Genetic Determinants of Arterial Stiffness

Results from the Rotterdam Study

M.P.S. Sie

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Results from the Rotterdam Study

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Resultaten van het ERGO Onderzoek

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Chapter 2.1 Sie MP, Sayed-Tabatabaei FA, Oei HH, Uitterlinden AG, Pols HA, Hofman A, van Duijn CM, Witteman JC. Interleukin 6 -174 g/c promoter polymorphism and risk of coronary heart disease: results from the Rotterdam study and a meta-analysis. *Arterioscler Thromb Vasc Biol.* 2006;26:212-217.

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Chapter 3.3 Sie MP, Mattace-Raso FU, Uitterlinden AG, Arp PP, Hofman A, Hoeks AP, Reneman RS, Asmar R, van Duijn CM, Witteman JC. TGF-β1 polymorphisms and arterial stiffness; the Rotterdam Study. *J Hum Hypertens*, 2007 Mar 15, Epub ahead of print.

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Chapter 4.1. Sie MP, Yazdanpanah M, Mattace Raso FU, Uitterlinden AG, Hofman A, Hoeks AP, Reneman RS, Asmar R, Witteman JC, van Duijn CM. Genetic variation in the renin-angiotensin system and arterial stiffness; the Rotterdam Study (submitted).

Chapter 4.2 Mattace- Raso FU, Sie MP, van der Cammen TJ, Safar ME, Hofman A, van Duijn CM, Witteman JC. Insertion/Deletion gene polymorphism of the angiotensin-converting enzyme and blood pressure changes in older adults. The Rotterdam Study. *J Hum Hypertens*., accepted for publication.

Chapter 4.3 Sie MP, van Rooij FJ, de Maat MP, Isaacs A, Mattace-Raso FU, Uitterlinden AG, Hofman A, Hoeks AP, Reneman RS, Asmar R, van Duijn CM, Witteman JC. Genetic Variation in the matrix metallo-proteinase 3 gene in relation to arterial stiffness: the Rotterdam Study (submitted).

CONTENTS

Chapter 1
Introduction

INTRODUCTION

Cardiovascular disease is, and will remain so for many years to come, one of the leading causes or morbidity and mortality, especially in the Western World.[1] Improving and expanding insight into the pathogenesis of cardiovascular disease is an important part of managing, treating and preventing cardiovascular disease.

The cardiovascular system can be described as a closed conduit with a central pump with the principal function of supplying blood to all parts of the body. However simple, this description and nomenclature also indicate those components of the system, in which problems can arise: the blood vessels and the heart. Apart from delivery of blood throughout the body, the arterial system decreases the pulsatility of the flow, generated by the heart, resulting in a continuous flow at the level of the capillaries, so that a constant amount of blood is delivered to tissues and organs. A very important function of the (elastic) arteries is to store volume energy in order to limit the rise in the systolic blood pressure. This is achieved by the viscoelastic properties of the arterial wall. Key structural elements, that play a large role in this process, are innate structural components of the arterial wall, such as collagen and elastin. Throughout life physiological changes in the arterial wall result in stiffening of the vessel walls, which is more pronounced in the central (mostly elastic) arteries, compared to distal, more muscular, arteries.[2,3]

Increased arterial stiffness has been associated with hypertension, diabetes mellitus, renal disease and atherosclerosis and, by now, it has also been established as an independent risk factor for cardiovascular disease.[4-13] Arterial stiffness increases with age, independently of the presence of cardiovascular risk factors. Traditionally it was considered a physiological part of ageing, but the increase in stiffness may depend on several factors, environmental as well as genetic.[8] Indeed, heritability estimates, mostly ranging from 0.30-0.40, were found for arterial stiffness.[14] Insight into the pathogenesis of arterial stiffness may offer possibilities for further research into treatment and prevention or cardiovascular disease and its sequelae.

For long, non-invasive techniques to adequately measure arterial stiffness were not available, making large-scale research not feasible. However, nowadays stiffness can be measured with various non-invasive techniques, such as pulse wave velocity measurements for aortic stiffness, the distensibility coefficient determination of the carotid arteries and pulse pressure, as a more general measure of stiffness. Other currently used measures of stiffness include central pulse wave analyses, such as the Augmentation Index (AIx), and local indices as the carotid Young´s modulus.[15,16] These new techniques facilitate more extensive and in depth study into determinants of arterial stiffness.

The aim of the studies, described in this thesis is to examine the influence of variation in genes that may affect arterial stiffness and cardiovascular events.

The first part of this thesis focuses on genetic variation in pleiotropic genes that influence inflammation and cardiovascular events. Chapter 2.1 focuses on the relation of the interleukin 6 -174 G/C promoter polymorphism and coronary heart disease. In chapter 2.2 the study is described on the relation between polymorphisms in the transforming growth factor β1 gene and risk of myocardial infarction and stroke. The second part of this thesis focuses on genetic variation and the relation with arterial stiffness. First inflammatory genes are considered. In chapter 3.1 the association between the interleukin 6 -174 G/C promotor polymorphism, levels of interleukin 6 and C-reactive protein and arterial stiffness is described. In chapter 3.2 the association between several polymorphisms in the C-reactive protein and arterial stiffness is set out. Chapter 3.3 focuses on polymorphisms in the transforming growth factor β1 gene in relation to arterial stiffness. Chapter 3.4 describes the relation between genetic variation in the fibrinogen α and γ gene, fibrinogen levels and arterial stiffness. Also non-inflammatory genes that influence the structure of the vascular wall are considered. In chapter 4.1 the association between polymorphisms in the renin-angiotensin system genes and arterial stiffness is described. Chapter 4.2 aims to describe the relation between the angiotensin converting enzyme insertion/deletion polymorphism and blood pressure changes. Chapter 4.3 focuses on a study of polymorphisms in the matrix-metalloproteinase 3 gene and arterial stiffness. Finally, the main findings of this thesis and suggestions for future research are discussed.

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Chapter 2
Genetic Determinants of Cardiovascular Disease

Chapter 2.1

Interleukin 6 -174 G/C Promoter Polymorphism and Risk of Coronary Heart Disease

Background and purpose - Inflammation plays a pivotal role in the pathogenesis of atherosclerosis. Interleukin 6 (IL-6) has many inflammatory functions and the IL-6 -174 G/C promoter polymorphism appears to influence IL-6 levels. Findings of previous studies on the relation between this polymorphism and risk of cardiovascular diseases are inconsistent. We investigated this polymorphism in relation to risk of coronary heart disease (CHD) in a population-based study and meta-analysis.

Methods - 6434 participants of the Rotterdam Study were genotyped. Analyses on the relation between genotype and CHD were performed using Cox proportional hazards analyses, and the association between genotype and plasma levels of IL-6 and CRP was investigated. All analyses were adjusted for age, sex and common cardiovascular risk factors. A meta-analysis was performed, using a random effects model.

Results - No association between genotype and risk of CHD was observed. The polymorphism was not associated with IL-6 levels, but the C-allele was associated with higher CRP levels ($p < 0.01$). Our meta-analysis did not show a significant association between the genotype and risk of CHD.

Conclusions - We conclude that the polymorphism is not a suitable genetic marker for increased risk of CHD in subjects aged 55 years and older.

INTRODUCTION

Inflammatory processes play a pivotal role in the pathogenesis of atherosclerosis.[1-4] Within the inflammatory pathway, cytokines fulfill a multitude of functions. Interleukin 6 (IL-6) is a pleiotropic inflammatory cytokine. It plays an important part in the acute-phase response and inflammatory cascade, such as up-regulation of acute-phase proteins as C-reactive protein (CRP).[5,6] CRP levels have been found to be associated with risk of coronary heart disease (CHD).[7,8] Several studies have also shown an association between IL-6 plasma levels and cardiovascular pathology.[9-17] Ridker et al. found elevated IL-6 levels to be associated with increased risk of myocardial infarction (MI).[12] This finding was replicated in another large population by Bennet et al., who found an increased risk of MI for those in the upper quartile levels of IL-6 versus those in the lowest quartile levels, and by Cesari et al., when comparing subjects with highest and lowest IL-6 tertiles in an American population.[10,15] Fishman et al. detected a functional polymorphism (G->C) in the promoter region of the human IL-6 gene (174 base pairs upstream from the start site).[18] This polymorphism appears to influence the transcription of the IL-6 gene and also plasma levels of IL-6, and IL-6 is therefore a candidate gene for further study into its role in cardiovascular disease.[18] However, results from previous studies on the -174 G/C polymorphism and CHD were inconsistent.[15,19-26] Five studies, most of which case-control studies, conducted in Western, mainly Caucasian, populations, found the C-allele to be associated with (an increased risk of) CHD or cardiovascular disease.[21-24,26] Four other studies, however, did not find a significant association.[15,19,20,25] We studied the IL-6 -174 G/C polymorphism in relation to risk of CHD in a large population-based study. Also a meta-analysis was conducted including studies on this polymorphism and CHD.

METHODS

Study Population

The Rotterdam Study is an ongoing prospective cohort study including 7983 participants of 55 years and older. Its general aims are to investigate determinants of chronic diseases.[27] During the first phase of this study (1990-1993), all inhabitants of a Rotterdam suburban area (Ommoord) aged 55 years and over, were invited to participate in this study. The response rate was 78%. Baseline investigations included an interview and visits to the research center, where a number of clinical measurements were performed. Approval of the Medical Ethics Committee of the Erasmus University Rotterdam was obtained for the Rotterdam Study. From all participants written informed consent was acquired. A more in depth description of the Rotterdam Study was published previously.[27]

Clinical Characteristics

Trained investigators collected information using a computerized questionnaire during the home visits. The information included current health status, medical history, use of medication and smoking behavior. During the two subsequent visits to the center, blood samples were obtained and established cardiovascular risk factors were measured as described elsewhere.[28] Hypertension was defined as a systolic blood pressure of 160 mmHg and over, and/or a diastolic blood pressure of 100 mmHg and over, and/or use of antihypertensive medication (with indication hypertension). Diabetes mellitus (DM) was defined as a non-fasting serum glucose level of 11.1 mmol/l and over, and/or use of antidiabetic medication. A 12-lead electrocardiogram was recorded and analyzed by the Modular ECG Analysis System (MEANS).[29] Evaluation of the atherosclerotic status of participants was accomplished using ultrasonography (carotid arteries), radiographic detection (aorta), ankle-arm index (AAI) (via blood pressure measurements); these methods have been extensively described previously.[30]

Measurement of IL-6 and CRP Plasma Levels

A venapuncture was performed by application of minimal stasis with a 21-gauge Butterfly needle with tube (Surflo winged infusion set, Terumo). Non-fasting blood was collected in tubes containing 0.129 mol/L sodium citrate at 4°C. The ratio of blood to sodium citrate was 9:1. Plasma was collected after centrifugation for 10 minutes at 3000 rpm. Subsequently, platelet-free plasma was obtained by centrifugation for 10 minutes at 10000 rpm, immediately frozen in liquid nitrogen, and stored at -20 degrees centigrade. All tubes were stored on ice before and after blood sampling. Plasma levels of IL-6 were measured by using a commercially available enzyme-linked immunosorbent assay (Quantikine HS from R&D Systems Europe, Oxon, United Kingdom). CRP plasma levels were measured in samples stored at -20 degrees centigrade, by sensitive immunological methods based on rate near infrared particle immunoassays (IMMAGE from Beckman Coulter Netherlands).

Genotyping

Genotyping of the IL-6 -174 G/C polymorphism was performed using samples stored earlier at -80 degrees centigrade. DNA was isolated using standard procedures. 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 in 2 µl reaction volume.

Follow-up Procedures and Definition of Events

General practitioners (GPs) in the research district, with whom 85% of the participants of the Rotterdam Study were enlisted, reported fatal and non-fatal cardiovascular events. Research assistants verified all information by checking medical records at the GPs' offices. All medical records of the participants under the care of general practitioners outside the study area 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, the cause and circumstances of death were established by a questionnaire from the GPs. Two research physicians independently coded all reported events according to the International Classification of Diseases, 10th edition (ICD-10).[31] Codes on which the research physicians disagreed were discussed in order to reach consensus. Finally, a medical expert in cardiovascular disease, whose judgement was considered final, reviewed all events. CHD was defined (based upon ICD-10) as the occurrence of an MI (I21), revascularization procedure (percutaneous transluminal coronary angioplasty or coronary artery bypass graft) (Z95.5 & Z95.1), ischaemic heart disease (I20, I22-25), sudden cardiac death (I46), ventricular fibrillation or tachycardia (I49), congestive heart failure (I50) or sudden death undefined (R96) during follow-up. Sudden death was defined as death occurring instantaneously or within 1 hour after onset of symptoms. Incident MI was defined as the occurrence of a fatal or non-fatal MI after the baseline examination.

Meta-analysis

For the meta-analysis, published data were used from previously published studies, concerning the IL-6 -174 G/C polymorphism and CHD, until December 2004. In addition our own data were also included. Studies were found with Pubmed/Medline using key words: interleukin 6, -174, polymorphism, cardiovascular disease, myocardial infarction, coronary heart disease, and using references from retrieved articles. Eight studies were identified, of which seven were included in the analysis.[15,19-25] One study was excluded because no genotype frequencies were available specified for cases and controls.[23] All studies were conducted in European populations. As endpoint in the analyses, we used CHD, defined as the occurrence of an MI, revascularization procedure (percutaneous transluminal coronary angioplasty or coronary artery bypass graft), ischaemic heart disease, (sudden) cardiac death, ventricular fibrillation or tachycardia, sudden death undefined, or congestive heart failure.

Statistical Analyses

Baseline characteristics were tested for differences between the genotypes using ANOVA analyses for continuous variables (age, BMI [body mass index], SBP [systolic blood pressure], cholesterol levels, IL-6 plasma levels, CRP plasma levels) and Pearson chi-square tests for discrete variables (sex, smoking, diabetic status, history of MI). All values above mean plus

three times the standard deviation were excluded, as correction for outliers. Natural-log transformed (ln-transformation) values of IL-6 and CRP plasma levels were used to normalize the distribution of these variables; presented data are back-transformed. Cox proportional hazards analyses were performed in order to obtain relative risks. All analyses were adjusted for age and sex, and additionally for BMI, SBP, high-density lipoprotein (HDL-) and total cholesterol levels, baseline smoking and DM. Subjects with prevalent MI were excluded from the analyses. Additional analyses were performed in various age strata (10 year strata starting from 55 years, and above and below 75 years), in strata of sex, smoking, and DM, and in various strata of atherosclerosis (tertiles of aorta calcification and carotid plaques, and AAI above and below 0.9). A p-value of 0.05 and smaller was considered significant in all analyses. The statistical analyses were performed using SPSS 11 and S-Plus 6.0 for MS-Windows.

For the meta-analysis the method of moments has been used to calculate the relative risks in a random-effects model for the pooled data.[32] We used the funnel plots to examine publication bias of reported associations. The meta-analysis and heterogeneity analysis were performed using Review Manager 4.2.7 (RevMan Analyses version 1.0; Cochrane Collaboration - Wintertree Software Inc.).

RESULTS

Rotterdam Study Results

Genotyping of the IL-6 -174 G/C polymorphism was successfully performed in 6434 subjects. The main characteristics of the study population are summarized in Table 1 (2612 male and 3822 female). Mean age was 69.5 (± 9.1) years. 2301 participants (36%) had the GG-genotype (wildtype), 3050 (47%) had the GC-genotype and 1083 (17%) had the CC-genotype. Genotype and allele proportions were in Hardy Weinberg equilibrium ($p = 0.18$).

IL-6 plasma levels were determined in a random subgroup of 641 subjects, after exclusion of outliers (2%). No significant difference in mean IL-6 plasma levels between the genotypes was observed (Figure 1). CRP plasma levels were successfully determined in 5924 cases, after exclusion of outliers (1%). The level of CRP was significantly higher in CC ($p < 0.01$) and GC ($p < 0.01$) individuals as compared to the individuals with the wildtype genotype GG (Figure 1). The C-allele was significantly associated with higher CRP plasma levels ($p < 0.01$).

During a mean follow-up period of 6.8 (± 2.3) years, there were 648 cases of CHD and 280 cases of MI. After exclusion of patients with previous MI, there were 463 newly diagnosed cases of CHD and 208 cases of incident MI.

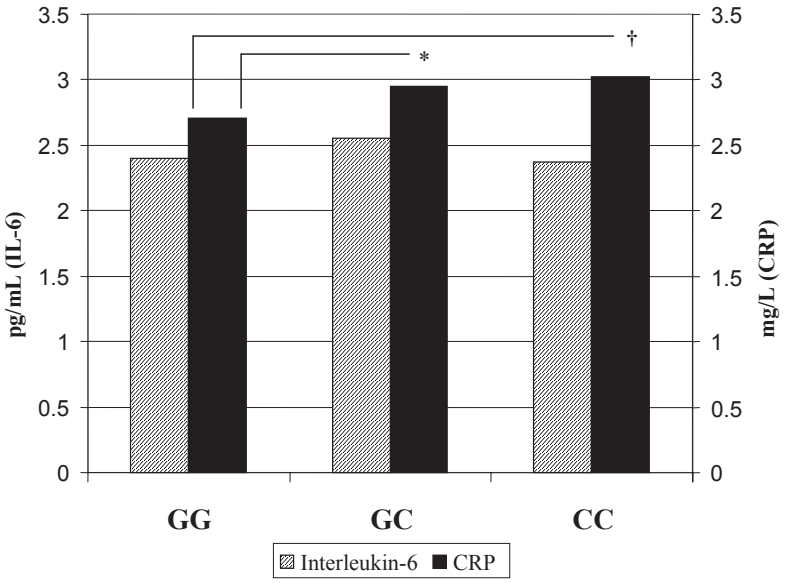
There was no significant difference in relative risk of CHD or MI when comparing the GC- or CC-genotypes and C-allele carriership with the -174 GG wildtype genotype, although there appeared to be a slightly, but not significantly, increased risk for the subjects with the GC-

Table 1. Baseline Characteristics

Baseline Characteristics				
Characteristic	Overall	IL-6 –174 Genotype		
		GG	GC	CC
Number (%)	6434 (100)	2301 (36)	3050 (47)	1083 (17)
Age – yrs	69.5 ± 9.1	69.2 ± 9.0	69.5 ± 9.1	69.9 ± 9.3
Male sex – number (%)	2612 (41)	958 (42)	1234 (41)	420 (39)
BMI – kg/m^2	26.3 ± 3.7	26.1 ± 3.7	26.4 ± 3.8	26.2 ± 3.6*
SBP – mm Hg	139 ± 22.2	139 ± 22.3	139 ± 22.4	140 ± 21.6
Total cholesterol – mmol/l	6.6 ± 1.2	6.6 ± 1.2	6.6 ± 1.2	6.6 ± 1.2
HDL-cholesterol – mmol/l	1.3 ± 0.4	1.4 ± 0.4	1.3 ± 0.4	1.3 ± 0.4
Smoking – number (%)				
- Current	1423 (21)	506 (22)	696 (23)	221 (20)
- Former	2618 (41)	950 (41)	1223 (40)	445 (41)
- Never	2214 (34)	781 (34)	1044 (34)	389 (36)
Diabetes – number (%)	657 (10)	222 (10)	333 (11)	102 (9)
History of MI (%)	750 (12)	255 (11)	377 (12)	118 (11)

BMI = body mass index
SBP = systolic blood pressure
HDL = high density lipoprotein
MI = myocardial infarction
Continuous values are depicted as mean ± SD
* = GC different from GG (p = 0.04)

Figure 1. Plasma Levels of IL-6 and CRP per Genotype



* Significant difference from GG (p < 0.01)
† Significant difference from GG (p < 0.01)

Table 2. Risk of CHD and MI by IL-6 Genotype

		CHD				MI			
		Events/ total	RR	95% CI	p	Events/ total	RR	95% CI	p
Males	GG*	84/801	1	-	-	44/801	1	-	-
	GC	116/1019	1.10	0.83-1.46	0.49	54/1019	0.96	0.65-1.44	0.86
	CC	43/352	1.19	0.83-1.72	0.35	21/352	1.10	0.66-1.86	0.71
	Carrier	159/1371	1.13	0.86-1.47	0.38	75/1371	1.00	0.69-1.45	1.00
Females	GG*	74/1245	1	-	-	29/1245	1	-	-
	GC	106/1654	1.05	0.78-1.41	0.76	43/1654	1.09	0.68-1.75	0.71
	CC	40/613	1.02	0.70-1.50	0.92	17/613	1.14	0.63-2.08	0.67
	Carrier	146/2267	1.04	0.79-1.38	0.78	60/2267	1.11	0.71-1.72	0.66

* = Reference genotype
CI = Confidence interval
CHD = coronary heart disease
MI = myocardial infarction
Data are age adjusted

and CC-genotype (Table 2). Adjusting for age, BMI, SBP, total and HDL-cholesterol, smoking and DM did not alter these results.

No difference in survival and event-free survival was found between the genotypes CC, GC and GG for CHD in men (p = 0.36) or women (p = 0.79). There was also no difference in survival between the genotypes for MI in men (p = 0.81) or women (p = 0.63). Further adjustment for covariates did not alter these findings.

No significant associations between the -174 genotype and risk of CHD or MI were observed in strata of age, smoking, diabetic status and levels of atherosclerosis (data not shown).

