkste bevindingen in het perspectief geplaatst van huidige inzichten. Klinische implicaties, zoals het nut van biomarkers voor risicostratificatie, worden besproken. Daarnaast wordt de richting van toekomstig onderzoek, waaronder klinische trials en genoomwijde associatie studies, bediscussieerd.

Dankwoord

Graag wil ik op deze plaats iedereen bedanken die heeft bijgedragen aan de totstandkoming van dit proefschrift, met op de eerste plaats de duizenden deelnemers aan het ERGO-onderzoek, die zich jarenlang hebben laten ondervragen en onderzoeken, en de huisartsen en apothekers die de data beschikbaar hebben gesteld. Hun bereidwilligheid was onmisbaar voor dit onderzoek.

Een aantal mensen wil ik persoonlijk bedanken voor hun bijdrage:

Mijn promotoren, Prof.dr. Jacqueline C.M. Witteman en Prof.dr. Albert Hofman, en mijn co-promotor, Dr. Moniek P.M. de Maat. Beste Jacqueline, bedankt dat je mij de kans hebt gegeven om mijn promotie-onderzoek te verrichten bij jouw onderzoeksgroep, en dat je me de gelegenheid hebt gegeven om aan meerdere projecten te werken. Dit zorgde voor afwisseling en voor uitdaging. Ik heb veel geleerd van jouw inzicht en kritische blik. Het is een eer om bij de eersten te behoren die jou als promotor mogen hebben. Beste Bert, mijn kennismaking met de epidemiologie was reeds in het tweede jaar van de geneeskunde studie, toen je mij toeliet tot de Master of Science opleiding in de Klinische Epidemiologie. Ik heb zeer veel aan je te danken als hoofd van de afdeling Epidemiologie en initiator van het ERGO-onderzoek. Bovendien was jouw enthousiasme voor het vak erg motiverend. Beste Moniek, hartelijk dank voor de begeleiding bij de SNP bepalingen en de bijbehorende papers, en voor de frequente discussies. Je was altijd bereikbaar en las de stukken in recordtijd. Jouw bijdrage aan dit proefschrift was essentieel.

Prof.dr. Dirk-Jan G.M. Duncker, Prof.dr. C. Erik Hack en Prof.dr. Maarten L. Simoons wil ik bedanken voor hun bereidheid om zitting te nemen in de kleine commissie en voor de inhoudelijke beoordeling van dit proefschrift. Prof.dr. Maarten L. Simoons wil ik tevens bedanken voor het in mij gestelde vertrouwen door mij toe te laten tot de opleiding cardiologie. Dr. Folkert J. ten Cate, Dr. Jaap W. Deckers en Prof.dr. Pieter H. Reitsma wil ik bedanken voor hun bereidheid om zitting te nemen in de grote commissie.

De co-auteurs van mijn manuscripten wil ik bedanken voor hun intellectuele bijdrage en voor hun waardevolle commentaar. Een speciaal woord van dank gaat uit naar Dr. André G. Uitterlinden, voor het gebruik van zijn genetische laboratorium en de leerzame discussies, naar Prof.dr. Bruno H. Ch. Stricker, voor zijn betrokkenheid bij de hartfalen-stukken, naar Dr. Jaap W. Deckers voor de supervisie van de codering van de events, en naar Dr. Caroline C.W. Klaver, Prof.dr. Paulus T.V.M. de Jong en Prof. dr. Arthur A. Bergen voor de samenwerking betreft CFH. Suzette Elias wil ik bedanken voor haar werk aan de CRP-atherosclerose paper. Laura van Vark voor haar werk aan de Lp-PLA2-hartfalen paper. Voorts een woord van dank voor Irene van der Meer, Hok-Hay Oei en Rozemarijn Vliegenthart, die ik reeds leerde kennen tijdens mijn MSc tijd, en die nu mijn co-auteurs zijn.

Prof. Paul M. Ridker, thank you very much for giving me the opportunity to visit your research group at Brigham and Women's Hospital. This has been an important scientific learning experience to me. I have enjoyed working on the HSP27 paper and I am very glad that it is a part of this thesis.

Dr. Jeanenne J. Nelson, thank you for your valuable input and for the pleasant collaboration on Lp-PLA2.

Mijn kamergenoten Abbas, Annette, David, Inge, Laura van Vark, Milad, Mohammed, Saskia, en Toos, en mijn collega's van de hart- en vaatgroep Annemarieke, Arlette, Charlotte, Francesco, Frank, Germaine, Jan, Karen, Mark, Susanne, Suzette en Quirijn, voor de interessante discussies en prettige samenwerking, en natuurlijk voor de gezelligheid.

De vele collega's van de afdeling epidemiologie die ik in de afgelopen drie jaren heb gekend, Aaron, Albert-Jan, Alejandro, Angela, Annelous, Arfan, Christiane, Claire, Cornelis, Dika, Dominiek, Elizabeth, Fakhredin, Fernando, Frank-Jan, Jan-Jaap, Julia, Kamran, Laura de Vries, Lintje, Lonneke, Marieke van Oijen, Marieke Dekker, Meike, Mariëtte, Matthijs, Martina, Mendel, Michiel, Miranda, Mojgan, Monika, Monique, Nahid, Sabine, Sharmila, Simone, Sjoerd, Vishal, Yannick, en Ylian, voor het delen van alle epi-perikelen, de goede werksfeer en de gezellige lunches en borrels.