Figure 3. Meta-analysis Funnel Plot

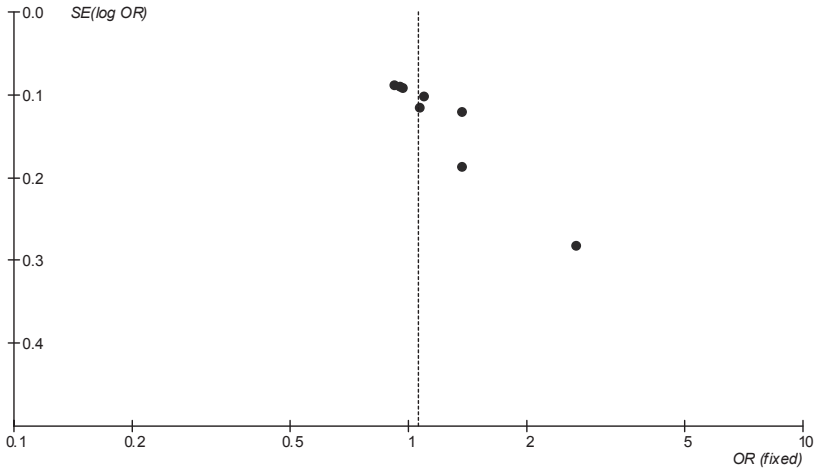
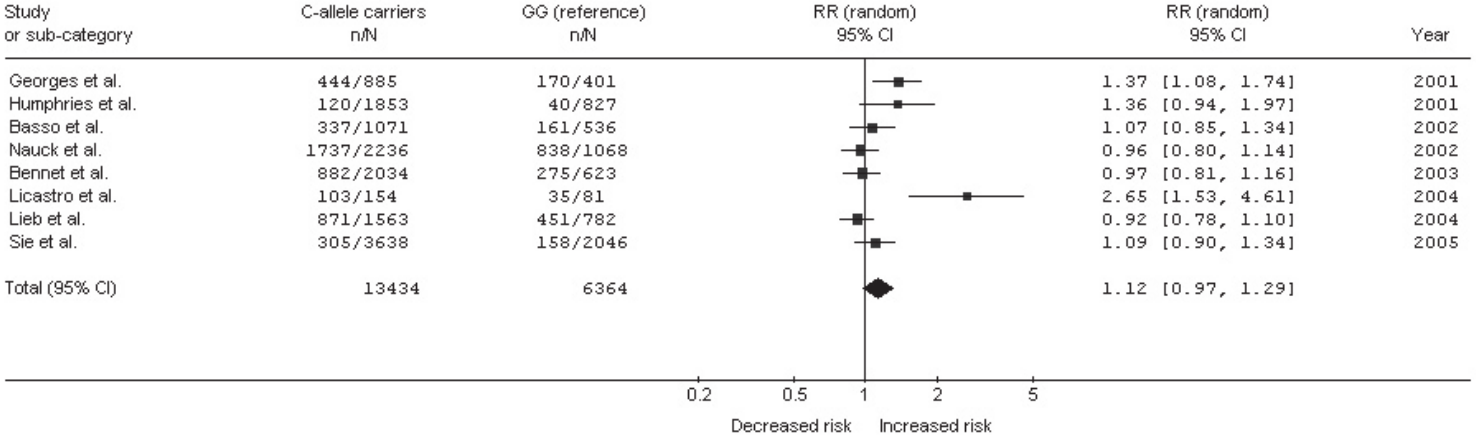


Figure 2. Meta-Analysis of CHD Risk for Carriers of the C-allele versus the GG Genotype



Meta-Analysis Results

In the meta-analysis were a total of 13434 C-allele carriers of whom 36% (n = 4799) were CHD cases; and 6364 subjects with the wildtype GG genotype of whom 33% (n = 2128) were CHD cases.

The meta-analysis did not show a significant association between the polymorphism and CHD. Subjects with the CC-genotype compared to individuals with the GG-genotype had a relative risk of 1.03 (0.92-1.16) (p = 0.59). Also when carriers of the C-allele were compared to the GG-genotype, there was no significant difference in risk, 1.12 (0.97-1.29) (p = 0.12) (Figure 2). There was evidence for heterogeneity (p = 0.003). This was caused by the study of Licastro et al., which was an outlier with a very high odds ratio (Figures 2 and 3).[24] We did not exclude this study, as the exclusion would only shift the non-significant results further towards the null value, and would not lead to a significant difference in the overall outcome.

DISCUSSION

In this prospective population-based study no significant association between the IL-6 -174 G/C genotype and CHD or MI was found, nor was there an association with plasma levels of IL-6. A higher CRP plasma level was found among subjects with the C-allele. Adjusting for possible confounders and analyses in various substrata, did not alter these findings. A meta-analysis, including our own study, showed no significant association between the genotype and risk of CHD.

Our study is based on a large ongoing population based study in a relatively homogeneous population, as 98% of the participants in our study are Caucasians and are all living in the same area, a suburb of Rotterdam. In contrast to case control studies, the prospective nature of our study makes our results less prone to survival bias. We adjusted all analyses for established risk factors, but this did not change the estimates.

Results from previous studies on this IL-6 -174 G/C polymorphism and CHD were inconsistent (Table 3).[15,19-26] In the LURIC cohort and several other studies no association between this polymorphism and CHD was found.[15,19,20,25] Other studies as the ECTIM Study and a study based on the CHS cohort, found a higher risk of CHD associated with the C-allele.[21-24,26] All studies on IL-6 genotype and CHD were performed in Western populations with predominantly male subjects with an average age of 50 years and over. It is therefore unlikely that differences in findings are due to ethnicity, gender or age differences between the studies.

In order to provide a better overview of (inconsistent) findings of various studies, we also studied the association between the IL-6 genotype and risk of CHD by performing a meta-analysis, which made it possible to study a very large number of events. This meta-analysis also did not show an association between the genotype and risk of CHD. Publication bias is always an important potential source of bias in meta-analyses. However, studies have been

Table 3. Overview of Studies on the IL-6 –174 G/C Polymorphism and Risk of Coronary Heart Disease

Study	**Population**		RR		
			GG	GC	CC
Georges et al., 2001 (ref. 22)	Cases: Controls: Population: Age:	640 719 UK, France – males 25-64 yrs	1.0 *	1.35 † (1.05-1.73)	1.31 (0.94-1.84)
Humphries et al., 2001 (ref. 21)	Follow-up: Population: Age (mean):	2751 (2560 cases and 160 controls) UK – males 57 yrs (cases) / 56 yrs (controls)	1.0 *	1.54 † (1.06-2.22)	1.11 (0.67-1.83)
Basso et al., 2002 (ref. 20)	Cases: Controls: Population: Age (mean):	498 1109 Scotland – males 56 yrs (cases & controls)	1.0 *	1.07 (0.77-1.48)	1.26 (0.83-1.91)
Nauck et al., 2002. (ref. 19)	Cases: Controls: Population: Age (mean):	2559 729 Germany – males & females 64 yrs (cases) / 58 yrs (controls)	1.0 *	0.98 (0.79-1.16)	0.95 (0.75-1.20)
Jenny et al., 2002 (ref. 23)	Cases: Controls: Population: Age (mean):	770 + 250 500 USA (Caucasians + African) – males & females 73 yrs	1.0 *	1.50 † (1.05-2.14)	
Bennet et al., 2003 (ref. 15)	Cases: Controls: Population: Age (mean):	1179 1528 Sweden – males & females 59 yrs (males, cases & controls) 63 yrs (females, cases & controls)	1.0 * 1.0 *	- -	1.1 ‡ (0.8-1.4) 0.8 § (0.6-1.3)
Licastro et al., 2004 (ref. 24)	Cases: Controls: Population: Age (mean):	139 198 Italy - males 65 yrs (cases) / 57 yrs (controls)	1.0 *	2.65 † (1.53-4.62)	
Lieb et al., 2004 (ref. 25)	Cases: Controls: Population: Age (mean):	1322 1023 Germany – males & females 58 yrs & 56 yrs (2 case groups) / 52 yrs (controls)	1.0 *	0.92 ‖ (0.78-1.10)	

* reference, † significant, ‡ men, § women, || not provided in original article; the relative risk mentioned is derived from our meta-analysis

published both with positive and negative findings on the association between the polymorphism and risk of CHD and the funnel plot does not suggest a strong publication bias.

There is also no consensus on the effect of the genotype on plasma levels of IL-6 and CRP.[14,15,19-21,23,25,26] The C-allele in our study was significantly associated with higher CRP plasma levels. In most studies showing an effect of the C-allele on plasma levels, the C-allele was associated with higher plasma levels of both IL-6 and CRP. [20,21,26,33] This is pathophysiologically plausible as CRP is produced in the liver and IL-6 is a hepatocyte stimulant, so elevated IL-6 levels will result in higher CRP levels. However, IL-6 has been described to be too unstable in time (plasma half-life of less than 2 hours) to be measured precisely.[34,35] In

addition, there was a limited sample size of IL-6 plasma levels. Given the short half-life, larger numbers would have been needed to detect a relatively small difference in IL-6 levels. This might explain the lack of association between the genotype and IL-6 plasma levels in our study.

In our study, we did not find a clear association between genotype and risk of CHD. This may be related to the fact that the functionality of the polymorphism, at least with respect to the extent of the influence on IL-6 plasma levels, has not been definitively established. Although an effect on transcription and IL-6 levels was described, the view presented by Terry et al., whereby the effect is cell-specific and dependent on complex interactions between several polymorphisms, rather then on an individual polymorphism, might be more applicable.[18,33,36] This implies that the solitary -174 G/C genotype might influence plasma levels, but not in a substantial way.

There is evidence for the IL-6 -174 G/C polymorphism to be in linkage disequilibrium (LD) with other functional, but less frequently investigated, polymorphisms -597 G/A and -572 G/C, and with possibly functional polymorphisms in the -373 AT run.[37-42] Because of this LD, these polymorphisms were not considered in our analyses, as analyzing these would provide similar information.

In conclusion, we did not find a significant relation between the IL-6 promoter polymorphism -174 G/C and risk of CHD. Based on our analyses and results from our meta-analysis, we conclude that the polymorphism does not have a prominent role in the pathogenesis of CHD and is therefore not a suitable genetic marker for increased risk of CHD.

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Chapter 2.2

Transforming Growth Factor-β1 Polymorphisms and Myocardial Infarction and Stroke

Background and purpose - Inflammation plays a pivotal role in the pathogenesis of atherosclerosis and of cardio- and cerebrovascular complications. Transforming growth factor β1 (TGF-β1) is a pleiotropic cytokine with a central role in inflammation. Little is known of the relation of variations within the gene and risk of cardio- and cerebrovascular disease. We therefore investigated five polymorphisms in the TGF-β1 gene (-800 G/A, -509 C/T, codon 10 Leu/Pro, codon 25 Arg/Pro and codon 263 Thr/Ile) in relation to risk of myocardial infarction and stroke in a population-based study.

Methods - 6456 participants of the Rotterdam Study were included in the current study. Analyses on the relations of the genotypes with risk of myocardial infarction and stroke were performed using Cox proportional hazards methods. All analyses were adjusted for age, sex, conventional cardiovascular risk factors and medical history.

Results - We found no association with risk of myocardial infarction. A significantly increased risk of stroke was found, associated with the T-allele of the -509 C/T polymorphism (RR = 1.26, CI 1.06-1.49) and with the Pro-variant of the codon 10 polymorphism (RR = 1.24, CI 1.04-1.48).

Conclusions - No association of the TGF-β1 polymorphisms and myocardial infarction was observed; however, the -509 C/T and codon 10 Leu/Pro polymorphisms were associated with risk of stroke.

INTRODUCTION

Inflammation is an essential part in the pathogenesis of atherosclerosis and consequently, of coronary heart disease and cerebrovascular disease.[1,2] Inflammation is influenced by many different cytokines, such as transforming growth factor β1 (TGF-β1), the most common variant of three isoforms.[3] TGF-β has many different functions, both pro- and anti-atherogenic. Some consider the overall effect of TGF-β to be protective, reducing risk of cardio- and cerebrovascular disease.[4-10] Others describe TGF-β as inducing or facilitating cardio- and cerebrovascular pathology such as vascular stenosis and thrombogenesis.[11-16]

The TGF-β1 gene is located on chromosome 19q13.2. There are several commonly known (potentially) functional polymorphisms in this gene. Cambien et al. described the -988 C/A, -800 G/A, -509 C/T polymorphisms (promoter region); a C-insertion at position +72 (non-translated region); and codons 10 Leu/Pro (c10) and 25 Arg/Pro (c25) (signal peptide sequence) and 263 Thr/Ile (c263) (precursor part of the protein).[17] Strong linkage disequilibrium (LD) between the polymorphisms was described.[17,18] The +72 was in almost complete linkage disequilibrium (with c25); -988 C/A was extremely rare.[17] Grainger et al. described the -509 C/T polymorphism to be associated with levels of TGF-β1.[19]

The c25 polymorphism was associated with cardiovascular disease in several studies, as was the c10 polymorphism.[17,20-22] However, other studies reported no association with cardiovascular disease.[17,18,20,23] To our knowledge, none of the polymorphisms was ever studied in relation to risk of stroke in a general population.

We studied the TGF-β1 -800 G/A, -509 C/T, c10, c25 and c263 polymorphisms in relation to the risk of myocardial infarction and stroke in a large population-based study.

METHODS

Study Population

The Rotterdam Study is an ongoing prospective cohort study including 7983 participants of 55 years and older. Its general aims are to investigate determinants of chronic diseases.[24] During the first phase (1990-1993), all inhabitants of the Rotterdam suburban area (Ommoord) aged 55 years and over were invited to participate. Baseline investigations included an interview and visits to the research center, where a varied number of measurements were performed. Approval of the Medical Ethics Committee of the Erasmus University Rotterdam was obtained for the Rotterdam Study. From all participants written informed consent was acquired. An in depth description of the Rotterdam Study was given in previously published literature.[24]

Clinical Characteristics

Skilled investigators collected information using a computerized questionnaire. The information included current health status, medical history, drug and smoking behavior. Blood samples were obtained and established cardiovascular risk factors were measured as described elsewhere.[25] Diabetes mellitus (DM) was defined as a non-fasting / post-load serum glucose level of 11.1 mmol/l and over, and/or use of antidiabetics. A 12-lead electrocardiogram was recorded and was analyzed by the Modular ECG Analysis System (MEANS).[26] A diagnosis of atrial fibrillation was based on ECG data and/or confirmation by a subject's general practitioner (GP).

Follow-up Procedures and Definition of Events

General practitioners in the research district, with whom 85% of the participants were enlisted, reported fatal/non-fatal cardiovascular events. Research assistants verified all information by checking medical records at the GPs' offices. All medical records of the participants under the care of general practitioners outside the study area were checked annually. Letters and discharge reports from medical specialists were obtained. Information on the vital status of participants was obtained regularly from the municipal health authorities in Rotterdam. After notification, cause and circumstances of death were established by a questionnaire from the GPs. Two research physicians independently coded all reported cardiovascular events according to the International Classification of Diseases, 10th edition (ICD-10) (World Health Organization, 1992).[27] Codes on which the research physicians disagreed were discussed in order to reach consensus. Finally, a medical expert in cardiovascular disease, whose judgement was considered final, reviewed all events. Incident myocardial infarction (MI) was defined as the occurrence of a fatal or non-fatal MI (ICD-10 code I21) after the baseline examination. Medical records of subjects with history of stroke were verified. For reported events, additional information was obtained from hospital records. Information on all potential strokes and transient ischaemic attacks were reviewed by both a research physician and an experienced stroke neurologist (P.J.K.) to verify all diagnoses. Subarachnoid hemorrhages and retinal strokes were excluded. Stroke was subclassified as ischaemic when a CT or MRI scan, made within 4 weeks after the stroke occurred, ruled out other diagnoses, or when indirect evidence (deficit limited to 1 limb or completely resolved within 72 hours, atrial fibrillation in absence of anticoagulants) pointed at an ischaemic nature of the stroke. A stroke was subclassified as hemorrhagic when a relevant hemorrhage was shown on CT or MRI scan, or when the subject lost consciousness permanently or died within hours after onset of focal signs. If a stroke could not be subclassified, it was called unspecified.

Genotyping

Genotyping of the TGF-β1 polymorphisms (-800 G/A [rs1800468], -509 C/T [rs1800469], codon 10 Leu/Pro [T/C] [rs1982073], codon 25 Arg/Pro [G/C] [rs1800471] and codon 263 Thr/Ile [C/T]

[rs1800472]) was performed, regardless of disease status, using blood samples, acquired by venepuncture, stored earlier at -80 degrees centigrade. DNA was isolated using standard procedures. 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 in 2 µL reaction volume. The polymorphisms were selected based on (potential) functionality (-800 G/A and -509 C/T located in promotor; codons 10, 25 and 263 resulting in an amino-acid change) and literature, making comparison and replication feasible.

Measurement of IL-6 and CRP Plasma Levels

Levels of IL-6 and CRP were determined in samples obtained at baseline. These methods have been described previously.[28]

Population for Analysis

The Rotterdam Study is comprised of 7983 subjects. The current study included participants based on the largest successfully genotyped group, i.e., 6456 participants (for the -800 G/A polymorphism). Of these, 6392 subjects were successfully genotyped for the -509 C/T polymorphism, and 6187 for codons 10, 25 and 263.

Statistical Analyses

Chi-square tests were performed to test for deviations from HWE. Missing data were imputed using Expectation-Maximization algorithms. Baseline characteristics were tested using analyses of variance and logistic regression analyses adjusted for age and sex. As correction for outliers in serum measurements all values above mean plus three times the standard deviation were excluded. Natural-log transformed (ln-transformation) values of IL-6 and CRP levels were used to normalize the distribution of these variables. Cox proportional hazards analyses were performed in order to obtain relative risks. All analyses were adjusted for age and sex, and additionally for body mass index (BMI), systolic blood pressure, high-density lipoprotein (HDL) and total cholesterol levels, baseline smoking and DM, and additionally with adjustment for a history of MI/stroke/atrial fibrillation, or with exclusion of subjects with a history of MI/stroke. A p-value of 0.05 and smaller was considered significant. Statistical analyses were performed using SPSS version 11.0.1 for MS-Windows.

RESULTS

A total of 6456 participants were included. Baseline characteristics are described in Table 1. Few and small differences in clinical characteristics between the genotypes were found (Table 2). During a mean follow-up of 9.2 (± 3.0) years, 358 incident cases of MI and 540 incident cases of stroke, of which 312 ischaemic strokes and 51 haemorrhagic strokes were identified. At baseline 756 (12%) subjects had a history of MI and 191 (3%) of stroke.

Genotyping of the TGF-β1 polymorphisms was performed for all five polymorphisms: -800 G/A (n = 6456), -509 C/T (n = 6392), c10 (n = 6187), c25 (n = 6187) and c263 (n = 6187). All

Table 1. Baseline Characteristics

Characteristic	Overall	MI	Stroke	Ischaemic Stroke
Total number	6456	358	540	312
Age – yrs	69.5 ± 9.1	70.3 ± 7.8	74.3 ± 8.5	71.9 ± 7.5
Male sex – %	41	61	42	46
Body mass index – kg/m^2	26 ± 4	26 ± 3	26 ± 4	27 ± 3
Systolic blood pressure – mm Hg	139 ± 22	143 ± 21	149 ± 22	147 ± 20
Diastolic blood pressure – mm Hg	74 ± 11	74 ± 11	75 ± 12	75 ± 11
Total cholesterol – mmol/l	6.6 ± 1.2	6.9 ± 1.2	6.5 ± 1.2	6.6 ± 1.2
HDL-cholesterol – mmol/l	1.3 ± 0.4	1.2 ± 0.3	1.3 ± 0.4	1.3 ± 0.4
Smoking – % Current	22	24	24	27
Former	43	51	40	43
Never	34	24	36	31
Diabetes – %	10	17	19	16
CRP*† – mg/L	1.8 (0.8-3.5)	1.9 (1.6-3.7)	1.7 (0.9-3.0)	2.1 (1.0-3.3)
IL-6*‡ – pg/L	1.9 (1.2-2.9)	2.1 (1.0-4.3)	2.3 (1.6-3.7)	2.0 (1.5-3.7)
Incident events				
Incident MI – no. (%)	358 (6)	- -	33 (6)	21 (7)
Incident stroke – total – no. (%)	540 (8)	33 (9)	- -	- -
Incident stroke – ischaemic – no. (%)	312 (5)	21 (6)	312 (58)	- -
Data on CT/MRI available – no. (%)	- -	- -	326 (60)	280 (90)
History				
History of MI – no. (%)	756 (12)	84 (24)	91 (17)	59 (19)
History of stroke – no. (%)	191 (3)	17 (5)	47 (9)	25 (8)
History of atrial fibrillation – no (%)	327 (5)	17 (5)	55 (10)	24 (8)

Continuous variables: mean ± SD
CRP = C-reactive protein
IL-6 = interleukin 6
MI = myocardial infarction
* Median (interquartile range)
† Based on n = 5941
‡ Based on n = 643

Table 2. Baseline Characteristics by Genotype

Characteristic	-800 G/A			-509 C/T			c10 Leu/Pro			c25 Arg/Pro		
	GG	*GA*	*AA*	*CC*	*CT*	*TT*	*Leu/Leu*	*Leu/Pro*	*Pro/Pro*	*Arg/Arg*	*Arg/Pro*	*Pro/Pro*
Total number	5359	1050	47	3214	2597	581	2457	2862	868	5289	868	30
Age – yrs	69.5 ± 9.1	69.5 ± 9.2	69.8 ± 9.0	69.6 ± 9.1	69.5 ± 9.2	68.4 ± 8.6[**]	69.5 ± 9.1	69.4 ± 9.0	68.7 ± 8.9[§§]	69.3 ± 9.0	69.4 ± 9.0	68.5 ± 8.6
Male sex – %	2201 (41)	396 (38)	21 (45)	1291 (40)	1029 (40)	265 (46)	959 (39)	1166 (41)	387 (45)	2118 (40)	380 (44)	14 (47)
Body mass index – kg/m^2	26 ± 4	26 ± 4[§]	27 ± 4	26 ± 4	26 ± 4	26 ± 4	26 ± 4	26 ± 4	26 ± 4	26 ± 4	26 ± 4	26 ± 3
Systolic blood pressure – mm Hg	140 ± 22	138 ± 22[‖]	139 ± 21	139 ± 22	139 ± 22	138 ± 22	139 ± 22	139 ± 22	139 ± 22	139 ± 22	140 ± 22	138 ± 25
Diastolic blood pressure – mm Hg	74 ± 11	73 ± 11	75 ± 11	74 ± 11	74 ± 11	74 ± 12	74 ± 11	74 ± 11	74 ± 11	74 ± 11	74 ± 11	70 ± 8
Total cholesterol – mmol/l	6.6 ± 1.2	6.6 ± 1.3[#]	6.6 ± 1.2	6.6 ± 1.2	6.6 ± 1.2	6.6 ± 1.2	6.6 ± 1.2	6.6 ± 1.2	6.6 ± 1.2	6.6 ± 1.2	6.6 ± 1.2	7.0 ± 1.1
HDL-cholesterol – mmol/l	1.3 ± 0.4	1.4 ± 0.4	1.3 ± 0.4	1.3 ± 0.4	1.3 ± 0.3	1.4 ± 0.4[††]	1.4 ± 0.4	1.3 ± 0.4[‡‡]	1.3 ± 0.3	1.3 ± 0.4	1.3 ± 0.4	1.3 ± 0.3
Smoking – % Current	1182 (22)	237 (23)	7 (15)	684 (21)	588 (23)	144 (25)	532 (22)	639 (22)	207 (24)	1183 (22)	184 (21)	9 (30)
Former	2349 (44)	439 (41)	20 (42)	1393 (43)	1116 (43)	266 (46)	1063 (43)	1208 (42)	403 (46)	2279 (43)	380 (44)	14 (47)
Never	1828 (34)	374 (36)	20 (43)	1137 (35)	893 (34)	171 (29)	862 (35)	1015 (36)	258 (30)	1824 (35)	304 (35)	7 (23)
Diabetes – %	538 (10)	113 (11)	3 (6)	327 (10)	271 (10)	51 (9)	258 (11)	281 (10)	82 (9)	542 (10)	77 (9)	2 (7)
CRP*[†] – mg/L	1.8 (0.9-3.4)	1.6 (0.7-4.0)	1.7 (0.6-3.6)	1.7 (0.8-3.3)	1.9 (0.9-3.6)	1.8 (1.0-4.2)	1.7 (0.7-3.4)	1.8 (0.9-3.4)	1.8 (1.0-4.1)	1.8 (0.8-3.5)	1.5 (0.8-3.2)	2.1 (1.2-3.3)
IL-6*[‡] – pg/L	1.8 (1.2-2.9)	1.8 (1.2-3.2)	2.7 (1.5-5.0)	1.8 (1.2-2.9)	1.9 (1.2-3.0)	1.7 (1.2-3.3)	1.8 (1.2-2.8)	1.9 (1.2-3.0)	1.9 (1.2-3.1)	1.8 (1.2-2.9)	2.0 (1.3-3.2)	1.5 (1.2-1.9)
Incident events												
Incident myocardial infarction – no. (%)	288 (5)	66 (6)	4 (9)	171 (5)	156 (6)	28 (5)	135 (5)	164 (6)	44 (5)	297 (6)	45 (5)	1 (3)
Incident stroke – total – no. (%)	450 (8)	89 (8)	1 (2)	243 (8)	241 (9)	49 (8)	185 (8)	259 (9)	76 (9)	445 (8)	74 (9)	1 (3)
Incident stroke – ischaemic – no. (%)	263 (5)	48 (5)	1 (2)	138 (4)	140 (5)	31 (5)	109 (4)	142 (5)	48 (6))	261 (5)	37 (4)	1 (3)
History												
History of myocardial infarction – no. (%)	631 (12)	120 (11)	5 (11)	369 (12)	318 (12)	63 (11)	291 (12)	335 (12)	92 (11)	626 (12)	91 (11)	1 (3)
History of stroke – no. (%)	160 (3)	29 (3)	2 (4)	90 (3)	89 (3)	12 (2)	70 (3)	91 (3)	18 (2)	159 (3)	18 (2)	2 (7)
History of atrial fibrillation – no (%)	270 (5)	55 (5)	2 (4)	179 (6)	125 (5)	22 (4)	131 (5)	147 (5)	38 (4)	264 (5)	49 (6)	3 (10)

Continuous variables: mean ± SD
CRP = C-reactive protein
IL-6 = interleukin 6
* Median (interquartile range)
† Based on n = 5941
‡ Based on n = 643
§ -800 GA > GG (p = 0.05)
|| -800 GA < GG (p = 0.04)
-800 GA < GG (p = 0.05)
** -509 TT < CC (p = 0.09) and TT < CT (p = 0.02)
†† -509 TT > CT (p = 0.01)
‡‡ c10 Leu/Pro < Leu/Leu (p = 0.04)
§§ c10 Pro/Pro < Leu/Leu (p=0.03)
All analyses are age and sex adjusted

genotype and allele proportions were in HWE, except for c263 (p = 0.00078), which was not included in the analyses.

For any of the four analyzed polymorphisms, no significant associations with risk of MI were found, when comparing individuals heterozygous and homozygous for the risk allele with those with the wildtype genotype (Table 3). Also when comparing homozygotes versus non-homozygotes, and carriers versus non-carriers, no evidence for an association with risk of MI was found.

Table 3. Relative Risk of Myocardial Infarction by Genotype

Myocardial Infarction				
Polymorphism	**Genotype**	**Events/total (%)**	**RR**	**95% CI**
-800 G/A	*GG*	288/5359 (5)	1.00	
	GA	66/1050 (6)	1.22	0.93-1.59
	AA	4/47 (9)	1.59	0.59-4.26
	A-Carrier	70/1097 (6)	1.23	0.95-1.60
-509 C/T	*CC*	171/3214 (5)	1.00	
	CT	156/2597 (6)	1.15	0.93-1.43
	TT	28/581 (5)	0.88	0.59-1.31
	T-Carrier	184/3178 (6)	1.10	0.89-1.35
c10 Leu/Pro	*Leu-Leu*	135/2457 (5)	1.00	
	Leu-Pro	164/2862 (6)	1.04	0.83-1.31
	Pro-Pro	44/868 (5)	0.88	0.63-1.24
	Pro-Carrier	208/3730 (6)	1.00	0.81-1.24
c25 Arg/Pro	*Arg-Arg*	297/5289 (6)	1.00	
	Arg-Pro	45/868 (5)	0.89	0.65-1.22
	Pro-Pro	1/30 (3)	0.49	0.07-3.49
	Pro-Carrier	46/898 (5)	0.88	0.64-1.20

Wildtype is reference
Analyses are age/sex adjusted
RR = Relative risk

Table 4. Relative Risk of Stroke by Genotype

Stroke				
Polymorphism	**Genotype**	**Events/total (%)**	**RR**	**95% CI**
-800 G/A	*GG*	450/5359 (8)	1.00	
	GA	89/1050 (8)	1.03	0.82-1.29
	AA	1/47 (2)	0.25	0.04-1.78
	A-Carrier	90/1097 (8)	1.00	0.79-1.25
-509 C/T	*CC*	243/3214 (8)	1.00	
	CT	241/2597 (9)	1.27	1.06-1.51 *
	TT	49/581 (8)	1.21	0.89-1.65
	T-Carrier	290/3178 (9)	1.26	1.06-1.49 †
c10 Leu/Pro	*Leu-Leu*	185/2457 (8)	1.00	
	Leu-Pro	259/2862 (9)	1.24	1.03-1.50 ‡
	Pro-Pro	76/868 (9)	1.23	0.94-1.61
	Pro-Carrier	335/3730 (9)	1.24	1.04-1.48 §
c25 Arg/Pro	*Arg-Arg*	445/5289 (8)	1.00	
	Arg-Pro	74/868 (9)	1.02	0.80-1.30
	Pro-Pro	1/30 (3)	0.35	0.05-2.48
	Pro-Carrier	75/898 (8)	0.99	0.78-1.27

Wildtype is reference
Analyses are age/sex adjusted
RR = Relative risk
* p = 0.01
† p = 0.01
‡ p = 0.03
§ p = 0.02

Subjects with the -509 CT genotype had a significantly increased risk of stroke compared with those with the wildtype genotype (CC) (relative risk (RR) 1.27, 95% confidence interval (CI) 1.06-1.51, p=0.01) (Table 4). Also T-allele-carriers had a significantly increased relative risk of 1.26 (CI 1.06-1.49, p=0.01) compared with non-carriers. For ischaemic stroke, we found an increased risk for both subjects with the CT genotype compared with the wildtype CC (RR 1.29, CI 1.02-1.63, p=0.03) and T-allele-carriers in comparison with non-carriers (RR 1.29, CI 1.03-1.61, p=0.03) (Table 5).

For the c10 polymorphism, a significantly increased risk of stroke of 1.24 (CI 1.03-1.50, p=0.03) for subjects with the Leu/Pro genotype was found, compared with the wildtype genotype (Leu/Leu) (Table 4). Pro-carriers also were at increased risk when compared with non-carriers: RR 1.24 (CI 1.04-1.48, p=0.02). For ischaemic stroke a consistently, but non-significantly, increased risk was observed for subjects with the Leu/Pro genotype and Pro/Pro genotype, as well as for Pro-carriers (Table 5).

The -800 G/A and c25 polymorphisms were not associated with risk of (ischaemic) stroke (Tables 4 & 5).

Table 5. Relative Risk of Ischaemic Stroke by Genotype

Ischaemic Stroke				
Polymorphism	**Genotype**	**Events/total (%)**	**RR**	**95% CI**
-800 G/A	*GG*	263/5359 (5)	1.00	
	GA	48/1050 (5)	0.95	0.70-1.30
	AA	1/47 (2)	0.43	0.06-3.03
	A-Carrier	49/1097 (4)	0.93	0.69-1.26
-509 C/T	*CC*	138/3214 (4)	1.00	
	CT	140/2597 (5)	1.29	1.02-1.63 *
	TT	31/581 (5)	1.28	0.86-1.88
	T-Carrier	171/3178 (5)	1.29	1.03-1.61 †
c10 Leu/Pro	*Leu-Leu*	109/2457 (4)	1.00	
	Leu-Pro	142/2862 (5)	1.15	0.89-1.47
	Pro-Pro	48/868 (6)	1.26	0.90-1.77
	Pro-Carrier	190/3730 (5)	1.17	0.93-1.49
c25 Arg/Pro	*Arg-Arg*	261/5289 (5)	1.00	
	Arg-Pro	37/868 (4)	0.86	0.61-1.21
	Pro-Pro	1/30 (3)	0.58	0.08-4.14
	Pro-Carrier	38/898 (4)	0.85	0.60-1.19

Wildtype is reference
Analyses are age/sex adjusted
RR = Relative risk
* p = 0.03
† p = 0.03

All analyses were adjusted for age and sex. Further adjustments for BMI, systolic blood pressure, HDL- and total cholesterol levels, smoking and diabetes mellitus, did not essentially change our estimates (data not shown). Additional analyses, either with adjustment for history of cardiovascular disease, or with exclusion of subjects with a history of cardiovascular disease, yielded essentially similar results (data not shown).

Separate analyses for males and females yielded no consistent results different from the overall analyses (data not shown).

DISCUSSION

In this prospective population-based study we found no significant association between four common TGF-β1 polymorphisms and risk of MI. We did find, however, an increased risk of stroke, associated with the risk alleles of the -509 C/T and c10 Leu/Pro polymorphisms.

To date, few studies have been published on the association of the TGF-β1 polymorphisms -800 G/A, -509 C/T, c10 and c25 and risk of myocardial infarction. To our knowledge, there are no previous studies on the association of these polymorphisms with risk of stroke in a general population.

Table 6. Literature Overview on Studies of TGF-β Polymorphisms and Coronary Heart Disease

Study	N (cases)	Population	Polymorphism	Events	Result	Remarks
Cambien et al. (ECTIM study, 1996)	1192 (563)	France/UK	-988 C/A -800 G/A -509 C/T +72 C-insertion c10 Leu/Pro c25 Arg/Pro c263 Thr/Ile	Myocardial infarction	For c25 Pro-carriers vs non-carriers overall risk of MI: 1.40 ($p<0.05$)	
Rao et al. (HEMO Study, 2004)	183 (115)	USA (44% African-American)	c10 Leu/Pro c25 Arg/Pro	Vascular disease	For c25 heterozygotes vs. wildtype risk of vascular disease: 3.05 ($p<0.01$)	End-stage renal disease patients
Syrris et al. (1998)	899 (655)	UK	-800 G/A -509 C/T c10 Leu/Pro c25 Arg/Pro c263	CAD	No association with CAD ($p>0.05$ for all polymorphisms)	
Wang et al. (1998)	371	Australia	-509 C/T	CAD severity and myocardial infarction	No association with severity of CAD ($p=0.646$) or history of MI	Number of events and p-value for MI not specified
Yokota et al. (2000)	906 (315)	Japan	c10 Leu/Pro	Myocardial infarction	For male c10 Leu-carriers vs. non-carriers risk of MI: 3.5 ($p<0.001$)	
Holweg et al. (2001)	252 (72 of 236 surviving patients)	Netherlands	c10 Leu/Pro c25 Arg/Pro	Graft vascular disease (GVD) (form of accelerated CAD)	For c10 Pro-Pro vs. wildtype risk of GVD: 7.7 ($p=0.03$) For c25 no associations ($p=0.09$)	Heart transplantation patients

CAD = coronary artery disease, MI = myocardial infarction

The TGF-β polymorphisms have been studied before in relation to coronary heart disease (CHD) (Table 6): the c25 polymorphism was associated with risk of CHD in several larger and smaller studies, as was the c10 polymorphism.[17,20-22] However, (other) studies reported no associations with risk of CHD.[17,18,20,23] Overall, the sparse results available are not consistent.

TGF-β is a pleiotropic cytokine with a diversity of effects, both pro- and anti-atherogenic. It is not known yet what the overall effect of TGF-β is on atherogenesis and associated morbidity. Some consider an adequate level of TGF-β to be protective, reducing risk of cardio- and cerebrovascular disease.[4-10] Indeed, Cipollone et al. postulated a stabilizing effect of increased expression of TGF-β on atherosclerotic plaques.[29] Others assume that high levels are the cause of adverse events, inducing or facilitating cardio- and cerebrovascular pathology such as vascular stenosis and thrombogenesis.[11-16]

The -800 G/A and -509 C/T polymorphisms are located in the promoter region. Their precise effect is still unknown, but due to their location, they are considered possible modulators of expression of the TGF-β gene and levels.[17,19] The c10 and c25 polymorphisms are located in the signal peptide sequence; this sequence is involved in export of synthesized proteins across membranes of the endoplasmatic reticulum.[17] They are also located at potentially

important positions that influence activation of the TGF-β protein.[18] We found an association with increased risk of (ischaemic) stroke for the -509 C/T and c10 Leu/Pro polymorphisms. For c10 Leu/Pro the findings in the overall stroke group were somewhat stronger than in the ischaemic stroke group; we have no explanation for this. Overall, the associations between the polymorphisms and (ischaemic) stroke may very well be due to changes in expression or activity of TGF-β. It is unclear why similar effects on risk of MI were not observed.

Our study is based on a large ongoing population-based study in a relatively homogeneous population, as 98% of the participants are Caucasian and living in the same area. In contrast to case-control studies, the prospective nature of our study makes our results less prone to survival bias. We adjusted for common cardiovascular risk factors, including history of cardiovascular disease. Because of high linkage disequilibrium between the four polymorphisms ($D' \geq 0.97$, data not shown), we did not use haplotypes/haplotype-based analyses. Unfortunately, no levels of TGF-β were determined. Therefore we were not able to elucidate the effect of the polymorphisms on levels. Despite the fact that the Rotterdam Study is a large study, (small) effects may have been missed due to the limited number of cases.

In conclusion, we found no association between the TGF-β1 -800 G/A, -509 C/T, c10 and c25 polymorphisms and risk of MI. We observed, however, a significant but small association between the -509 C/T and c10 Leu/Pro polymorphisms and risk of stroke. These results warrant further studies, with larger numbers, to consolidate these findings, to investigate the functional effects of these polymorphisms and to study the underlying pathophysiological mechanism. Only after replication in other studies implications of these findings can be discussed.

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Chapter 3

Genetic Determinants of Arterial Stiffness

Inflammatory Genes

Chapter 3.1

Interleukin 6 -174 G/C Promoter Polymorphism and Arterial Stiffness

Background and purpose - Arterial stiffness increases with age and is a precursor of cardiovascular disease. Interleukin 6 is an inflammatory cytokine influencing the acute-phase response and inflammatory cascade, such as up-regulation of C-reactive protein. The interleukin 6 -174 G/C promoter polymorphism influences levels of inflammatory markers, which are associated with arterial stiffness. We studied the association of this polymorphism and plasma levels of interleukin 6 and C-reactive protein with arterial stiffness.

Methods - The study (n=3849) was embedded in the population-based Rotterdam Study. Analyses on the association between the -174 G/C polymorphism and arterial stiffness were performed using analyses of variance. Analyses on the plasma levels of inflammatory markers and arterial stiffness were performed using linear regression analyses. All analyses were adjusted for age, sex, mean arterial pressure, heart rate, known cardiovascular risk factors and atherosclerosis.

Results - We found pulse wave velocity to be 0.3 m/s higher for CC vs. GG-homozygotes (p=0.024) with evidence for an allele-dose effect (p=0.029), and a similar, although non-significant, pattern for pulse pressure. No apparent consistent association with the distensibility coefficient was found. C-reactive protein plasma levels were associated with pulse wave velocity (p=0.007), and inversely, although not significantly, with the distensibility coefficient. Interleukin 6 plasma levels were similarly, but not significantly, associated with arterial stiffness.

Conclusions - The interleukin 6 -174 G/C polymorphism and CRP plasma levels are associated with increased arterial stiffness.

INTRODUCTION

Arterial stiffness increases with age and has been associated with hypertension, diabetes mellitus (DM), end-stage renal disease and atherosclerosis.[1-6] Arterial stiffness has recently been established as a predictor of cardiovascular events.[7-10] The extent of the increase in stiffness may depend on various factors, such as genetic variations.

Inflammatory markers, such as interleukin 6 (IL-6) and C-reactive protein (CRP), and general systemic inflammation have been found to be associated with arterial stiffness.[11-14] However, it is not clear whether this is a causal relation, or a (confounding) effect via atherosclerosis. After all, inflammation has an important role in atherosclerosis and the atherosclerotic process itself implies increased stiffness. Therefore, further study into genetic variation in the inflammatory pathway in relation to arterial stiffness may provide more information on potential pathophysiological mechanisms. Significant heritability estimates have been found for arterial stiffness, suggesting a role of genetic factors.[15]

Interleukin 6 is a pleiotropic cytokine with many different functions. It plays an important role in the acute-phase response and inflammatory cascade, such as up-regulation of acute-phase proteins as CRP.[16-19] An association between IL-6 levels and increased arterial stiffness has been described.[12,20,21] Also elevated CRP levels have been shown to be associated with increasing pulse pressure and increased arterial stiffness.[11-14] Fishman et al. detected a functional polymorphism in the promoter region of the human IL-6 gene (G -> C, 174 bp upstream from the start site).[22] This polymorphism appears to influence the IL-6 gene transcription and is associated with elevated CRP levels.[22-26]

To the best of our knowledge, the IL-6 gene has not been studied in relation to arterial stiffness. We therefore studied the association of the interleukin 6 -174 G/C promoter polymorphism, arterial stiffness and plasma levels of interleukin 6 and C-reactive protein within the Rotterdam Study.

METHODS

The Rotterdam Study is an ongoing prospective cohort study including 7983 participants of 55 years and older. Its general aims are to investigate determinants of chronic diseases.[27] During the first phase of this study (1990-1993), all inhabitants from a Rotterdam suburban area (Ommoord) aged 55 years and over were invited to participate in this study. The response rate was 78%. The third examination phase took place from 1997-1999, during which measures of arterial stiffness were performed. Approval of the Medical Ethics Committee of the Erasmus University Rotterdam was obtained for the Rotterdam Study. From all participants written informed consent was acquired. A more in depth description of the Rotterdam Study was published previously.[27]

Genotyping

Genomic DNA was extracted from samples of peripheral venous blood according to standard procedures. Genotyping methods were described more in-depth previously.[24]

Measurement of IL-6 and CRP Plasma Levels

Levels of IL-6 and CRP were determined in plasma samples obtained at baseline during the first phase of the Rotterdam Study. The methods were described previously.[24]

Arterial stiffness

Arterial stiffness was measured using the carotid-femoral pulse wave velocity (PWV) as measure of aortic stiffness and the distensibility coefficient (DC) of the common carotid artery as measure of common carotid arterial stiffness, and in addition also pulse pressure (PP). Validation studies have been performed.[6,28] All measures were obtained during the same session. The methods were described more in-depth previously.[9,14,29]

Clinical Characteristics

Information on cardiovascular risk factors was collected during the third follow-up examination by trained investigators. At the research center, measurements were performed by skilled, as described previously.[27] Blood pressure was measured twice on the right arm using a random-zero sphygmomanometer. The average of the two blood pressure values was used in the analyses. Length and weight were measured and body mass index (kg/m^2) (BMI) was calculated. Serum total cholesterol and high-density lipoprotein (HDL) cholesterol values were determined by an automated enzymatic procedure (Boehringer Mannheim System). Diabetes mellitus (DM) was defined as use of anti-diabetic medication and/or a fasting serum glucose level of equal to or above 7.0 mmol/L.[30] Evaluation of the atherosclerotic status of participants was accomplished using ultrasonography (carotid intima-media thickness [IMT]) and radiography (aortic calcification); the methods used, are similar to those that have been extensively described and validated previously.[6,31-33]

Population for Analysis

A total of 4024 subjects, generally Caucasian, underwent the physical examination of the third phase and PWV was measured in 3550 subjects; 69 subjects (1.9%) were excluded from the analyses because of poor quality of the PWV index recordings, leaving 3481 subjects (3207 successfully genotyped) whereas common carotid distensibility was measured in 3098 subjects (2836 successfully genotyped). PP measurements could be determined for all subjects participating in the third phase (3833 successfully genotyped). For 3849 genotyped subjects, data were available on one or more measures of arterial stiffness. Missing information on measures of arterial stiffness was almost entirely due to logistic reasons.

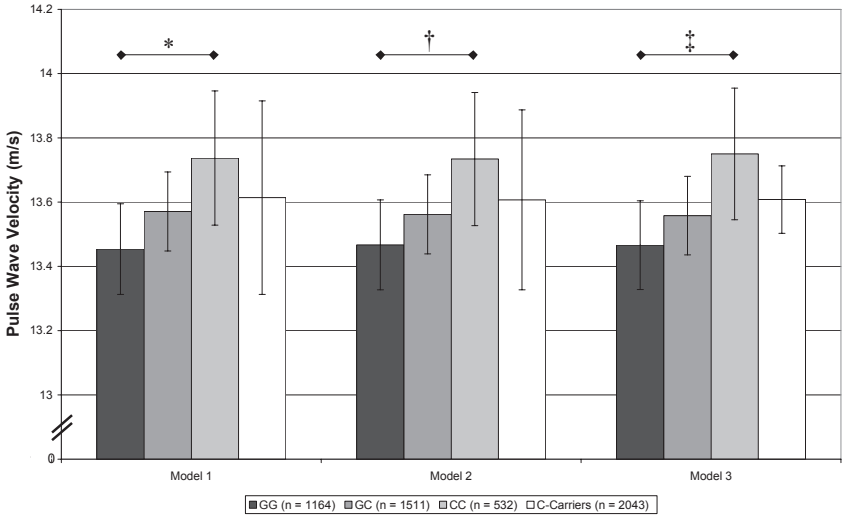
Statistical Analyses

Chi-square tests were performed to test for deviations from Hardy-Weinberg equilibrium. Missing data in clinical characteristics were imputed using Expectation-Maximization algorithms available in SPSS. For serum measurements all values above mean plus three times the standard deviation were excluded, as correction for outliers. Natural-log transformed (ln-transformation) values of IL-6 and CRP plasma levels were used to normalize the distribution of these variables. The association between genotype and arterial stiffness was investigated using analyses of variance. Analyses on the association of IL-6 and CRP levels and arterial stiffness were performed using linear regression. All analyses were adjusted for age and sex, and (if applicable) additionally for mean arterial pressure (MAP) and heart rate (model 1), and additionally for body mass index, high-density lipoprotein (HDL) and total cholesterol levels, smoking and diabetes mellitus (model 2) and finally also for atherosclerosis (carotid IMT and aortic calcification) (model 3). A p-value of 0.05 and smaller was considered significant in all analyses. The statistical analyses were performed using SPSS version 11.0.1 for MS-Windows.

RESULTS

General characteristics are described in Table 1 (ln-transformed data are back transformed). Genotype and allele proportions were in Hardy Weinberg equilibrium (p=0.18).