Van de afdeling Hematologie: Lamberto Felida, voor zijn hulp bij de genotyperingen en Goran Rudez, voor mijn "inwijding" in de genetische databases. Van de afdeling Interne Geneeskunde: Lisette Stolk, Pascal Arp, en Mila Jhamai voor advies en tips bij het genotyperen. Jeannette Vergeer van het laboratorium van de Epidemiologie voor het beantwoorden van mijn lab-vragen. Joep Kurstjens van de Klinische Chemie voor de CRP bepalingen.

Prof.dr. T. Stijnen en de collega's van de biostatistiek (Lydia Arends, Bettina Hansen, Paul Mulder, Maria de Ridder, en Maarten Schipper), voor de hulp bij de statistische problemen.

De dames van het ERGO-centrum, onder leiding van Anneke Korving, en de ERGO fup-sters, voor de inzet bij het verwerven van de gegevens en voor de gezellige dagen op het ERGO-centrum. In het bijzonder Toos Stehmann, Inge Haumersen en Saskia Hoornweg, voor het verzamelen van de velerlei cardiovasculaire data.

De dames van het secretariaat (Kabita Asray, Esther Bruining, Cerisette van Houwelingen, Marjolijn Kasi, Petra Rikxoort, Marti von Stein), voor de ondersteuning in de afgelopen jaren.

De heren van het datamanagement en de automatisering (Alwin Koedoot, René Molhoek, Eric Neeleman, Marcel Rond, Frank van Rooij, Nano Suwarno, René Vemeeren), voor de technische ondersteuning. Nano: super dat je altijd voor me klaarstond bij een figure-emergency! Jolande Verkroost en Anneke de Koning, voor de hulp bij het verwerken van de events en de overige zaken.

Boston 2002: een leerzame en tevens onvergetelijke ervaring als voorbode van dit proefschrift. Meiden en Michiel, bedankt!

Mijn paranimfen, Renata Schouw en Abbas Dehghan. Lieve Renata, sinds de eerste geneeskunde colleges zijn we vriendinnen. Je systematische aanpak, zowel qua carrière als qua voorraadkast, en je doorzettingsvermogen heb ik altijd bewonderd. Ik ben erg blij dat je naast me staat. Dear Abbas, for almost three years we have shared a room at the department. In these years I have gotten to appreciate your excellent intellectual abilities, your subtle but exhilarating sense of humour, and above all your understanding and kind personality. Thank you for everything!

Velen verdienen dank voor hun indirecte bijdrage en voor de nodige afleiding naast het promotieonderzoek. Mijn (schoon)familie wil ik bedanken voor de nimmer aflatende interesse in mijn werkzaamheden en voor de leuke zondagen en uitstapjes. Mijn vriendinnen voor hun interesse en goede raad, en tevens de etentjes, shopping, vakanties, sportieve activiteiten en alle andere dingen naast het werk.

Mijn ouders: Lieve Mama, je hebt altijd voor me klaargestaan en alles voor me overgehad. Jouw kracht is voor mij een grote bron van inspiratie, en jouw steun en stimulans waren onontbeerlijk om te komen waar ik nu ben. Lieve Papa, jij hebt me altijd gestimuleerd om me te ontwikkelen, en de tijd genomen om mij vele dingen te leren. Bedankt voor alles.

Lieve Hans, er is er maar één zoals jij…Door jouw bemoedigende woorden en je overtuiging van mijn kunnen leek alles een stuk makkelijker. Bedankt voor je liefde, vriendschap en geduld. Het leven met jou is mooi!

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Isabella Kardys was born on 14 May 1979 in Poznań, Poland. In 1997 she graduated from the 'Sint-Montfortcollege' (presently 'NOVA college-Montfort'), Rotterdam. Hereafter she studied medicine at the 'Rijksuniversitair Centrum Antwerpen', Antwerp, Belgium, for one year, obtaining the 'First Candidature'. In 1998 she started medical school at Erasmus University Rotterdam. During medical school, she participated in an elective in oncologic surgery in Heraklion, Greece, for six weeks, and she completed the Master of Science program in Clinical Epidemiology at the Netherlands Institute for Health Sciences. As part of this program, she studied for four weeks at the Harvard School of Public Heath, Boston, USA. From January 2002 until June 2002 she participated in research on the predictive value of the spatial QRS-T angle for coronary heart disease at the Department of Epidemiology & Biostatistics (head: Prof.dr. A. Hofman). After obtaining her medical degree in August 2004, she started working on this thesis in September 2004 within the Cardiovascular Epidemiology group (Prof.dr. J.C.M. Witteman) of the same department. From September 2006 to December 2006, she visited Boston once again to work as a research fellow at the Center for Cardiovascular Disease Prevention (director: Prof. P.M. Ridker), Brigham and Women's Hospital (Harvard Medical School). In October 2007, she started working as a resident at the department of Internal Medicine of 'Medisch Centrum Rijnmond-Zuid', Rotterdam (head: Dr. A. Berghout) as part of her Cardiology training. She will continue her training in cardiology at the Department of Cardiology of Erasmus MC, Rotterdam (head: Prof.dr. M.L. Simoons).