Figure 1. Association of IL-6 –174 G/C and Pulse Wave Velocity



Vertical lines depict the 95% confidence interval
* CC significantly different from GG (p=0.028); p for trend 0.027
† CC significantly different from GG (p=0.036); p for trend 0.040
‡ CC significantly different from GG (p=0.024); p for trend 0.029
Models 1-3 are described in the methods section

Table 1. Population Characteristics by IL6 –174 G/C Genotype

Characteristic	GG	GC	CC
Total number – No. (%)	1390 (36)	1830 (48)	629 (16)
Age – yrs	72 ± 7	72 ± 7	73 ± 7
Male sex – %	42	43	41
Body mass index – kg/m^2	27 ± 4	27 ± 9	27 ± 4
Systolic blood pressure – mm Hg	143 ± 21	143 ± 22	144 ± 21
Diastolic blood pressure – mm Hg	75 ± 11	75 ± 11	75 ± 11
Mean Arterial Pressure – mm Hg	106 ± 13	107 ± 13	107 ± 13
Total cholesterol – mmol/l	5.8 ± 1.0	5.8 ± 1.0	5.8 ± 0.9
HDL-cholesterol – mmol/l	1.4 ± 0.4	1.4 ± 0.4	1.4 ± 0.4
Smoking – % Current	28	32	30
Former	39	37	38
Never	33	31	32
Diabetes – %	8	9	10
Intima Media Thickness – mm	0.77 ± 0.14	0.78 ± 0.12	0.78 ± 0.14
Aortic calcifications – %*	22	23	20
Pulse Wave Velocity – m/s	13.4 ± 2.9	13.5 ± 3.1	13.8 ± 3.2
Distensibility Coefficient – 1/MPa	10.4 ± 4.2	10.6 ± 4.4	10.1 ± 4.3
Pulse Pressure – mm Hg	68 ± 17	68 ± 18	69 ± 17
C-reactive protein – mg/L†§	2.2 ± 2.3	2.6 ± 2.8	2.5 ± 2.7
Interleukin-6 – pg/mL‡§	2.1 ± 1.5	2.3 ± 1.7	2.2 ± 1.8

Continuous values are depicted as mean ± SD
Numbers based on genotyped subjects with data on either pulse wave velocity (PWV) and/or distensibility coefficient (DC) and/or pulse pressure (PP)
* Percentage of subjects with aortic calcification over a length of ≥ 2.5 cm
† Data available for 3556 subjects (n = 1285 [GG], n = 1694 [GC], n = 577 [CC])
‡ Based on random subgroup of 433 subjects (n = 158 [GG], n = 212 [GC], n = 63 [CC])
§ Measured in samples obtained during first phase of Rotterdam Study

The C-allele of the -174 G/C polymorphism (frequency: 40%) was significantly associated with an increased PWV, with evidence for an allele-dose effect (Figure 1). After adjustment for age, gender, MAP and heart rate, this trend was significant (model 1, p=0.027), and remained significant after further adjustment for cardiovascular risk factors (model 2, p=0.040) and atherosclerosis (model 3, p=0.029). The association was lost after additional adjustment for plasma levels of IL-6 and CRP (data not shown). CC-homozygotes had a 0.3 m/s higher PWV than wildtype GG homozygotes (model 1, p=0.028; model 2 p=0.036; model 3 p=0.024) (Figure 1).

The association of the IL-6 polymorphism and the carotid DC is less clear; it was increased in the heterozygotes (-174 GC) compared with the wildtype genotype, but this was not shown in CC-homozygotes (Figure 2). No significant trend was observed (model 1 p=0.564, model 2 p=0.483, model 3 p=0.487).

Table 2. Association of Pulse Wave Velocity and Distensibility Coefficient with Levels of C-reactive protein

Pulse Wave Velocity (n=2995)	**β**	**95% CI**	**p**
Model 1	0.208	0.119 – 0.298	< 0.000
Model 2	0.159	0.067 – 0.250	0.001
Model 3	0.124	0.034 – 0.215	0.007
Distensibility Coefficient (n=2634)	**β**	**95% CI**	**p**
Model 1	-0.219	-0.347 – -0.091	0.001
Model 2	-0.098	-0.231 – 0.034	0.147
Model 3	-0.058	-0.191 – 0.074	0.389

Presented values are natural-log transformed
Models 1-3 are described in the methods section

Table 3. Association of Pulse Wave Velocity and Distensibility Coefficient with Levels of Interleukin-6

Pulse Wave Velocity (n=316)	**β**	**95% CI**	**p**
Model 1	0.411	-0.135 – 0.956	0.139
Model 2	0.259	-0.300 – 0.818	0.362
Model 3	0.149	-0.402 – 0.701	0.594
Distensibility Coefficient (n=281)			
Model 1	-0.696	-1.316 – -0.076	0.028
Model 2	-0.631	-1.281 – 0.018	0.057
Model 3	-0.593	-1.237 – 0.051	0.071

Presented values are natural-log transformed
Models 1-3 are described in the methods section

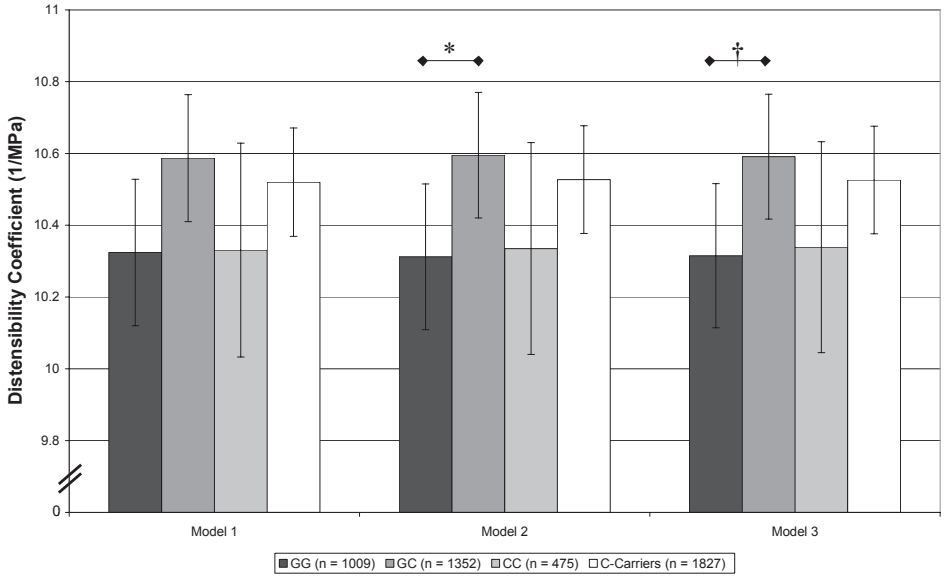
The -174 G/C polymorphism appeared to be related to increased PP in a similar pattern as for PWV, although these findings were not significant (age/gender-adjusted trend p=0.061, after further adjustment trend p=0.094) (Figure 3).

CRP plasma levels were significantly associated with PWV (regression coefficient [natural log transformed] 0.208 mg/L, 95% CI 0.119 - 0.298) (Table 2). This association remained significant after full adjustment (Table 2). An inverse, but after full adjustment non-significant, relation of CRP levels and the DC was shown (Table 2).

Analyses of IL-6 plasma levels showed a positive, although not significant, association with PWV (Table 3). A significant inverse association was found between IL-6 plasma levels and the DC within model 1 (Table 3). After full adjustment, this association remained near significant (Table 3).

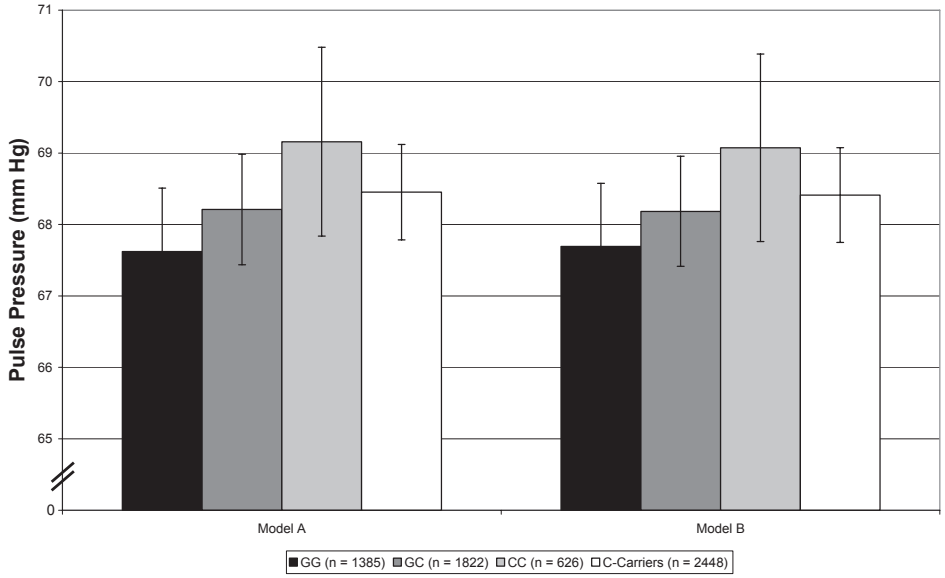
All analyses stratified by gender yielded no results essentially different from the overall analyses.

Figure 2. Association of IL-6 –174 G/C and Distensibility Coefficient



Vertical lines depict the 95% confidence interval
* GC significantly different from GG (p=0.038)
† GC significantly different from GG (p=0.042)
Models 1-3 are described in the methods section

Figure 3. Association of IL-6 –174 G/C and Pulse Pressure



Vertical lines depict the 95% confidence interval
Model A: adjusted for age and sex
Model B: adjusted for age, sex, body mass index, total cholesterol, HDL-cholesterol, diabetes mellitus and smoking

DISCUSSION

We studied a well-known functional polymorphism in the promoter region of the Interleukin 6 gene, -174 G/C in relation to arterial stiffness. The C-allele of the polymorphism was significantly associated with increased PWV and showed a similar relation with PP, although not significantly. CRP plasma levels were significantly associated with PWV. Plasma levels of IL-6 also appeared to be related (although not significantly) with arterial stiffness.

The -174 G/C polymorphism is located in the promotor of the IL-6 gene and may influence gene-transcription. In some studies no effect of this polymorphism on IL-6 and CRP levels was found.[34-37] However, other studies describe it to influence IL-6 and of CRP levels.[22,23,25,26,38,39] Indeed, in a previous study in the Rotterdam Study the C-allele was associated with increased CRP plasma levels, but not with IL-6 plasma levels.[24] However, we consider an association with IL-6 levels biologically likely, and contribute our finding to a very limited number of IL-6 samples.[24]

Interleukin 6 (IL-6) is a pleiotropic cytokine with many different inflammatory functions, e.g. upregulation of CRP.[16-19] IL-6 levels have been associated with arterial stiffness. Diamant et al. described a positive association of IL-6 and CRP levels and increased arterial stiffness in a small Dutch population (n=32) in both type 2 DM patients as well as in healthy controls.[20] Mahmud et al. described an association of IL-6 and CRP levels and PWV in a study of Irish hypertensives (n=78).[21] In a Greek healthy population (n=100) Vlachopoulos et al. also showed IL-6 and CRP levels to be correlated with PWV.[12] In a much larger study of 9867 healthy Americans, CRP levels were associated with PP, a manifestation of arterial stiffness.[40] Within the Rotterdam Study an association between CRP plasma levels and PWV was shown in 866 subjects, which was confirmed in our larger sample of 2995 subjects.[14] Further, Kullo et al. found a relation between CRP levels and PWV in an American population (n=214).[11]

As CRP and IL-6 plasma levels are associated with arterial stiffness, our findings of a significant association of the polymorphism and increased PWV, is biologically plausible. Although not significant, the similar findings for PP are in concordance herewith. We consider the higher DC for heterozygous a chance finding and unlikely to reflect the true relation of the -174 G/C polymorphism and the DC, since literature and our other analyses suggest an inverse association (i.e., increased arterial stiffness).

We also found a significant association of CRP plasma levels and PWV. The number of currently available CRP levels within the Rotterdam Study was more than three times larger than the number available at the time of our previous analyses; the results of our present study are, also after additional adjustment for atherosclerosis, in concordance with these earlier findings.[14] Our findings suggest IL-6 levels to be related to PWV, although this association was not significant. IL-6 has been described to be too unstable in time (plasma half-life of less than 2 hours) to be measured precisely.[41,42] In addition, the sample size of subjects with IL-6

measurements was limited. This might explain the lack of a significant association between PWV and IL-6 plasma levels.

Our study is based on a large ongoing population-based study in a relatively homogeneous population, as 98% of the participants in our study are Caucasian and are all living in the same area, a suburb of Rotterdam. We adjusted all analyses for established cardiovascular risk factors. The DC has a strong correlation with mean arterial pressure; a higher mean arterial pressure in the artery stretches the elastin and collagen fibres in the arterial wall, making the arteries less distensible. Therefore, the analyses were adjusted for mean arterial pressure.

Some additional issues need to be addressed: findings on PWV were significant, but on the DC were not. To interpret these findings, several methodological aspects of the measures of stiffness need to be discussed. First, using the distance between the carotid and the femoral arteries leads to an overestimation of the real distance travelled by the pulse wave, resulting in higher mean values of aortic PWV. Second, the use of a tape measure over the surface of the body may lead to an overestimation of the distance travelled by the pulse wave, and therefore we adjusted for body mass index. Third, in computing the carotid DC, we used the brachial PP rather than the carotid PP. Information on comparisons between carotid and brachial PPs indicates that there is a difference between these pressures.[43] These findings indicate that using brachial artery PP instead of carotid artery PP may have led to an underestimation of the distensibility. This may lead to an underestimation of the association with the genotype. It has been suggested to derive carotid artery PP with the use of brachial artery PP.[44] However, to perform this procedure, brachial mean pressure should be measured directly, whereas in our study this was computed from blood pressure components by the Korotkoff sound method. Therefore, we cannot exclude the possibility that other methods for assessing carotid distensibility show a clearer association with the genotype. The methodological aspects, however, are not likely to be related to the genotype and will thus not have biased our results.

Furthermore some general remarks: data on measures of stiffness were not available for all subjects who visited the research center. However, missing information was primarily due to logistic reasons, which is likely to be random and thus will not have biased our results. Second, the cross-sectional design may limit the ability to infer a causal relationship between the IL-6 -174 G/C polymorphism and arterial stiffness. Furthermore, because our study was performed in a population of predominantly elderly Caucasian subjects, the generalizability of our findings to younger individuals or other ethnicities remains uncertain. Finally, there are more polymorphisms in the IL-6 gene in addition to the -174 G/C polymorphism. Indeed, there is evidence for the IL-6 -174 G/C polymorphism to be in linkage disequilibrium (LD) with other functional, but less frequently investigated, polymorphisms -597 G/A and -572 G/C, and with possibly functional polymorphisms in the -373 AT run.[45-50] Because of this LD, these polymorphisms were not considered in our analyses.

In conclusion, the C-allele of the interleukin 6 -174 G/C promoter polymorphism is associated with increased PWV, independently of atherosclerosis. Our results consolidate the finding that CRP plasma levels are associated with PWV in an elderly population. It is likely, that the gene influences stiffness through its association with CRP and IL-6 plasma levels, as the effect was attenuated when the analyses were adjusted for levels.

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Chapter 3.2

Variation in the C-reactive Protein Gene and Arterial Stiffness

Background and purpose - With age arterial stiffness increases and has been found to predict cardiovascular disease. C-reactive protein (CRP) is an inflammation marker and has been found to be associated with arterial stiffness and risk of cardiovascular disease. Genetic factors account for part of the variance in CRP serum level. We studied the association of the total common variation in the CRP gene by polymorphisms 1184 C/T, 2042 C/T, 2911 C/G and haplotypes with arterial stiffness within the Rotterdam study.

Methods - The study (n=3615) was embedded in the Rotterdam Study, a prospective, population-based study among subjects aged 55 years and older. Associations of genotypes and haplotypes with the CRP serum level and measures of arterial stiffness were examined using linear regression and analyses of variance. Measures of arterial stiffness included aortic pulse wave velocity, carotid distensibility and pulse pressure. Analyses were adjusted for age, sex, mean arterial pressure, heart rate, known cardiovascular risk factors and measures of atherosclerosis.

Results – The CRP serum level was significantly associated with pulse wave velocity ($p<0.001$) and pulse pressure ($p<0.05$), also after adjusting for cardiovascular risk factors. CRP level was also increased for carriers of the 1184 T-allele and 2911 G-allele and decreased for carriers of the 2042 T-allele (all $p<0.001$). Genotype and haplotype analyses showed no consistent associations of genetic variation with pulse wave velocity, carotid distensibility and pulse pressure.

Conclusions - No consistent associations of the CRP polymorphisms 1184 C/T, 2042 C/T, 2911 C/G and corresponding haplotypes were found with measures of arterial stiffness, although CRP serum levels were associated with pulse wave velocity and pulse pressure, and the individual polymorphisms were associated with the CRP serum level.

INTRODUCTION

With age arterial stiffness increases and has been associated with hypertension, diabetes mellitus, end-stage renal disease and atherosclerosis.[1-6] Arterial stiffness has been found to predict cardiovascular events in various populations.[7-10] The extent of the increase in stiffness may also depend on genetic variation.

Arterial stiffness has been found to be associated with inflammatory markers such as C-reactive protein (CRP).[11-16] However, it is not clear whether the association is causal, or whether the effect is mediated through atherosclerosis. After all, inflammation has been established as marker of atherosclerosis and the atherosclerotic process itself affects arterial stiffness. Studies of genetic variation in the inflammatory pathway in relation to arterial stiffness may provide more insight into the underlying mechanisms. Heritability estimates for levels of CRP vary from 27% up to 40%, which suggests genetic variation to have an effect on bioavailability of CRP.[17,18] If genetic variation in CRP can be found to be related to arterial stiffness, this would support evidence for a causal role of CRP in the loss of elasticity of the vessel wall.

The CRP gene is located on chromosome 1 (1q21-q23). SeattleSNPs (part of the National Heart Lung and Blood Institute's Programs for Genomic Applications) reports that four CRP gene haplotypes are present in populations of European descent. These haplotypes represent all common variation across the CRP gene in these populations and can be inferred from three tagging polymorphisms, 1184 C/T, 2042 C/T and 2911 C/G.

In order to clarify the role of genetic variation in the CRP gene in the development of arterial stiffness, we set out to investigate CRP polymorphisms 1184 C/T, 2042 C/T and 2911 C/G and resultant haplotypes in relation to arterial stiffness within the Rotterdam Study.

METHODS

The Rotterdam Study is an ongoing prospective cohort study including 7983 participants of 55 years and older. Its general aims are to investigate determinants of chronic diseases, including cardiovascular disease, dementia and osteoporosis.[19] During the first phase of this study (1990-1993), all inhabitants of a Rotterdam suburban area (Ommoord) aged 55 years and over, were invited to participate in this study. The response rate was 78%. The third examination phase took place from 1997-1999, during which measurements of arterial stiffness were performed. Approval of the Medical Ethics Committee of the Erasmus University Rotterdam was obtained for the Rotterdam Study. From all participants written informed consent was acquired. A more in depth description of the design of the Rotterdam Study was published previously.[19]

Measurement of CRP Serum Levels

Serum levels of CRP were determined in blood samples obtained during the third examination phase of the Rotterdam Study and stored at -80 degrees centigrade. High-sensitivity CRP measurements were performed using rate near-infrared particle immunoassay (Immage Immunochemistry System; Beckman Coulter, San Diego, CA).

Genotyping and Haplotyping

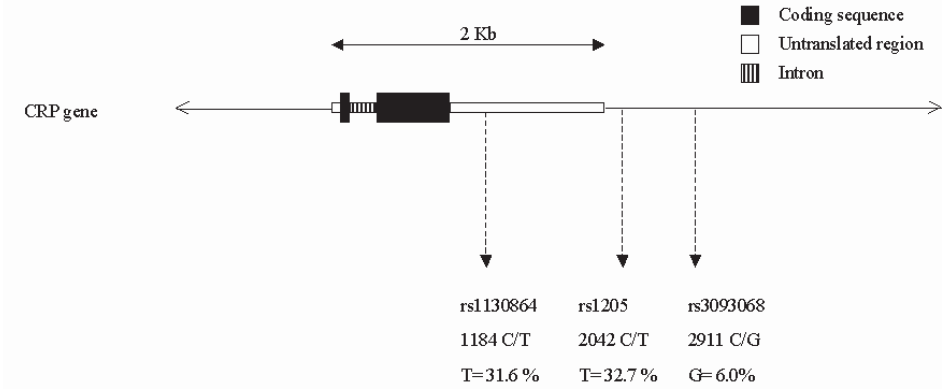
The Seattle SNPs Program for Genomic Applications has identified 31 SNPs in the CRP gene and has established that, based on SNPs with overall frequencies above 5%, four common CRP gene haplotypes are present in 23 unrelated individuals of European descent from the CEPH pedigrees (http://pga.gs.washington.edu/data/crp). These four haplotypes are identified by "haplotype tagging" SNPs. By genotyping three haplotype tagging SNPs we were able to infer all four haplotypes and consequently to describe the total common variation across the CRP gene. These three tagging SNPs were chosen partly based on their presence in existing literature and on their proximity to the CRP gene. Genotyping of the CRP 1184 C/T, 2042 C/T and 2911 C/G polymorphisms [also 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), and also at http://www.ncbi.nlm.nih.gov/projects/SNP/ under identification numbers rs1130864 (1184C/T), rs1205 (2042C/T) and rs3093068 (2911C/G)] was performed using samples stored earlier at -80 degrees centigrade. DNA was isolated using standard procedures. 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 in 2 µl reaction volume.

Haplotypes were estimated using PHASE software (http://archimedes.well.ox.ac.uk).[20] Haplotypes with a frequency of <0.001% were not used in the analyses. The remaining 4 haplotypes were coded from 1 to 4 in decreasing order of their population frequency (coding from 1184C/T, 2042C/T and 2911C/G, haplotype 1=C-T-C, 2=T-C-C, 3=C-C-C and 4=C-C-G) (Figure 1).

Arterial Stiffness

In this study three measures of arterial stiffness were used: the carotid-femoral pulse wave velocity (PWV) as a measure of aortic stiffness and the distensibility coefficient (DC,) of the common carotid artery as a measure of common carotid arterial stiffness. In addition, pulse pressure (PP) was assessed as an indicator of arterial stiffness. All measures were obtained on the same day, during the same session, during the third follow-up examination. Details on all measures of stiffness have been described previously.[6,7]

Figure 1. CRP Gene and 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

Reproduced with permission from the European Heart Journal (Kardys et al.[28])

Carotid-femoral PWV (m/s) was measured using an automatic device (Complior, Colson) and was calculated as the ratio between the distance traveled by the pulse wave and the foot-to-foot time delay.

Common carotid artery distensibility was assessed by measuring the vessel wall motion of the right common carotid artery using a duplex scanner (ATL Ultramark IV, operating frequency 7.5 MHz) connected to a vessel wall movement detector system.[21,22] The cross-sectional arterial wall DC (1/MPa) was calculated as a measure of arterial stiffness.[23] A decreased DC implies increased carotid stiffness.

PP (mmHg) was defined as the difference between systolic and diastolic blood pressure, using the mean systolic and diastolic blood pressure of two measurements obtained by measuring blood pressure on the right arm using a random-zero sphygmomanometer.

Clinical Characteristics

Information on cardiovascular risk factors was collected during the third follow-up examination. Data on drug use and smoking habits were obtained during the home interview.

Smoking was classified as never, former or current smoking. At the research center, blood pressure was measured twice on the right arm using a random-zero sphygmomanometer. The average of the two blood pressure values was used in the analyses. Length and weight

were measured and body mass index (weight/height^2) (BMI) was calculated. Serum total cholesterol and high-density lipoprotein (HDL) cholesterol values were determined by an automated enzymatic procedure (Boehringer Mannheim System). Diabetes mellitus (DM) was defined as use of anti-diabetic medication and/or a fasting serum glucose level of equal to or above 7.0 mmol/L.[24] Evaluation of the atherosclerotic status of participants was accomplished using ultrasonography (carotid intima-media thickness [IMT]) and radiography (aorta calcification); these methods have been extensively described previously.[25,26]

Population for Analysis

The Rotterdam Study comprises 7893 subjects, of which 6007 subjects were successfully and completely genotyped for the CRP polymorphisms. A total of 4024 subjects underwent the physical examination of the third phase. PWV was measured in 3550 subjects; 69 subjects (1.9%) were excluded from the analyses because of poor quality of the PWV recordings, leaving 3481 subjects (3023 successfully genotyped for all polymorphisms). Common carotid distensibility was measured in 3098 subjects (2672 successfully genotyped for all polymorphisms). PP measurements could be determined for all subjects participating in the third phase (3601 successfully genotyped for all polymorphisms). For 2402 completely genotyped subjects, full data on both PWV, the distensibility coefficient and pulse pressure were available. For 3615 completely genotyped subjects data was available on one or more measures of arterial stiffness. CRP serum levels were successfully measured during the third phase in 3824 subjects. Missing information on measures of arterial stiffness was almost entirely due to logistic reasons.

Statistical Analyses

Chi-square tests were performed to test for deviations from Hardy-Weinberg equilibrium. Missing data on clinical characteristics were imputed using Expectation-Maximization algorithms available in SPSS. For CRP measurements all values above mean plus three times the standard deviation were excluded, as correction for outliers. Natural-log transformed (ln-transformation) CRP serum levels were used to normalize the distribution of this variable. Haplotype analyses were allele-based: each haplotype-allele is considered individually in the analyses. The most common haplotype-allele is the reference (i.e., haplotype 1). Analyses on the associations of CRP levels and arterial stiffness were performed using linear regression. Analyses on genotypes and haplotypes in relation to CRP levels and arterial stiffness were performed using linear regression and analyses of variance. The analyses were adjusted for age and sex (and for PWV and DC also for mean arterial pressure (MAP) and heart rate), additionally for systolic blood pressure (only when MAP was not in the model), BMI, HDL and total cholesterol levels, smoking, diabetes mellitus, and in the full model also for measures of atherosclerosis. A p-value of 0.05 and smaller was considered significant in all analyses. The statistical analyses were performed using SPSS version 11.0.1 for MS-Windows.

RESULTS

General characteristics of the subjects are described in Table 1. Genotype and allele proportions were in Hardy Weinberg equilibrium. Haplotypes are described in Figure 1.

CRP Serum Level and Arterial Stiffness

The CRP serum level was positively associated with pulse wave velocity ($p<0.001$), also after full adjustment (Table 2). The CRP serum level was inversely associated with the distensibility coefficient ($p<0.001$), but only in the age and sex-adjusted analyses. The CRP serum level was positively associated with pulse pressure ($p<0.001$), which remained significant after additional adjustment for cardiovascular risk factors, but was borderline significant after additional adjustment for measures of atherosclerosis ($p=0.06$).

Table 1. General Characteristics of the Study Population

Characteristic	
Total number	3615
Age – yrs	72.4 ± 7.0
Male sex – %	43
Body mass index – kg/m^2	27
Systolic blood pressure – mm Hg	144 ± 21
Diastolic blood pressure – mm Hg	75 ± 11
Mean Arterial Pressure – mm Hg	107 ± 13
Total cholesterol – mmol/l	5.8 ± 1.0
HDL-cholesterol – mmol/l	1.4 ± 0.4
Smoking – % Current	30
Former	38
Never	32
Diabetes – %	9
Pulse Wave Velocity* – m/s	13.6 ± 3.0
Distensibility Coefficient† – 1/MPa	10.4 ± 4.3
Pulse Pressure – mm Hg	68 ± 18
Intima Media Thickness – mm	0.77 ± 0.13
Aorta calcifications‡ – %	22
CRP§ – mg/L	2.4 (1.2-4.5)

Continuous values are depicted as mean ± SD
CRP = C-reactive protein
* Available for 3023 subjects
† Available for 2672 subjects
‡ Percentage of subjects with aorta calcification over a length of ≥ 2.5 cm
§ Median (interquartile range)

Table 2. Relation between CRP Level and Arterial Stiffness

	Pulse Wave Velocity (n=3252)		**Distensibility Coefficient (n=2876)**		**Pulse Pressure (n=3721)**	
	β-coefficient (SE)	*p*	*β-coefficient (SE)*	*p*	*β-coefficient (SE)*	*p*
Model 1	0.206 (0.04)	<0.001	–0.222 (0.063)	<0.001	1.103 (0.279)	<0.001
Model 2	0.168 (0.046)	<0.001	–0.098 (0.067)	0.141	0.809 (0.289)	0.046
Model 3	0.143 (0.045)	<0.001	–0.081 (0.066)	0.221	0.538 (0.283)	0.058

β-coefficient: regression coefficient based on ln-transformed CRP (C-reactive protein) levels

Model 1: adjusted for age, sex (and for pulse wave velocity and the distensibility coefficient also for mean arterial pressure and heart rate)

Model 2: model 1 + body mass index (BMI), total & HDL-cholesterol, smoking, diabetes mellitus

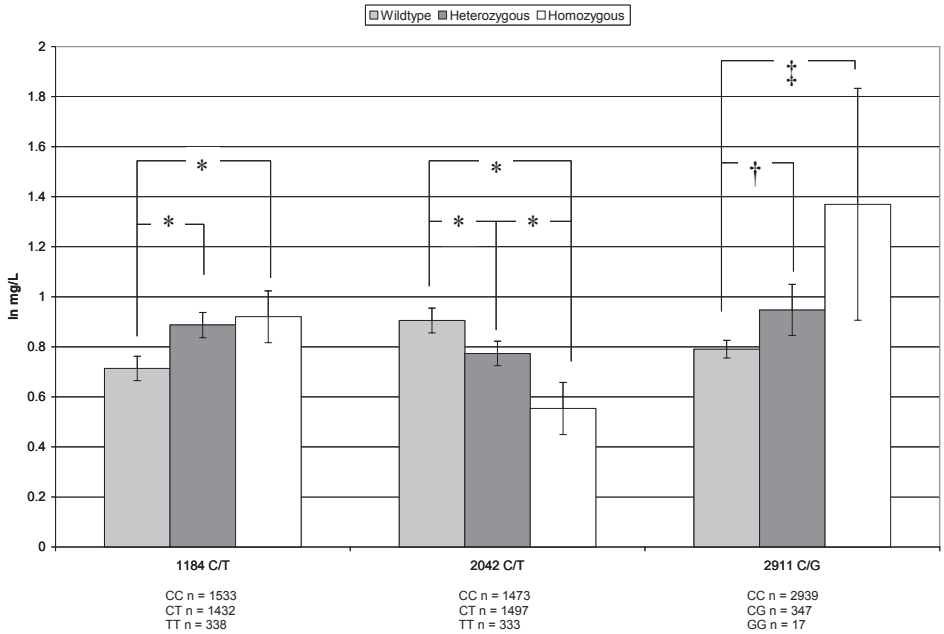
Model 3: model 2 + measures of atherosclerosis

CRP Serum Level by Genotype and Haplotype

CRP serum levels according to the genotype of the three CRP polymorphisms are shown in Figure 2. For all three polymorphisms we observed an allele dose effect (all p<0.001).

Differences in CRP serum level according to haplotypes are shown in Table 3. In comparison to the reference haplotype 1, the other haplotypes had a significantly higher serum level of

Figure 2. CRP level by CRP Genotype



Age and sex adjusted

Trend p<0.001 (all polymorphisms)

* p < 0.001

† p=0.005

‡ p=0.015

Table 3. CRP levels by Haplotype of the CRP gene

Haplotype	n	CRP Level (mg/L)	95% CI
1*	1873	0.67	0.63 – 0.72
2	1809	0.87†	0.83 – 0.91
3	1716	0.79†	0.74 – 0.83
4	334	0.92†	0.81 – 1.02

Based on ln-transformed CRP (C-reactive protein) levels
Age and sex adjusted
*Haplotype 1 is reference
†Significant difference in comparison with reference haplotype 1, all p<0.001

CRP (all p<0.001). Haplotype 2, 3 and 4 all contained one or more of the alleles associated with an increased CRP serum level in the genotype analyses.

CRP Gene and Pulse Wave Velocity

No significant associations between genotypes of the 1184 C/T or 2024 C/G polymorphisms and pulse wave velocity were found. Those with the 2911-GG genotype (n=18, mean 14.7 m/s, CI 13.6-15.9) had a higher PWV compared with CG heterozygotes (n=336, mean 13.4 m/s, CI 13.1-13.7) (p=0.03), but no significant trend was found, p=0.66. The haplotype-based analyses yielded no significant relations with arterial stiffness (Table 4).

Table 4. Measures of Arterial Stiffness by CRP Haplotype

		Pulse Wave Velocity			**Distensibility Coefficient**			**Pulse Pressure**		
Model	**Haplotype**	***n***	**m/s**	**CI**	***n***	**1/MPa**	**CI**	***n***	**mmHg**	**CI**
1	1	1961	13.57	13.46 - 13.68	1732	10.38	10.22 - 10.54	2346	68.3	67.6 - 69.0
	2	1903	13.67	13.56 - 13.78	1703	10.41	10.25 - 10.57	2285	68.5	67.8 - 69.2
	3	1817	13.55	13.44 - 13.67	1597	10.42	10.26 - 10.58	2142	67.9	67.2 - 68.6
	4	359	13.54	13.28 - 13.80	307	10.50	10.13 - 10.87	423	68.7	67.1 - 70.3
2	1	1961	13.57	13.46 - 13.68	1732	10.38	10.23 - 10.54	2346	68.3	67.6 - 68.9
	2	1903	13.66	13.55 - 13.77	1703	10.41	10.26 - 10.57	2285	68.5	67.8 - 69.1
	3	1817	13.56	13.45 - 13.67	1597	10.42	10.26 - 10.59	2142	67.9	67.2 - 68.6
	4	359	13.53	13.27 - 13.78	307	10.45	10.08 - 10.81	423	68.7	67.1 - 70.3
3	1	1961	13.57	13.47 - 13.68	1732	10.39	10.23 - 10.54	2346	68.2	67.6 - 68.9
	2	1903	13.67	13.56 - 13.78	1703	10.42	10.26 - 10.57	2285	68.5	67.8 - 69.2
	3	1817	13.55	13.44 - 13.66	1597	10.41	10.25 - 10.57	2142	67.9	67.2 - 68.6
	4	359	13.52	13.27 - 13.77	307	10.45	10.08 - 10.81	423	68.7	67.2 - 70.3

All differences in measures of stiffness between the genotypes were not significant
CRP = C-reactive protein
Model 1: age, sex (and for pulse wave velocity and the distensibility coefficient also for mean arterial pressure and heart rate)
Model 2: model 1 + body mass index, total + HDL-cholesterol, smoking, diabetes mellitus
Model 3: model 2 + measures of atherosclerosis

CRP Gene and Distensibility Coefficient

Subjects with the 1184 TT genotype had a lower distensibility coefficient than heterozygotes (p = 0.04), also after adjustment for cardiovascular risk factors (Figure 4). The association, however, did not remain significant after additional adjustment for atherosclerosis. For polymorphisms 2042 C/G and 2911 C/T no significant differences in the distensibility coefficient between the genotypes were found. In the haplotype-based analyses no significant differences were found (Table 4).

CRP Gene and Pulse Pressure

No significant associations were found of 1184 C/T (p=0.38), 2042 C/T (p=0.96) or 2911 C/G (p=0.49) with pulse pressure. The haplotype analyses also yielded no significant associations (Table 4).

Overall, analyses specified by gender yielded no essentially different results (data not shown).

DISCUSSION

We studied the association of the CRP polymorphisms 1184 C/T, 2042 C/T, 2911 C/G and haplotypes with arterial stiffness within the Rotterdam study. We found the CRP serum level to be positively associated with pulse wave velocity and with pulse pressure, independent of cardiovascular risk factors. The minor alleles of the 1184 C/T and 2911 C/G polymorphisms were positively associated with the CRP serum level, and the minor allele of the 2042 C/T polymorphism inversely. Overall, no consistent significant differences between the genotypes and haplotypes of the CRP gene in pulse wave velocity, the distensibility coefficient or pulse pressure were found.

The CRP 1184 C/T, 2042 C/T and 2911 C/G polymorphisms were chosen because together, they represent the total common genetic variation across the CRP gene. By genotyping these three haplotype tagging SNPs we were able to infer all four haplotypes, that describe the common variation across the CRP gene. Few studies have used this approach.[27-29] Miller et al. and Carlson et al. used more tagging SNPs than in our study to describe common variation in the CRP gene.[27,29] In these studies, however, ethnic diverse populations were used. For example, the population used in the study by Carlson et al. consisted of participants partly of European descent and partly of African descent. Therefore, more and other haplotypes were found and compared with the population of European descent used in our study. Both Miller et al. and Carlson et al. report associations between haplotypes and CRP levels that are in agreement with the associations between haplotypes and CRP levels in our study.[28]

While the studied genotypes and haplotypes were associated with CRP serum level, we found no consistent relation of the geno- and haplotypes of the CRP gene with arterial

stiffness. Only 2911-GG homozygotes had a higher pulse wave velocity in comparison to heterozygotes. Although this is in line with the association of the polymorphism with an increased CRP serum level, one has to take into account that the number of homozygous subjects was limited; this finding may therefore have been a chance finding.

The absence of a relation between variation in the CRP gene with measures of arterial stiffness might suggest that the relation between the CRP serum level and arterial stiffness is not causal. The effect of variation in the CRP gene on CRP serum level, however, was only modest. Haplotype 2, 3 and 4 were associated with a higher CRP serum level compared with haplotype 1. The differences, however, varied only from 0.12-0.25 mg/L. In general, effects of genetic variation on a trait with a complex and multifactorial pathogenesis, such as arterial stiffness, are generally modest. Therefore, a small effect of genetic variation in CRP on arterial stiffness may have gone undetected and a judgment about causality cannot be given with certainty.

The associations between the CRP polymorphisms/haplotypes and the level of CRP was described earlier in the Rotterdam Study.[28] The same applies for the association between the CRP level (n=866) and pulse wave velocity.[14] These studies, however, were based on CRP levels obtained at baseline. In the current study, we have used CRP levels obtained during the third examination phase of the Rotterdam Study. Our results are in concordance with these earlier results.

Although we found no clear association of genetic variation in the CRP gene and arterial stiffness, an association between the CRP level and arterial stiffness is nonetheless biologically plausible and may be explained in several ways.[14] Increased CRP levels and impaired endothelial dysfunction have found to be related before.[30-34] Fichtlscherer et al. described CRP to be associated with decreased endothelial vasodilator function.[34] CRP level is also described to be associated with various markers of endothelial dysfunction.[32,33] Inflammatory processes inhibit endothelium-dependent vasodilatation.[14,35] The endothelium itself produces vasoactive substances, such as nitric oxide, which have been demonstrated to play a significant role in peripheral resistance, blood pressure and vascular reactivity.[36,37] Furthermore, agonists that stimulate endothelial nitric oxide release, such as acetylcholine, reduce stiffness of muscular arteries in vivo.[38,39] Basal nitric oxide production has been demonstrated to influence muscular arteries distensibility in vivo positively and the effect of acetylcholine on large arteries is also mainly nitric oxide-mediated.[40] Increased CRP levels decrease nitric oxide production.[31] Therefore, as endothelium obviously plays an important role in the regulation of arterial stiffness, CRP-related impairment of endothelial function may very well lead to changes in arterial stiffness.[14]

Our study is based on a large ongoing population-based study, in a relatively homogeneous population, as 98% of the participants in our study are Caucasian and are all living in the same area, a suburb of Rotterdam. We used both genotype and haplotype based analyses. We adjusted all analyses for established risk factors and measures of atherosclerosis.

To interpret the findings correctly, several methodological aspects of the measures of arterial stiffness need to be discussed. First, pulse waves in the carotid artery and the femoral artery travel in opposite directions, while measurements of carotid-femoral pulse wave velocity is based on the assumption that the pulse wave travels from the carotid artery to the femoral artery. In this way, measuring the distance between the carotid and the femoral artery lead to an overestimation of the distance between the sites of the pulse waves resulting in overestimation of the velocity of the pulse waves. However, variations in anatomy are limited and this error may be considered similar for all subjects examined, therefore we do not think it has seriously biased our results. Second, the distance between the carotid and the femoral artery may be overestimated in (especially obese) subjects when this distance is measured by tape. To avoid this error we adjusted the analyses for body mass index. Third, in computing the carotid distensibility coefficient, we used the brachial pulse pressure rather than the carotid pulse pressure. Information on comparisons between the carotid and the brachial pulse pressure indicates that the carotid pulse pressure is lower than the brachial pulse pressure, but differences are relatively small.[41]

Data on measures of stiffness were not available for all subjects who visited the research center. Missing information was primarily due to logistic reasons, which is likely to be random and thus will not have biased our results. Furthermore, the advantage of the allele-based haplotype analysis is the increase in power in comparison with genotype-based analyses. However, the allele-based haplotype analyses offer no information on possible dominance or recessiveness. Finally, because our study was performed in a population of predominantly elderly Caucasian subjects, the generalizability of our findings to younger individuals or other ethnicities remains uncertain.

In conclusion, genetic variation in the CRP gene, as expressed by polymorphisms 1184 C/T, 2042 C/T and 2911 C/G and concurrent haplotypes, is not consistently and significantly associated with arterial stiffness. However, we cannot exclude a small effect, which was not detected in our study. Further and larger studies are needed for confirmation and to further elucidate the role of genetic variation in the CRP gene in arterial stiffness.

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Chapter 3.3

Variation in the Transforming Growth Factor-β1 Gene and Arterial Stiffness

Background and purpose - Arterial stiffness is a risk factor for cardiovascular disease. Transforming growth factor β1 is a pleiotropic cytokine, with many functions, including influence on the vascular wall (e.g., on angiogenesis, endothelial cells and the extra-cellular matrix). We investigated five functional polymorphisms in the Transforming Growth Factor β1 gene (-800 G/A, -509 C/T, codon 10 Leu/Pro, codon 25 Arg/Pro and codon 263 Thr/Ile) in relation to arterial stiffness in a population-based study.

Methods - A total of 3863 participants of the Rotterdam Study, a prospective population-based study, were included in the current study. The relations of the genotypes and haplotypes with arterial stiffness (pulse wave velocity, distensibility coefficient and pulse pressure) were studied using analyses of variance and linear regression. The analyses were adjusted for age, sex, mean arterial pressure, heart rate, conventional cardiovascular risk factors and measures of atherosclerosis.

Results - There were no associations between pulse wave velocity and -800 G/A (p=0.56), -509 C/T (p=0.29), codon 10 (p=0.98), codon 25 (p=0.28). These polymorphisms were not associated with the distensibility coefficient or with pulse pressure. The haplotype based analyses yielded similar results.

Conclusions - The results of this study show that the TGF-β1 -800 G/A, -509 C/T, codon 10 Leu/Pro and codon 25 Arg/Pro polymorphisms are not associated with arterial stiffness.

INTRODUCTION

With increasing age, also arterial stiffness increases. Increased arterial stiffness is also associated with hypertension, diabetes mellitus (DM), end-stage renal disease and atherosclerosis.[1-6] Recently, it has also been shown to be associated with increased risk of cardiovascular disease.[7-9] The extent of the increase in stiffness may depend on various factors; it is likely that genetic variations play a role. Indeed, a recent study described heritability estimates of arterial stiffness (varying from 0.26 - 0.36).[10]

Transforming growth factor β (TGF-β) is a pleiotropic cytokine. In man, three isoforms (with largely similar functions) exist, of which TGF-β1 is the most common.[11] Its effects include influence on T-cells, on the release of (other) cytokines and fibroblast growth factors, but it also exerts influence on the vascular wall, e.g. in angiogenesis, on endothelial cells and on modulation of the extra-cellular matrix.[12-17] TGF-β also regulates (vascular) smooth muscle cell differentiation.[18-21] Robertson et al. described that disruption of TGF-β signaling in mice led to decreased collagen in plaques. Others also found TGF-β to be associated with collagen upregulation in the vascular wall.[13,15,18] Also Mallat et al. described TGF-β as a pro-fibrotic cytokine.[14] Increased TGF-β has also been implicated in the fibrosclerotic process in varicose vessel walls.[22] Variations in bioavailability of TGF-β levels may therefore influence arterial stiffness and the TGF-β gene is a candidate gene for further study of variation in arterial stiffness.

The TGF-β1 gene (23.5 kb) is located on chromosome 19q13.2. There are several commonly known (potentially) functional polymorphisms in this gene. Cambien et al. described the -988 C/A, -800 G/A, -509 C/T polymorphisms (promoter region); a C-insertion at position +72 (non-translated region); and in addition codons 10 Leu/Pro (c10) and 25 Arg/Pro (c25) (signal peptide sequence) and 263 Thr/Ile (c263) (precursor part of the protein).[23] Of these, +72 was in almost complete linkage disequilibrium with c25, while -988 C/A was extremely rare.[23] Grainger et al. described the -509 C/T polymorphism to be associated with levels of TGF-β1.[24]

The TGF-β1 -800 G/A and -509 C/T polymorphisms are located in the promoter region; the full extent of their effect is still unknown, but due to their location, they are considered possible modulators of expression of the TGF-β gene and levels.[23-25] The c10 and c25 polymorphisms are located in the signal peptide sequence, which is involved in export of synthesized proteins across membranes of the endoplasmatic reticulum, thereby possibly influencing TGF-β levels.[23] The c10 and c25 polymorphisms are also located at potentially important positions that influence activation of the TGF-β protein.[25]

To the best of our knowledge, these (potentially) functional polymorphisms have not been investigated in association with arterial stiffness before. We studied the association of the TGF-β1 -800 G/A, -509 C/T, c10, c25 and c263 polymorphisms and arterial stiffness in a large population-based study.

METHODS

The Rotterdam Study is an ongoing prospective cohort study including 7983 participants of 55 years and older. Its general aims are to investigate determinants of chronic diseases.[26] During the first phase of this study (1990-1993), all inhabitants of a Rotterdam suburban area (Ommoord) aged 55 years and over were invited to participate in this study. The response rate was 78%. The third examination phase took place from 1997-1999, during which measures of arterial stiffness were performed. Approval of the Medical Ethics Committee of the Erasmus University Rotterdam was obtained for the Rotterdam Study. From all participants written informed consent was acquired. A more in depth description of the Rotterdam Study was published previously.[26]

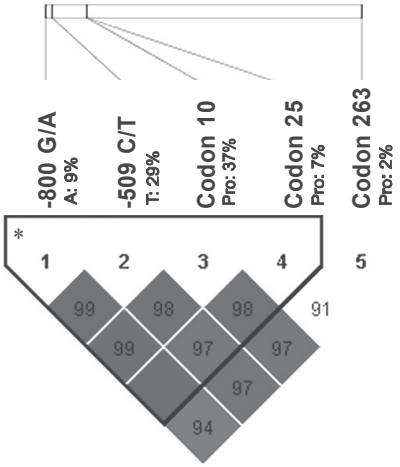
Genotyping

Genomic DNA was extracted from samples of peripheral venous blood according to standard procedures. 1-2 ng genomic DNA was dispensed into 384-wells plates using a Caliper Sciclone ALH3000 pipetting robot (Caliper LS, Mountain View, CA, USA). Genotypes were determined using the Taqman allelic discrimination assay. The Assay-by-Design service (www.applied-biosystems.com) was used to set up assays for the 800 G/A *[rs1800468]*, -509 C/T *[rs1800469]*, codon 10 Leu/Pro [T/C] *[rs1982073]*and codon 263 Thr/Ile [C/T] *[rs1800472]* polymorphisms. For codon 25 Arg/Pro [G/C] *[rs1800471]*an assay was set up using primer express 2.0 software (Applied Biosystems Inc., Foster City, CA, USA). The assays-by-design consisted of 5 ng of genomic DNA in a 2 ul volume and the following reagents: FAM and VIC probes (400 nM), primers (1,8 uM), 2x Taqman PCR master mix (ABgene). The assay for codon 25 consisted of 5 ng of genomic DNA in a 4 ul volume and the following reagents: FAM probes (250 nM), TET probe (500 nM), primers (300 nM), 2x Taqman PCR master mix (ABgene). Reagents were dispensed in a 384-well plate using the Deerac Equator NS808 (Deerac Fluidics, Dublin, Ireland). PCR cycling reaction were performed in 384 wells PCR plates in an ABI 9700 PCR system (Applied Biosystems Inc., Foster City, CA, USA) and consisted of initial denaturation for 15 minutes at 95° C, and 40 cycles with denaturation of 15 seconds at 95° C and annealing and extension for 60 seconds at 60° C. Results were analysed by the ABI Taqman 7900HT using the sequence detection system 2.22 software (Applied Biosystems Inc., Foster City, CA, USA). To confirm the accuracy of genotyping results, 332 (5%) randomly selected samples were re-genotyped with the same method. All polymorphisms had an error rate lower than 1%. The polymorphisms were selected based on (potential) functionality (-800 G/A and -509 C/T located in promotor; codons 10, 25 and 263 resulting in an amino-acid change) and literature, describing previous research on these polymorphisms and cardiovascular disease.

Haplotyping

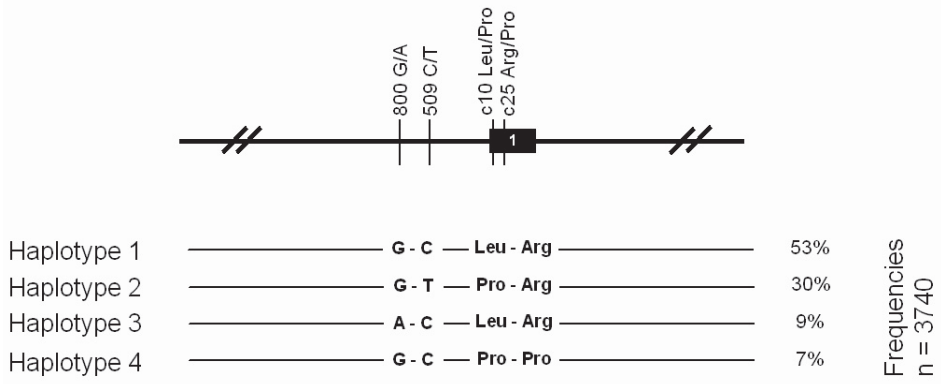
Haplotypes were reconstructed using PHASE software (http://archimedes.well.ox.ac.uk). Genotype data of the four TGF-β1 polymorphisms in Hardy Weinberg equilibrium (-800 G/A, -509 C/T, codon 10 Leu/Pro and, codon 25 Arg/Pro) enabled us to distinguish the four most common haplotypes. They were coded as 1 to 4 in order of decreasing frequency in the population (coding from -800 G/A, -509 C/T, c10 Leu/Pro, c25 Arg/Pro: haplotype 1 = G-C-Leu-Arg, haplotype 2 = G-T-Pro-Arg, haplotype 3 = A-C-Leu-Arg, haplotype 4 = G-C-Pro-Pro) (Figures 1 & 2).

Figure 1. Linkage Disequilibrium between TGF-β1 Polymorphisms



LD coefficient (D') between TGF-β1 Polymorphisms
Allele frequencies of risk allele (%)
* Polymorphisms in analyses

Figure 2. Overview of TGF-β1 Gene, Polymorphisms and Haplotypes



Exon 1 is depicted by the block
Individual polymorphisms are depicted by the vertical lines

Arterial Stiffness

In this study three measures of arterial stiffness were used: the carotid-femoral pulse wave velocity (PWV, or aortic stiffness) as a measure of aortic stiffness, the distensibility coefficient (DC, or carotid stiffness) of the common carotid artery as a measure of common carotid arterial stiffness and, in addition, pulse pressure (PP) was assessed as an indicator of arterial stiffness. All measures were obtained on the same day, during the same session, during the third follow-up examination.

Carotid-femoral PWV (m/s) was measured using an automatic device (Complior, Colson) and was calculated as the ratio between the distance travelled by the pulse wave and the foot-to-foot time delay.

Common carotid artery distensibility was assessed by measuring the vessel wall motion of the right common carotid artery using a duplex scanner (ATL Ultramark IV, operating frequency 7.5 MHz) connected to a vessel wall movement detector system.[27,28] The cross-sectional arterial wall distensibility coefficient (DC) (1/MPa) was calculated as a measure of arterial stiffness.[29] A decreased distensibility coefficient implies increased carotid stiffness.

Pulse pressure (PP) (mmHg) was defined as the difference between systolic and diastolic blood pressure, using the mean systolic and diastolic blood pressure of two measurements obtained by measuring blood pressure on the right arm using a random-zero sphygmomanometer.

Details on all measures of stiffness, have been described previously.[6,9]

Clinical Characteristics

We collected information on cardiovascular risk factors during the third follow-up examination. During the home interview information was collected on medication use and smoking habits. Smoking was classified as never, former or current smoking. At the research centre, blood pressure was measured twice on the right arm using a random-zero sphygmomanometer. The average of the two blood pressure values was used in the analyses. Hypertension was defined as having a systolic blood pressure of >= 160 mmHg and/or diastolic blood pressure of >= 100 mmHg and/or blood pressure lowering medication with an indication for hypertension. Height and weight were measured and body mass index (kg/m^2) (BMI) was calculated. Serum total cholesterol and high-density lipoprotein (HDL) cholesterol values were determined by an automated enzymatic procedure (Boehringer Mannheim System). Diabetes mellitus (DM) was defined as use of anti-diabetic medication and/or a fasting serum glucose level of equal to or above 7.0 mmol/L.[30] Evaluation of the atherosclerotic status of participants was accomplished using ultrasonography (carotid intima-media thickness [IMT]) and radiography (aorta calcification); these methods have been extensively described previously.[31,32]

Population for Analysis

A total of 4024 subjects underwent the physical examination of the third phase and PWV was measured in 3550 subjects; 69 subjects (1.9%) were excluded from the analyses because of poor quality of the PWV recordings, leaving 3481 subjects, whereas common carotid distensibility was measured in 3098 subjects. Information on the -800 G/A polymorphism and PWV measurements were available for 3221 subjects and on distensibility for 2845 subjects. For the -509 C/T polymorphism the corresponding numbers were 3174 and 2804 subjects. For codon 10, codon 25 and codon 263 corresponding numbers were 3113 and 2750 subjects. Data on pulse pressure was available for 3847 genotyped subjects. A total of 3863 subjects were successfully genotyped for at least one of the polymorphisms and information was available on either PWV, DC or PP. Of these, 3740 subjects were successfully haplotyped. Missing information on measures of arterial stiffness was almost entirely due to logistic reasons.

Statistical Analyses

Chi-square tests were performed to test for deviations from Hardy-Weinberg equilibrium (HWE). Missing data in clinical characteristics were imputed using Expectation-Maximization algorithms available in SPSS. The associations between the genotypes and haplotypes and arterial stiffness were investigated using analyses of variance and linear regression. The haplotype analyses were allele-based: each haplotype-allele (as opposed to a a haplotype-allele pair) is considered and analyzed separately, using the outcomes and characteristics of the subject carrying that allele. The most common haplotype-allele is the reference (i.e., haplotype 1). All analyses were adjusted for age and sex, and (if applicable) additionally for mean arterial pressure (MAP), heart rate, body mass index, high-density lipoprotein (HDL) and total cholesterol levels, smoking, diabetes mellitus and measures of atherosclerosis (carotid IMT and aorta calcification). A p-value of 0.05 and smaller was considered significant in all analyses. The statistical analyses were performed using SPSS version 11.0.1 for MS-Windows.

RESULTS

Genotype and allele proportions were in Hardy Weinberg Equilibrium, except for c263 ($p = 0.00078$). Therefore, this polymorphism genotype was not included in the analyses. Haplotypes are shown in Figures 1 & 2. General characteristics are described in Table 1.

No significant association was found between PWV and any of the polymorphisms, either in unadjusted analyses (model 1) or adjusted analyses (models 2 and 3) (Table 2). None of the tests for trend were significant (-800 G/A $p = 0.559$, -509 C/T $p = 0.294$, c10 $p = 0.977$ or c25 $p = 0.282$). Full adjustment did not alter the findings. The haplotype-based analyses also showed no differences in PWV between the haplotypes (Table 3).

Table 1. General Characteristics

Characteristic	
Total number	3863
Age – yrs	72.4 ± 7.0
Male sex – %	43
Body mass index – kg/m^2	27 ± 7.0
Systolic blood pressure – mm Hg	143 ± 21
Diastolic blood pressure – mm Hg	75 ± 11
Mean Arterial Pressure – mm Hg	98 ± 13
Hypertension – %	33
Use of antihypertensives* – %	39
Total cholesterol – mmol/l	5.8 ± 1.0
HDL-cholesterol – mmol/l	1.4 ± 0.4
Smoking – % Current	30
Former	38
Never	32
Diabetes – %	9
Pulse Wave Velocity† – m/s	13.6 ± 3.0
Distensibility Coefficient‡ – 1/MPa	10.4 ± 4.3
Pulse Pressure – mm Hg	68.2 ± 17.5
Intima Media Thickness – mm	0.78 ± 0.13
Aorta calcifications§ – %	22

Continuous values are depicted as mean ± SD

*Based on any use of diuretics, β-blockers, Ca-antagonists, ACE-inhibitors and other antihypertensives

† Based on all participants with PWV information available (n = 3221)

‡ Based on all participants with DC information available (n = 2845)

§ Percentage of subjects with aorta calcification over a length of ≥ 2.5 cm

The distensibility coefficient showed no association with any of the polymorphisms, except for a lower DC for subjects with the -509 TT in comparison to those with the CT-genotype (Table 2). No overall trends were shown for the -800 G/A polymorphism (p = 0.320), the -509 C/T (p = 0.376), c10 (p = 0.215) or c25 (p = 0.866) in the unadjusted analyses. Further adjustment did not alter these results. Also the haplotype-based analyses showed no differences in distensibility coefficient between any of the haplotypes (Table 3).

No association between pulse pressure and any of the genotypes was shown (Table 2). Also no trends were shown (-800 G/A p = 0.895; -509 C/T p = 0.549; c10 p = 0.824 and c25 p = 0.433). Further adjustment did not alter these results. Also the haplotype-based analyses showed no differences in pulse pressure between any of the haplotypes (Table 3).

The gender-stratified analyses showed no results essentially different from the overall analyses (data not shown).

Table 2. Measures of Arterial Stiffness by TGF-β Genotype

		n	Pulse Wave Velocity (m/s)	p trend	n	Distensibility Coefficient (1/MPa)	p trend	n	Pulse Pressure (mm Hg)	p trend
–800 G/A	**GG**	2669	13.58 (13.49-13.68)	0.559	2349	10.40 (10.27-10.54)	0.320	3203	68.20 (67.62-68.79)	0.895
	GA	525	13.54 (13.33-13.76)		472	10.57 (10.27-10.87)		613	68.12 (66.78-69.46)	
	AA	27	13.24 (12.31-14.17)		24	10.56 (9.24-11.89)		31	67.94 (61.98-73.90)	
–509 C/T	**CC**	1576	13.56 (13.44-13.68)	0.294	1392	10.43 (10.26-10.60)	0.376	1887	68.37 (67.60-69.13)	0.549
	CT	1279	13.59 (13.46-13.73)		1118	10.54 (10.34-10.73)		1526	68.07 (67.22-68.92)	
	TT	319	13.74 (13.47-14.01)		294	10.06 (9.68-10.44)		385	67.93 (66.24-69.62)	
Codon 10 Leu/Pro	**Leu/ Leu**	1231	13.63 (13.49-13.77)	0.977	1087	10.49 (10.29-10.69)	0.215	1482	68.14 (67.28-69.00)	0.824
	Leu/ Pro	1413	13.53 (13.40-13.66)		1233	10.46 (10.27-1064)		1677	68.27 (67.46-69.08)	
	Pro/ Pro	469	13.69 (13.47-13.92)		430	10.22 (9.91-10.54)		565	68.30 (66.90-69.69)	
Codon 25 Arg/Pro	**Arg/ Arg**	2670	13.61 (13.52-13.71)	0.282	2353	10.43 (10.30-10.56)	0.866	3199	68.15 (67.56-68.73)	0.433
	Arg/ Pro	432	13.46 (13.23-13.70)		381	10.51 (10.18-10.85)		507	68.55 (67.08-70.02)	
	Pro/ Pro	11	13.73 (12.27-15.19)		16	9.141 (7.52-10.77)		18	71.86 (64.06-79.66)	

Measurements are depicted as: mean (95% confidence interval)
Adjusted for age and sex (and for pulse wave velocity and the distensibility coefficient also for mean arterial pressure and heart rate)

Table 3. Measures of Arterial Stiffness by TGF-β Haplotype

		n	Pulse Wave Velocity (m/s)	p trend	n	Distensibility Coefficient (1/MPa)	p trend	n	Pulse Pressure (mm Hg)	p trend
Haplotype	**1**	3294	13.60 (13.52-13.69)	0.239	2889	10.46 (13.34-13.58)	0.937	3961	68.23 (67.70-68.75)	0.813
	2	1832	13.63 (13.51-13.74)		1630	10.35 (10.19-10.51)		2187	68.11 (67.40-68.81)	
	3	563	13.50 (13.30-13.71)		504	10.57 (10.28-10.862)		658	68.00 (66.72-69.29)	
	4	451	13.46 (13.23-13.68)		410	10.42 (10.10-10.74)		540	68.70 (67.28-70.12)	

Measurements are depicted as: mean (95% confidence interval)
Adjusted for age and sex (and for pulse wave velocity and the distensibility coefficient also for mean arterial pressure and heart rate)

DISCUSSION

We studied four well-known functional TGF-β1 polymorphisms (-800 G/A, -509 C/T, c10, c25) in relation to arterial stiffness. None of the polymorphisms were consistently and significantly associated with either pulse wave velocity, distensibility coefficient or pulse pressure in both genotype and haplotype-based analyses.

To the best of our knowledge, there are no previous studies on these polymorphisms and their association with arterial stiffness.

Although the TGF-β polymorphisms may influence expression or activity of TGF-β, and this, in turn, may influence arterial stiffness, we did not find these polymorphisms to be associated with arterial stiffness. We consider the finding of a significant difference in the distensibility coefficient between subjects with the -509 CT and TT genotype no valid evidence for an association between the polymorphism and arterial stiffness, especially because this isolated finding was not supported by a corroborating trend or findings for PWV or PP. We hypothesize that any effects that these TGF-β polymorphisms might have on the vascular wall, do not lead to significant changes in arterial stiffness.

Our study is based on a large ongoing population-based study in a relatively homogeneous population, as 98% of the participants in our study are Caucasian and are all living in the same area, a suburb of Rotterdam. We used both genotype and haplotype based analyses. The distensibility coefficient has a strong correlation with mean arterial pressure. A high mean arterial pressure in the artery especially stretches the collagen fibers in the arterial wall, making the arteries less distensible. Consequently, the analyses were also adjusted for mean arterial pressure. We adjusted all analyses for established risk factors. As atherosclerosis and arterial stiffness are associated, we additionally adjusted for atherosclerosis.[6]

To interpret the findings correctly, several methodological aspects of the measures of arterial stiffness need to be discussed. First, pulse waves in the carotid artery and the femoral artery travel in opposite directions, while measurements of carotid-femoral pulse wave velocity is based on the assumption that the pulse wave travels from the carotid artery to the femoral artery. In this way, measuring the distance between the carotid and the femoral artery lead to an overestimation of the distance between the sites of the pulse waves resulting in overestimation of the velocity of the pulse waves. However, variations in anatomy are limited and this error may be considered similar for all subjects and genotypes examined, therefore we do not think it has seriously biased our results. Second, the distance between the carotid and the femoral artery may be overestimated in (especially obese) subjects when this distance is measured by tape; to avoid this error we adjusted the analyses for body mass index.[9] Third, in computing the carotid distensibility coefficient, we used the brachial pulse pressure rather than the carotid pulse pressure.[9] Information on comparisons between the carotid and the brachial pulse pressure indicates that the carotid pulse pressure is lower than the brachial pulse pressure but differences are relatively small.[33] Fourth, large topographic/anatomical

differences exist in the structure and function of the arterial system. As a consequence, arterial stiffness cannot be assessed by a single measurement at any random site within the arterial system. In the Rotterdam Study we use three diverse measures of arterial stiffness. We used pulse wave velocity, as this is the most generally accepted and robust measure of arterial stiffness in general.[34] Since the local measure of the carotid distensibility coefficient may also be of prognostic importance and offer additional information, we also used the carotid distensibility coefficient as a measure of stiffness.[34] Additionally we used pulse pressure. This is not a direct measurement of stiffness, but it can be used as an additional non-invasive and readily obtainable indicator for arterial stiffness.

Furthermore some general remarks need to be made. The effects of genetic variation on a trait with a complex and multifactorial pathogenesis, such as arterial stiffness, are generally modest. Power estimates for all analyses in this study were generally low (<80%). Our negative findings, therefore, do not exclude the possibility that small effects were not detected, however, it is unlikely that large effects were missed. Further, data on measures of stiffness were not available for all subjects. However, missing information was primarily due to logistic reasons, which is likely to be random and is unlikely to have introduced bias in our results. Furthermore, the cross-sectional design may limit the ability to infer a causal relationship between the TGF-β1 polymorphisms and arterial stiffness. Also, the generalizability of our findings to younger individuals or persons of other ethnicities remains uncertain, due to the fact that our population consisted of (mostly) elderly Caucasion subjects. Furthermore, the advantage of the allele-based haplotype analysis is the increase in power in comparison with genotype-based analyses. Also, the polymorphisms used in this study were selected based on potential functionality and literature. They were not selected as 'tagging' polymorphisms to cover all genetic variation in the TGF-β1 gene. Finally, the relation between the polymorphisms/haplotypes, arterial stiffness and plasma levels of TGF-β could not be investigated, because, unfortunately, no TGF-β levels were measured in the Rotterdam Study.

In conclusion: we found the TGF-β1 -800 G/A, -509 C/T, codon 10 Leu/Pro and codon 25 Arg/Pro polymorphisms not to be associated with arterial stiffness, either in genotype or haplotype-based analyses. However, we cannot exclude a small effect, which was not detected in our study. Additional studies are needed for confirmation and to further elucidate the effects of these potentially functional TGF-β1 polymorphisms on the vascular wall.

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Chapter 3.4

Variation in the Fibrinogen α and γ Genes and Arterial Stiffness

Background and purpose - Arterial stiffness increases with age and has been found to predict cardiovascular disease. Fibrinogen is an acute phase protein and some studies showed an association with arterial stiffness. We studied genetic variation in the fibrinogen α (FGA) and γ (FGG) gene, by means of seven SNPs (FGA: -58 G/A, 1374 G/A, 1526 T/C and 312 Thr/Ala, and FGG: 4288 G/A, 6326 G/A and 7792 T/C) and the resultant haplotypes in relation to arterial stiffness.

Methods - This study (n=3891) was embedded in the Rotterdam Study, a prospective, population-based study among subjects aged 55 years and older. Analyses on the associations of the fibrinogen plasma level, genotypes and haplotypes with aortic stiffness (pulse wave velocity), carotid stiffness (distensibility coefficient) and pulse pressure were performed using analyses of variance, linear regression and by using haplotypes analyses with haplotype 1 as reference. The analyses were adjusted for age, sex, and, additionally, for mean arterial pressure, heart rate, known cardiovascular risk factors and measures of atherosclerosis.

Results - Genotype analyses yielded significant associations of the FGA 1526 T/C (p=0.004), the FGA 312 Thr/Ala (p=0.008) and FGG 6326 G/A (p=0.024) polymorphisms with the fibrinogen plasma level. There were no consistent associations with measures of arterial stiffness at the genotype level. The fibrinogen plasma level was positively associated with aortic stiffness (p=0.015) and pulse pressure (p=0.049). Haplotype analyses showed FGA haplotype 4 to be associated with the fibrinogen plasma level (p=0.01) and pulse pressure (p<0.001). FGA haplotype 3 and FGG haplotype 4 were associated with aortic stiffness (p=0.03) and pulse pressure (p<0.001). No associations were found with carotid stiffness.

Conclusions - These findings indicate that the fibrinogen plasma level and genetic variation in the fibrinogen α and γ gene may influence arterial stiffness. Further research is needed to confirm these findings and to elucidate the underlying mechanisms.

INTRODUCTION

Normally, arterial stiffness increases with age and has been associated with hypertension, diabetes mellitus, end-stage renal disease and atherosclerosis.[1-6] Arterial stiffness was found to predict cardiovascular events in various populations.[7-10]

Acute-phase reactants and other inflammatory markers were found to be associated with arterial stiffness.[11-17] Fibrinogen is a well-known coagulation factor, but in addition, it is an important acute-phase protein and it influences several processes, such as inflammation, angiogenesis and matrix interactions.[18,19] Fibrinogen levels were previously associated with cardiovascular disease, which was recently reaffirmed in a large meta-analysis.[20] Fibrinogen levels were also been observed to be associated with arterial stiffness. Among 114 Japanese diabetics, fibrinogen levels were higher among those with a high cardio-ankle vascular index, a measure of arterial stiffness.[15] This was also shown in 2709 participants of the Strong Heart Study, where stiffness, assessed by pulse pressure/stroke index, was higher in those with elevated fibrinogen levels.[16] In 2262 healthy Swedish men from the Malmö Diet and Cancer Study, elevated levels of fibrinogen and other inflammation-sensitive proteins were associated with increased pulse pressure.[17] Overall, the heritability of the fibrinogen level was estimated at 45%, which suggests that genetic variation influences the bioavailability of fibrinogen.[21] Therefore, fibrinogen genes are candidate genes for further study of determinants of arterial stiffness.

Fibrinogen consists of two sets of three peptide chains (Aα, Bβ and γ) and is encoded by three genes (fibrinogen α [FGA], fibrinogen β [FGB] and fibrinogen γ [FGG]) located on chromosome 4q28 in a region of approximately 50 kb. The FGA and FGG genes are in close physical proximity and show a high degree of linkage disequilibrium ($D' \geq 0.94$) between them. Various polymorphisms were identified in the FGA and FGG genes. Common (haplotype-tagging) polymorphisms in the FGA gene are -58 G/A, 1374 G/A, 1526 T/C and 312 Thr/Ala and in the FGG gene 4288 G/A, 6326 G/A and 7792 T/C. Several of these were found to influence blood clot structure, clinical outcomes (such as embolisms, deep venous thrombosis and myocardial infarction) and fibrinogen levels.[22-28] To our knowledge, however, these fibrinogen polymorphisms were not previously studied in relation to arterial stiffness.

We studied genetic variation in the closely related FGA and FGG genes, using seven polymorphisms (FGA: -58 G/A, 1374 G/A, 1526 T/C and 312 Thr/Ala, and FGG: 4288 G/A, 6326 G/A and 7792 T/C) and the haplotypes they form, in relation to the fibrinogen plasma level and arterial stiffness in a large population-based study.

METHODS

The Rotterdam Study is an ongoing prospective cohort study including 7983 participants aged 55 years and older. Its general aims are to investigate determinants of chronic diseases, including cardiovascular disease, dementia and osteoporosis.[29] During the first phase of this study (1990-1993), all inhabitants of a Rotterdam suburb (Ommoord) aged 55 years and over were invited to participate in this study. The response rate was 78%. The third examination phase took place from 1997-1999, during which measurements of arterial stiffness were performed. Approval of the Medical Ethics Committee of the Erasmus University Rotterdam was obtained for the Rotterdam Study. All participants provided written informed consent. A more in depth description of the design of the Rotterdam Study was published previously.[29]

Fibrinogen Plasma Level

Platelet poor plasma was frozen in liquid nitrogen and stored at -80°C until analysis. Fibrinogen measurements were done in a random baseline sample (n=2968). The fibrinogen plasma level was 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).

Genotyping

Genomic DNA was isolated from peripheral venous blood, previously obtained at baseline during the first examination session, according to standard protocols. The Seattle SNPs Program for Genomic Applications identified common SNPs in the fibrinogen genes based on 23 unrelated individuals of European descent from the CEPH pedigrees and constructed haplotypes based on this dataset.[30] Tagging SNPs were selected, based on this database. All participants were genotyped for seven tagging SNPs. In this study, these SNPs have been coded according to the HUGO (Human Genome Organisation) guidelines: FGA: -58 G/A, 1374 G/A, 1526 T/C, 4253 A/G (more commonly known as: 312 Thr/Ala) and FGG: 4288 G/A, 6326 G/A and 7792 T/C (these correspond with Seattle SNP codes FGA: 2224 G/A, 3655 G/A, 3807 T/C and 312 Thr/Ala, and FGG: 5836 G/A, 7874 G/A and 9340 T/C). Genotypes were determined using 2-ng genomic DNA with Taqman allelic discrimination assay (Applied Biosystems, Foster City, California). Primer and probe sequences were optimized using the SNP assay-by-design service of Applied Biosystems (http://store.appliedbiosystems.com). Reactions were performed with the Taqman Prism 7900HT in 384-wells format. Details on genotyping were described previously.[31]

Haplotyping

Haplotypes were inferred by means of the haplo.em function of the Haplo Stats package (http://cran.r-project.org/src/contrib/Descriptions/haplo.stats.html) that functions as a

Table 1. Fibrinogen α and γ Haplotypes

	Fibrinogen α					**Fibrinogen γ**			
Haplotype	–58 G/A	1374 G/A	1526 T/C	312 Thr/Ala	n (%)	4288 G/A	6326 G/A	7792 T/C	n (%)
	rs2070011	*rs2070014*	*rs2070016*	*rs5060*		*rs2066860*	*rs2066861*	*rs1049636*	
1	G	G	T	Thr	1993 (30.6)	G	G	T	2677 (41.1)
2	**A**	G	T	**Ala**	1772 (27.2)	G	G	**C**	1896 (29.1)
3	G	**A**	T	Thr	1120 (17.2)	G	**A**	T	1687 (25.9)
4	G	G	**C**	Thr	827 (12.7)	**A**	G	T	241 (3.7)
5	**A**	G	T	Thr	775 (11.9)				
Other					27 (< 1)				13 (< 1)

The structure of each haplotype is depicted here, by way of displaying the alleles of each polymorphism, that are included in the haplotype (e.g. haplotype 1 consists of the alleles: –58-G, 1374-G, 1526-T and 312-Thr)
Minor alleles within the haplotypes are marked in bold
Based on n=6514 (total of successfully genotyped participants of the Rotterdam Study)

component of the statistical program R (http://www.r-project.org/).[32,33] Coding from FGA: -58 G/A, 1374 G/A, 1526 T/C and 312 Thr/Ala, and FGG: 4288 G/A, 6326 G/A and 7792 T/C, we denoted five FGA haplotypes (coded as haplotype 1 through 5 in order of decreasing frequency) and four FGG haplotypes (coded as 1 through 4 in order of decreasing frequency) (Table 1). The other haplotypes had a frequency of < 1% and were not considered in the analyses.

Arterial Stiffness

In this study, three measures of arterial stiffness were used: the carotid-femoral pulse wave velocity (PWV) as a measure of aortic stiffness and the distensibility coefficient (DC) of the common carotid artery as a measure of common carotid arterial stiffness. In addition, pulse pressure (PP) was assessed as an indicator of arterial stiffness. All measures were obtained during the same session, during the third follow-up examination. Details on all measures of stiffness were described previously.[6,7]

Carotid-femoral PWV (m/s) was measured using an automatic device (Complior, Colson) and was calculated as the ratio between the distance traveled by the pulse wave and the foot-to-foot time delay.

Common carotid artery distensibility was assessed by measuring the vessel wall motion of the right common carotid artery using a duplex scanner (ATL Ultramark IV, operating frequency 7.5 MHz) connected to a vessel wall movement detector system.[34,35] The cross-sectional arterial wall distensibility coefficient (1/MPa) was calculated as a measure of arterial stiffness.[36] A decreased distensibility coefficient implies increased carotid stiffness.

Pulse pressure (mmHg) was defined as the difference between systolic and diastolic blood pressure, using the mean systolic and diastolic blood pressure of two measurements obtained by measuring blood pressure on the right arm using a random-zero sphygmomanometer.

Clinical Characteristics

Information on cardiovascular risk factors was collected during all examination phases, including the third phase. Data on medication and smoking habits were obtained during a home interview. At the research center, blood pressure was measured twice on the right arm using a random-zero sphygmomanometer. The average of the two blood pressure values was used in the analyses. Length and weight were measured and body mass index (weight/height^2) (BMI) was calculated. Serum total cholesterol and high-density lipoprotein (HDL) cholesterol values were determined by an automated enzymatic procedure (Boehringer Mannheim System). Diabetes mellitus (DM) was defined as use of anti-diabetic medication and/or a fasting serum glucose level equal to or above 7.0 mmol/L.[37] Evaluation of the atherosclerotic status of participants was accomplished using ultrasonography (carotid intima-media thickness [IMT]) and radiography (aortic calcification); these methods were described previously.[38,39]

Population for Analysis

The Rotterdam Study comprises 7983 subjects. Of these, 6514 were (successfully) genotyped. A total of 4797 subjects participated in the third phase of the Rotterdam Study. Of these, 4024 subjects underwent the full physical examination in the third phase. PWV was measured in 3550 subjects; 69 subjects (1.9%) were excluded from the analyses because of poor quality of the PWV recordings, leaving 3481 subjects. Common carotid distensibility was measured in 3098 subjects. Data on successful genotyping of at least one polymorphism and on at least one measure of arterial stiffness were available for 3891 participants. Genotyping data on all polymorphisms and at least one measure of arterial stiffness were available for 3474 subjects. Missing information on measures of arterial stiffness was almost entirely due to logistic reasons. Fibrinogen plasma levels were available for 2968 participants, comprising a random sample from the baseline examination.

Statistical Analyses

Chi-square tests were performed to test for deviations from Hardy-Weinberg equilibrium. Missing data on clinical characteristics were imputed using Expectation-Maximization algorithms available in SPSS. Natural-log transformed (ln-transformation) values of the fibrinogen level were used to normalize the distribution. The associations of genotypes with the fibrinogen level and arterial stiffness were investigated using analyses of variance (using SPSS version 11.0.1 for MS-Windows). The associations of haplotypes with the fibrinogen plasma level and measures of arterial stiffness were analyzed using the programme Haplo.Stats, that functions as a component part of the statistical program R (http://www.r-project.org).[32,33,40] The haplo.glm analysis is based on a generalized linear model and computes the regression of a trait on haplotypes and other covariates. Analyses were adjusted for age and sex and, when applicable, additionally for mean arterial pressure (MAP) and heart rate, and furthermore for systolic and diastolic blood pressure, body mass index (BMI), high-density lipoprotein (HDL)

and total cholesterol levels, smoking, diabetes mellitus and measures of atherosclerosis. A p-value of 0.05 and smaller was considered significant in all analyses.

RESULTS

Genotype and allele frequencies were in Hardy Weinberg equilibrium. Haplotype reconstruction resulted in five FGA haplotypes with frequencies of >1% and four FGG haplotypes with frequencies of >1%. The structure of the haplotypes is described in Table 1. General characteristics of the population are described in Table 2.

Fibrinogen Plasma Level and Arterial Stiffness

The fibrinogen plasma level was positively associated with aortic stiffness (β 0.87, p=0.015, n=1169); this association remained after adjusting for traditional cardiovascular risk factors (Table 3). Only after additional adjustment for measures of atherosclerosis estimates were attenuated, but remained near significant (β 0.65, p=0.06). No association of the fibrinogen plasma level with carotid stiffness was found, but the fibrinogen plasma level was positively associated with pulse pressure (β 3.79, p=0.049, n=1641) and remained so after additional adjustments.

Table 2. General Characteristics of the Study Population

Characteristic	
Total number	3891
Age – yrs	72.4 ± 7.1
Male sex – %	42.5
Body mass index – kg/m^2	27 ± 7
Systolic blood pressure – mm Hg	143 ± 21
Diastolic blood pressure – mm Hg	75 ± 11
Mean Arterial Pressure – mm Hg	98 ± 13
Total cholesterol – mmol/l	5.8 ± 1.0
HDL-cholesterol – mmol/l	1.4 ± 0.4
Smoking Current – %	30
Former – %	38
Never – %	32
Diabetes – %	9
Pulse Wave Velocity – m/s	13.6 ± 3.0
Distensibility Coefficient – 1/MPa	10.4 ± 4.3
Pulse Pressure – mm Hg	68 ± 17
Intima Media Thickness – mm	0.78 ± 0.13
Aortic calcifications* – %	22

Values depicted as mean ± SD

* % Aortic calcifications of ≥ 2.5 cm length

Table 3. Fibrinogen Level and Arterial Stiffness

	Pulse Wave Velocity (n=1169)		**Distensibility Coefficient (n=1157)**		**Pulse Pressure (n=1641)**	
	β-coefficient (SE)	*p*	*β-coefficient (SE)*	*p*	*β-coefficient (SE)*	*p*
Model 1	0.865 (0.354)	0.015	– 0.714 (0.431)	0.098	3.793 (1.921)	0.049
Model 2	0.808 (0.353)	0.022	– 0.517 (0.433)	0.233	2.393 (1.038)	0.021
Model 3	0.654 (0.349)	0.061	– 0.510 (0.429)	0.235	2.209 (1.032)	0.032

β-coefficient: based on ln-transformed fibrinogen levels

Model 1: adjusted for age, sex (and for pulse wave velocity and distensibility coefficient also mean arterial pressure and heart rate)

Model 2: model 1 + body mass index, total and HDL-cholesterol, smoking, diabetes mellitus

Model 3: model 2 + measures of atherosclerosis

Table 4. Fibrinogen Levels by Genotype

Polymorphism	**Genotype**	**N**	**Level (g/L)**	**p***	**p for trend**
FGA –58 G/A	GG	972	2.82	ns	ns
	GA	1247	2.82	ns	
	AA	390	2.78	ns	
FGA 1374 G/A	GG	1772	2.81	ns	ns
	GA	731	2.80	ns	
	AA	86	2.91	ns	
FGA 1526 T/C	TT	1970	2.79	p=0.004	p=0.004
	TC	582	2.87		
	CC	36	2.87	ns	
FGA 312 Thr/Ala	Thr/Thr	1340	2.82	p=0.008†	ns
	Thr/Ala	1031	2.82	p=0.010	
	Ala/Ala	198	2.69		
FGG 4288 G/A	GG	2402	2.81	ns	ns
	GA	197	2.82	ns	
	AA	1	3.77	ns	
FGG 6326 G/A	GG	1404	2.82	p=0.001‡	p=0.024
	GA	998	2.82	p=0.002	
	AA	185	2.67		
FGG 7792 T/C	TT	1299	2.79	ns	ns
	TC	1104	2.83	ns	
	CC	205	2.80	ns	

Age and sex adjusted

*for differences in fibrinogen level between genotypes

ns = not significant

†compared with Ala/Ala

‡compared with AA

Fibrinogen Plasma Level and Genotype/Haplotype

Fibrinogen plasma levels were increased for those carrying the FGA 1526 C-allele (p for trend = 0.004) and decreased for those carrying the FGA 312 Ala variant (Ala-Ala homozygotes lower level than Thr-Thr homozygotes, p=0.008) and the FGG 6326 A-allele (p for trend = 0.024) (Table 4).

The regression analyses showed that the fibrinogen plasma level was associated with FGA haplotype 4 (containing the 1526 C-allele, which was associated with higher levels) (p=0.01) (Table 5). The fibrinogen plasma level was not associated with the other haplotypes.

Genotypes and Haplotypes and Arterial Stiffness

Genotype analyses showed no significant associations of the SNPs with any measure of arterial stiffness, except for FGA 312 Thr/Ala (Thr-Thr homozygotes had a higher carotid stiffness than heterozygotes, p=0.03), FGG 6326 G/A (GG homozygotes had a higher carotid stiffness than heterozygotes, in the fully adjusted model, p=0.05), and FGG 4288 G/A (GG-homozygotes had a lower pulse pressure than AA-homozygotes, p=0.03). This association, however, was based on only 3 subjects. No significant trends were observed.

In the haplotype regression analyses using haplotype 1 as reference, FGA haplotype 3 was associated with a lower aortic stiffness (PWV) (β coefficient of -0.20, p=0.03) and, concordantly, with a lower pulse pressure (β coefficient of -0.56, p<0.01) (Table 6). Significance was preserved after full adjustment. FGA haplotype 4, containing the 1526-C allele which was associated with a higher fibrinogen level, was associated with an increased pulse pressure (β coefficient of 0.43, p<0.01). FGG haplotype 4 was associated with a lower pulse pressure (β coefficient -1.71, p<0.01). Significance of these findings was retained after full adjustment (data not shown). The remaining haplotypes were not associated with arterial stiffness.

Table 5. FGA and FGG Haplotypes in Relation to the Fibrinogen Level

Haplotype	n	β-coefficient (SE)	p-value
Fibrinogen α gene			
1 (reference)	907		
2	816	-0.01 (0.01)	0.56
3	519	0.00 (0.01)	0.70
4	376	0.03 (0.01)	0.01
5	340	0.01 (0.01)	0.44
Fibrinogen γ gene			
1 (reference)	1208		
2	862	-0.00 (0.01)	0.86
3	784	-0.02 (0.01)	0.07
4	114	0.00 (0.02)	0.83

β-coefficient: based on ln-transformed fibrinogen levels
Based on total population with data on geno/haplotyping and fibrinogen levels (n=2968)
Analyses, using the Haplo.Stats haplo.glm function, are age and sex adjusted

Table 6. FGA and FGG Haplotypes in Relation to Arterial Stiffness

	Pulse wave velocity (n=2886)		**Distensibility coefficient (n=2550)**		**Pulse pressure (n=3459)**	
Haplotype	*β-coefficient (SE)*	*p*	*β-coefficient (SE)*	*p*	*β-coefficient (SE)*	*p*
FGA						
1 (reference)						
2	-0.06 (0.08)	0.44	0.14 (0.11)	0.22	-0.20 (0.37)	0.58
3	-0.20 (0.09)	0.03*	-0.06 (0.13)	0.66	-0.56 (0.15)	<0.001*
4	-0.14 (0.10)	0.17	0.15 (0.15)	0.33	0.43 (0.07)	<0.001*
5	-0.10 (0.11)	0.35	0.05 (0.16)	0.73	0.01 (0.05)	0.87
FGG						
1 (reference)						
2	-0.11 (0.07)	0.14	-0.06 (0.11)	0.55	-0.04 (0.45)	0.93
3	0.02 (0.08)	0.77	0.05 (0.11)	0.63	-0.30 (0.47)	0.52
4	0.18 (0.18)	0.30	-0.17 (0.26)	0.51	-1.71 (0.07)	<0.001*

Analyses, using Haplo.Stats haplo.glm function, were adjusted for age and sex, and for pulse wave velocity and the distensibility coefficient additionally for heart rate and mean arterial pressure
FGA = fibrinogen α
FGG = fibrinogen γ
* Significant also after adjusting for systolic and diastolic blood pressure, body mass index, total and HDL-cholesterol levels, smoking, diabetes mellitus and measures of atherosclerosis

Gender-stratified analyses on the association between genotypes and haplotypes with arterial stiffness yielded no findings essentially different from the overall analyses (data not shown).

DISCUSSION

In this population-based study, we found that the fibrinogen plasma level was associated with aortic stiffness and pulse pressure, independent of cardiovascular risk factors and atherosclerosis. Genotype analyses yielded significant associations of the FGA 1526 T/C, the FGA 312 Thr/Ala and FGG 6326 G/A polymorphisms with the fibrinogen plasma level, but revealed no overall and consistent associations with measures of arterial stiffness. Haplotype analyses showed FGA haplotype 4 to be associated with a higher fibrinogen plasma level and a higher pulse pressure. FGA haplotype 3 and FGG haplotype 4 were associated with a lower aortic stiffness and lower pulse pressure.

Several studies have demonstrated the association between the fibrinogen level and arterial stiffness.[15-17] Our findings of a significant association of the fibrinogen plasma level with aortic stiffness and pulse pressure are in concordance with these earlier findings. After adjustment for atherosclerosis, the observed association between the fibrinogen plasma level and arterial stiffness was borderline significant. Further research is needed to clarify

whether the relationship between the fibrinogen plasma level and pulse wave velocity is independent of atherosclerosis. The fibrinogen measurements were performed at baseline, while arterial stiffness was measured during the third examination phase. It is possible that the levels changed during the period between the first and third examination phase, which may have underestimated our estimates.

Strong heritability estimates for the fibrinogen level were previously found (0.20-0.50).[21] However, to date few studies have investigated the association between genetic variation in the FGA and FGG genes with respect to level of fibrinogen. In a recent study in nearly 4000 American subjects, several polymorphisms were associated with the fibrinogen level; the FGA 1526 C-allele was associated with higher levels, the 312-Ala-allele was associated with lower levels, as was the FGA -58 A-allele, which appears to be functional in vitro.[27,28] Our findings on the FGA 1526 C-allele and 312 Ala-allele are in concordance with these results. No association of the FGG 4288 G/A polymorphism and the fibrinogen plasma level was found in our study, but the 7792 C-allele was associated with higher levels.[27] In addition, we found that the risk allele of the FGG 6326 G/A polymorphism was associated with a lower fibrinogen plasma level. Overall, our findings on genotypes and the fibrinogen plasma level are in line with the literature.

We found no consistent trends for genotypes and measures of arterial stiffness, but the genotypic differences in stiffness that were statistically significant are in line with our findings on the fibrinogen plasma level. FGA haplotype 4 was associated with increased pulse pressure, which is in agreement with the fact that this haplotype contains the 1526 C-allele, which by itself was associated with a higher fibrinogen level. FGA haplotype 3 was associated with a lower aortic stiffness and lower pulse pressure and FGG haplotype 4 with a lower pulse pressure. The risk alleles in these haplotypes (FGA 1374-A and FGG 4288-A, respectively) were, by themselves, not associated with the fibrinogen level. The association between haplotypes and arterial stiffness may very well be due to an effect of other polymorphisms in these haplotypes. Generally, the precise functional effects of fibrinogen haplotypes and polymorphisms are largely unknown, certainly with respect to the mechanisms that underlie arterial stiffness.

Fibrinogen is probably best known as a coagulation factor. In addition, it is also an acute-phase protein and is associated with inflammation, angiogenesis and matrix interactions.[18,19] Through these processes fibrinogen may be associated with arterial stiffness. Indeed, the fibrinogen level was observed to be associated with arterial stiffness in some studies.[15-17] However, the precise mechanisms remain to be discovered. It is unclear whether the association between the fibrinogen level and arterial stiffness, is a causal association. From our findings on the influence of genetic variation in FGA and FGG on arterial stiffness we cannot conclude a causal relation between fibrinogen level and stiffness because genetic variation in these genes may also affect structure and thereby function of fibrinogen. Therefore, the effect on stiffness may also be through an effect on function of fibrinogen rather than through an effect on level of fibrinogen.

Our study is based on a large ongoing population-based study in a relatively homogeneous population, as 98% of the participants in our study are Caucasian and are all living in the same area, a suburb of Rotterdam. To interpret the findings correctly, several methodological issues should be considered. First, pulse waves in the carotid artery and the femoral artery travel in opposite directions, while measurements of carotid-femoral pulse wave velocity are based on the assumption that the pulse wave travels from the carotid artery to the femoral artery. In this way, measuring the distance between the carotid and the femoral artery lead to an overestimation of the distance between the sites of the pulse waves resulting in overestimation of the velocity of the pulse waves. However, variations in anatomy are limited and this error may be considered similar for all subjects examined, therefore we do not think it has seriously biased our results. Second, the distance between the carotid and the femoral artery may be overestimated in (especially adipose) subjects when this distance is measured by tape. To avoid this error we adjusted the analyses for body mass index. Third, in computing the carotid distensibility coefficient, we used the brachial pulse pressure rather than the carotid pulse pressure. Information on comparisons between the carotid and the brachial pulse pressure indicates that the carotid pulse pressure is lower than the brachial pulse pressure, but differences are relatively small.[41] Furthermore, some data on measures of stiffness were not available for all subjects who visited the research center. Missing information was primarily due to logistic reasons, which is likely to be random and thus will not have biased our results. Additionally, although this study included nearly 4000 subjects, the possibility cannot be excluded that a potential association remained undetected, especially as the effects of genetic variation on a trait with a complex and multifactorial pathogenesis, such as arterial stiffness, are generally modest. Finally, because our study was performed in a population of predominantly elderly Caucasian subjects, the generalizability of our findings to younger individuals or other ethnicities remains uncertain.

In conclusion, our findings indicate that fibrinogen plasma level and genetic variation in the fibrinogen α and γ gene are associated with arterial stiffness. Further studies are needed to confirm these findings and to elucidate the underlying pathogenetic mechanisms.

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Chapter 4

Genetic Determinants of Arterial Stiffness

Non-inflammatory Genes

Chapter 4.1

Variation in Renin Angiotensin System Genes and Arterial Stiffness

Background and purpose - Arterial stiffness has been found to predict the risk of cardiovascular disease and it generally increases with age. The renin-angiotensin system influences not only the circulating volume and blood pressure, but also arterial stiffness. We studied the associations of three renin-angiotensin system polymorphisms, ACE I/D, angiotensinogen 235 M/T and angiotensin II receptor type I 573 C/T, with arterial stiffness.

Methods - The study was embedded in the Rotterdam Study, a prospective, population-based study among subjects aged 55 years and older. Arterial stiffness was measured during the third phase of the Rotterdam Study (1997-1999). The association of the polymorphisms with pulse wave velocity, the carotid distensibility coefficient and pulse pressure was investigated in 3706 subjects using analyses of variance. All analyses were stratified by gender and adjusted for age, mean arterial pressure, heart rate, known cardiovascular risk factors and measures of atherosclerosis.

Results - We found no association of the ACE I/D polymorphism with pulse wave velocity, but the D-allele was associated with a lower distensibility coefficient (p=0.05) and higher pulse pressure (p=0.01). For the angiotensinogen 235 M/T polymorphism no significant associations with either pulse wave velocity (p=0.71), the distensibility coefficient (p=0.16) or pulse pressure (p=0.34) were found. Also, we found no significant associations of pulse wave velocity (p=0.32), the distensibility coefficient (p=0.08) and pulse pressure (p=0.09) with the angiotensin II receptor type 1 573C/T polymorphism.

Conclusions – Of the genetic variations in the renin-angiotensin system, that we studied, we only found the ACE I/D polymorphism, to be associated with reduced carotid distensibility and increased pulse pressure.

INTRODUCTION

Although arterial stiffness generally increases with age, vessel wall stiffness leads to an increased blood and pulse pressure and has been found to be independently associated with an increased risk of cardiovascular disease.[1-8]

The renin-angiotensin system (RAS) influences the vascular system in many ways. Renin converts angiotensinogen (AGT) into the inactive angiotensin I (AT-I); angiotensin-converting enzyme (ACE) converts the AT-I into the active angiotensin II (AT-II), which is the main product of the RAS.[9] This system is involved in regulation of blood pressure and circulating volume. AT-II is a potent vasoconstrictor and stimulates the release of aldosteron; this results in an increase in blood pressure. In addition, the RAS plays an important role in vascular regulation and atherogenesis, it influences cell growth and proliferation and endothelial dysfunction.[10-12]

Genetic variation in components of this system may affect the vascular wall and arterial stiffness. The most studied polymorphism in the RAS system is the ACE I/D polymorphism. This polymorphism concerns the insertion (or deletion) of a 287 bp sequence of DNA in the ACE gene and was described by Rigat et al.[13] Although it is located in an intron, the D-variant of this polymorphism is associated with increased ACE-levels, suggesting a tight link with other functional, but so far unknown, polymorphisms, and is commonly used as marker for increased ACE levels.[12-15] Indeed, the intron 16 ACE Insertion/Deletion (ACE I/D) polymorphism has been associated with carotid intima-media thickness and arterial stiffness in several studies.[16-21]

In our population we found the ACE I/D polymorphism to be associated with carotid stiffness (the distensibility coefficient) in an earlier study.[21] This prompted us to study genetic variation in the renin-angiotensin system in more detail, including the ACE I/D polymorphism and additional polymorphisms angiotensinogen (AGT) 235 M/T and angiotensin II receptor type I (AT1R) 573 C/T, in relation to pulse wave velocity, the distensibility coefficient, and additionally pulse pressure, in a large population-based study.

METHODS

The Rotterdam Study is an ongoing prospective cohort study including 7983 participants of 55 years and older.[22] During the first phase of this study (1990-1993), all inhabitants of a Rotterdam suburban area (Ommoord) aged 55 years and over were invited to participate in this study. The response rate was 78%. The third examination phase took place from 1997-1999, during which measurements of arterial stiffness were performed. Approval of the Medical Ethics Committee of the Erasmus University Rotterdam was obtained and written informed

consent was acquired. A more in depth description of the design of the Rotterdam Study has been published previously.[22]

Genotyping

Genomic DNA was isolated from blood samples using standard methods.[23] Methods of genotyping for the ACE I/D, angiotensinogen 235 M/T and angiotensin II receptor type I 573 C/T polymorphisms were described in detail previously.[21,24]

Arterial Stiffness

Arterial stiffness was measured by two methods, i.e. the carotid-femoral pulse wave velocity (PWV) as a measure of aortic stiffness and the distensibility coefficient (DC) of the common carotid artery as a measure of common carotid arterial stiffness. In addition, pulse pressure (PP) was assessed as an indicator of arterial stiffness. All measures were obtained during the same session during the third examination phase. Details on all measures of stiffness have been described previously.[5,8]

Carotid-femoral PWV (m/s) was measured using an automatic device (Complior, Colson) and was calculated as the ratio between the distance travelled by the pulse wave and the foot-to-foot time delay.

Common carotid artery distensibility was assessed by measuring the vessel wall motion of the right common carotid artery using a duplex scanner (ATL Ultramark IV, operating frequency 7.5 MHz) connected to a vessel wall movement detector system.[25,26] The cross-sectional arterial wall distensibility coefficient (DC) (1/MPa) was calculated as a measure of arterial stiffness.[27] A decreased distensibility coefficient implies increased carotid stiffness.

Pulse pressure (PP) (mmHg) was defined as the difference between systolic and diastolic blood pressure, using the mean systolic and diastolic blood pressure of two measurements obtained by measuring blood pressure on the right arm using a random-zero sphygmomanometer.

Clinical Characteristics

Information on cardiovascular risk factors was collected during the third follow-up examination. These data include information on drug use, smoking habits, blood pressure, height, weight, body mass index (weight/height^2) (BMI), serum total cholesterol and high-density lipoprotein (HDL) cholesterol, diabetes mellitus (DM) and on the atherosclerotic status of participants (carotid intima-media thickness [IMT]) and radiography (aorta calcification). All methods have been extensively described previously.[8,28-30]

Population for Analysis

The Rotterdam Study comprises 7983 subjects. A total of 4797 subjects participated in the third phase of the Rotterdam Study. Of these, 4024 subjects underwent a physical examina-

tion of the third phase. PWV was measured in 3550 subjects; 69 subjects (1.9%) were excluded from the analyses because of poor quality of the PWV recordings, leaving 3481 subjects. Common carotid distensibility was measured in 3098 subjects. Missing information on measures of arterial stiffness was almost entirely due to logistic reasons. Complete genotyping data on all polymorphisms and at least one measure of arterial stiffness was available for 3706 participants.

Statistical Analyses

Chi-square tests were performed to test for deviations from Hardy-Weinberg equilibrium. Missing data on clinical characteristics were imputed using Expectation-Maximization algorithms available in SPSS. The associations between genotypes and arterial stiffness were investigated using analyses of variance and linear regression. All analyses were adjusted for age, sex and (for PWV and DC) also for mean arterial pressure (MAP) and heart rate. Additionally, the analyses were adjusted for body mass index, high-density lipoprotein (HDL) and total cholesterol levels, smoking, diabetes mellitus and measures of atherosclerosis. A p-value of 0.05 or smaller was considered significant in all analyses. The statistical analyses were performed using SPSS version 11.0.1 for MS-Windows.

RESULTS

General characteristics have been described in Table 1. Genotype and allele proportions were in Hardy Weinberg equilibrium.

As reported earlier, the ACE D-allele was significantly associated with a decreased distensibility coefficient (i.e., increased carotid stiffness), p for trend = 0.05, also after full adjustment, p=0.03 (Table 2).[21] In the current study the D-allele was also associated with a significantly increased pulse pressure, p for trend = 0.01 (Table 2).

Analyses did not show evidence for an association of the AGT M/T polymorphism and pulse wave velocity, the distensibility coefficient or pulse pressure (Table 2).

For the AT1R 573 C/T polymorphism the analyses, although not significant, suggested a trend of an increased distensibility coefficient (i.e., a lower carotid stiffness) (p=0.08) and concordantly lower pulse pressure (p=0.09) for T-carriers (Table 2). However, differences between the genotypes were not significant. No associations with pulse wave velocity were found. Full adjustment did not alter these results.

All gender-stratified analyses yielded findings essentially similar to the overall analyses (data not shown).

Table 1. General Characteristics

Characteristic	
Total number	3706
Age – yrs	72.4 ± 7.1
Male sex – %	42
Body mass index – kg/m^2	27 ± 4
Systolic blood pressure – mm Hg	143 ± 21
Diastolic blood pressure – mm Hg	75 ± 11
Mean Arterial Pressure – mm Hg	98 ± 13
Pulse Pressure – mm Hg	68 ± 17
Total cholesterol – mmol/l	5.8 ± 1.0
HDL-cholesterol – mmol/l	1.4 ± 0.4
Smoking Current – %	27
Former – %	38
Never – %	35
Diabetes – %	9
Pulse Wave Velocity – m/s	13.6 ± 3.0
Distensibility Coefficient – 1/MPa	10.4 ± 4.4
Intima Media Thickness – mm	0.76 ± 0.13
Aortic calcifications* – %	22

* % Aortic calcifications of ≥ 2.5 cm length
Continuous values are depicted as mean ± SD

Table 2. Arterial Stiffness by Genotype

ACE I/D	**n**	**II**	**n**	**ID**	**n**	**DD**	**Trend**
Pulse Wave Velocity *(m/s)*	777	13.45 (13.26-13.65)	1704	13.53 (13.40-13.66)	925	13.57 (13.39-13.74)	p = 0.73*
Distensibility Coefficient *(1/MPa)*	675	10.65 (10.37-10.93)	1508	10.24 (10.06-10.43)	818	10.27 (10.02-10.52)	**p = 0.05***
Pulse Pressure *(mmHg)*	828	66.6 (65.4-67.7)	1876	68.5 (67.8-69.3)	986	68.6 (67.6-69.7)	**p = 0.01**
AGT 235 M/T		**MM**		**MT**		**TT**	
Pulse Wave Velocity *(m/s)*	1158	13.98 (13.74-14.22)	1447	14.00 (13.79-14.20)	494	14.20 (13.83-14.57)	p = 0.71
Distensibility Coefficient *(1/MPa)*	1022	11.25 (10.91-11.58)	1273	11.22 (10.93-11.51)	543	10.84 (10.35-11.33)	p = 0.16
Pulse Pressure *(mmHg)*	1352	67.1 (65.7-68.5)	1742	67.7 (66.5-68.9)	596	67.8 (65.7-69.9)	p = 0.34
AT1R C/T		**CC**		**CT**		**TT**	
Pulse Wave Velocity *(m/s)*	867	14.20 (13.92-14.47)	1501	13.97 (13.77-14.17)	731	13.93 (13.62-14.24)	p = 0.32
Distensibility Coefficient *(1/MPa)*	752	10.89 (10.51-11.27)	1332	11.30 (11.01-11.58)	664	11.23 (10.81-11.64)	p = 0.08
Pulse Pressure *(mmHg)*	1014	68.2 (66.6-69.7)	1799	67.2 (66.0-68.4)	877	67.3 (65.5-69.0)	p = 0.09

Presented values: mean (95% confidence interval)
Age and sex adjusted, and for pulse wave velocity and the distensibility coefficient also for heart rate and mean arterial pressure
*Results from Mattace-Raso et al. *Atherosclerosis*, 2004[21]

DISCUSSION

We studied the ACE I/D, the angiotensinogen 235 M/T and angiotensin II receptor type 1 573 C/T polymorphisms in relation to arterial stiffness in a large population-based study. We found no evidence for an association with pulse wave velocity for any of the studied polymorphisms. The ACE D-allele was significantly associated with a higher carotid stiffness (i.e., a decreased distensibility coefficient) and an increased pulse pressure. The AGT 235 M/T and AT1R 573 C/T polymorphisms were not significantly associated with carotid stiffness or pulse pressure, although our results suggested a (non-significant) trend for an association of the AT1R 573 C/T polymorphism with carotid stiffness and pulse pressure.

The renin-angiotensin system influences not only blood pressure and vascular tonus, but also arterial stiffness. The effects of the RAS appears not to be limited to the actions of angiotensin II; multiple other parts of the system, such as aldosteron and renin, may influence stiffness.[31] The potential effect on arterial stiffness is further supported by findings that medication acting on the RAS, such as ACE inhibitors and AT-II receptor blockers, reduce arterial stiffness.[31-33]

We found the D-allele of the ACE I/D polymorphism to be associated with a higher carotid stiffness (i.e., a lower distensibility coefficient) and a higher pulse pressure. The former association was already shown in the Rotterdam Study in a previous study.[21] Two other studies also evaluated this polymorphism in relation to arterial stiffness. In a relatively small French study no important influence of the I/D polymorphism on pulse wave velocity was found among 128 normotensives, while among 311 hypertensives a slightly higher PWV was present for those with the II-genotype.[20] Another study also found a higher PWV for those with the II-genotype, however, the association was only present among 137 type 2 diabetics and not among 260 non-diabetic controls.[19] Our study is much larger and is performed in a general population setting. In another, also more general, population-based study among 756 Flemish subjects decreased compliance (i.e., increased stiffness) was found to be associated with the D-allele.[18] It has been generally established that the D-allele is associated with increased ACE levels, which, in turn, may influence arterial stiffness.[12-15,21] Increased ACE levels, in turn, may influence arterial stiffness. Our findings are in line herewith.

We found no significant associations between the angiotensin 235 M/T polymorphism and arterial stiffness. The angiotensinogen polymorphism 235 M/T was first described by Jeunemaitre et al., and the T-variant has been associated with increased levels of angiotensinogen.[34,35] Previous studies on the AGT 235 M/T polymorphism and arterial stiffness were small and results were not consistent.[36,37] The T-allele was found to be associated with increased arterial stiffness in a relatively small study of 98 hypertensives.[36] Another study, however, among 441 European hypertensives failed to show an association between the 235 M/T polymorphism and pulse wave velocity.[37]

Variation in the angiotensin II type 1 receptor (AT1R) gene was found to be associated with pulse wave velocity.[37,38] Few studies have investigated the AT1R 573 C/T polymorphism, first described by Bonnardeaux et al., and it has also, to our knowledge, not been studied earlier in relation to arterial stiffness.[39,40] The 573 C/T polymorphism is in linkage disequilibrium (LD) with the AT1R 1166 A/C polymorphism, whereby the 573 T-allele is found to be linked with the 1166 A-allele.[41] Other studies have described a lower pulse wave velocity in association with the 1166 A-allele.[37,38] Our findings were not significant, but showed a tendency for the T-allele to be related to a lower arterial stiffness, especially in our analyses of carotid stiffness (the distensibility coefficient) and pulse pressure. Given the LD between the 573 C/T and 1166 A/C polymorphisms, our results appear in line with these earlier findings and support the need for further research, preferably with characterization of more markers in the AT1R gene.

To interpret the findings correctly, several methodological aspects of the measures of arterial stiffness need to be discussed. First, pulse waves in the carotid artery and the femoral artery travel in opposite directions, while measurements of carotid-femoral pulse wave velocity is based on the assumption that the pulse wave travels from the carotid artery to the femoral artery. In this way, measuring the distance between the carotid and the femoral artery lead to an overestimation of the velocity of the pulse waves. However, variations in anatomy are limited and this error may be considered similar for all subjects examined. Second, the distance between the carotid and the femoral artery may be overestimated in subjects with a large abdomen when measured by tape. To avoid this error we adjusted the analyses for body mass index. Third, in computing the carotid distensibility coefficient, we used the brachial pulse pressure rather than the carotid pulse pressure. Information indicates that the carotid pulse pressure is lower than the brachial pulse pressure, but differences are relatively small.[42]

Our study is based on a large ongoing cohort study. We adjusted for established risk factors and measures of atherosclerosis. Missing information on arterial stiffness was primarily due to logistic reasons, which is likely to be random. Also, in a large pathway such as RAS the effect of a single gene is often very small. Therefore, it is possible that small effects were not observed.

In conclusion, our findings suggest that of the genetic variations in the renin-angiotensin system, that we studied, only the ACE I/D polymorphism, is associated with carotid distensibility and pulse pressure. At this point, however, we do not exclude the possibility, that other polymorphism in the RAS system are involved in arterial stiffness. The findings support the need for further research on genetic variation in components of the RAS system and stiffness of the vascular wall.

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Chapter 4.2

Insertion/Deletion Gene Polymorphism of the Angiotensin Converting Enzyme and Blood Pressure Changes in Older Adults

Background and purpose - The insertion/ deletion (I/D) polymorphism of the ACE gene may be involved in determining blood pressure changes. Aim of the present study was to assess the relationship between the ACE I/D gene and the change of blood pressure levels during follow up. We calculated the difference between mean levels of SBP, DBP and PP obtained during the two observations as follows: BP mean levels obtained at third phase- BP mean levels at baseline, and subsequently we investigated the association of the ACE I/D polymorphism and the mean changes of SBP, DBP and PP levels.

Methods - The study was conducted within the Rotterdam Study, a population-based cohort study including subjects aged 55 years and older. Information on the II, ID and DD genotypes of the ACE gene and mean change of blood pressure levels were available in 3966 subjects.

Results - In adjusted models, subjects with the D allele had higher mean changes of systolic and pulse pressure than subjects with the I allele. The mean changes of systolic blood pressure were 6.1 (4.7-7.5), 8.2 (7.5-9.3) and 7.4 (5.9-8.5) mmHg in subjects with the II, ID and DD genotype, respectively. Corresponding mean changes of PP through genotypes were 4.3 (3.3-5.4), 6.0 (5.3-6.7) and 5.9 (4.9-6.9) mmHg, respectively. No difference was found for mean change of diastolic blood pressure among genotypes.

Conclusions - The results of this population-based study show that the ACE ID/DD genotypes are associated with increased mean changes of systolic and pulse pressure.

INTRODUCTION

There is increasing evidence that pulse pressure in older age is an independent predictor of risk of cardiovascular disease in the general population.[1-3] Findings from the Framingham Heart Study showed that with increasing age, there was a gradual shift in predictive value from diastolic blood pressure to systolic blood pressure and then to pulse pressure.[4] From 60 years and older pulse pressure became superior to systolic blood pressure and diastolic blood pressure in predicting coronary heart disease.

Genetic factors might influence blood pressure levels and several studies have investigated the possible relation between the angiotensin converting enzyme (ACE) I/D gene polymorphism and blood pressure levels; however, results have been controversial. Large population-based studies have shown the effect of the ACE I/D polymorphism on blood pressure levels in men and a higher prevalence of hypertension has been found in smokers with the D allele. [5,6,7,8] Recent studies suggested that the ACE I/D gene polymorphism might modulate independently age- related increases of pulse pressure; however considering the cross-sectional nature of the investigation these results require to be confirmed by longitudinal studies.[9,10] The aim of the present study was to investigate whether the ACE gene I/D polymorphism could modulate blood pressure changes in a large population-based study of older adults.

METHODS

This study was conducted within the framework of the Rotterdam Study, an ongoing prospective population-based cohort study among subjects aged 55 years or over, living in Ommoord, a suburb of Rotterdam, The Netherlands. The rationale and design of the Rotterdam Study have been described elsewhere.[11] The Medical Ethics Committee of Erasmus Medical Center approved the study and written consent was obtained from all participants. Baseline data were collected from 1990 to 1993 and the third examination phase took place from 1997 to 1999. In total, 7983 (response rate 78%) subjects agreed to participate and were interviewed at home.

Blood Pressure Measurements and Cardiovascular Risk Factors

Information on cardiovascular risk factors was collected during the baseline and the follow up examination. Data on use of blood pressure-lowering medication and smoking habits were obtained during the home interview. Blood pressure was measured twice, at the right upper arm using a random zero sphygmomanometer, with the individual in the sitting position after a minimum of 5-minutes rest. Participants were asked to abstain from smoking and drinking alcoholic or caffeine-containing beverages at least 2 hours before blood pressure

measurements were taken. The average of two measurements, obtained at a single visit, was used for analysis. Systolic (SBP), first Korotkoff phase and diastolic (DBP), fifth Korotkoff phase, blood pressure were measured. Pulse pressure (PP) was calculated (SBP-DBP). Body mass index [weight /height^2] was calculated. Serum total cholesterol and high-density lipoprotein (HDL) cholesterol values were determined by an automated enzymatic procedure (Boehringer Mannheim System). Diabetes mellitus was defined as the use of blood glucose lowering medication and/or a fasting serum glucose level equal to or greater than 7.0 mmol/l.[12] Hypertension was defined as a blood pressure level ≥160/90 mmHg and/or the use of antihypertensive medication.

Genotyping

The II, ID and DD genotypes were determined at baseline, in blood samples obtained at baseline, using the polymerase chain reaction technique (PCR) using a PE9600 PCR machine according to the method of Lindpaintner et al. with some modifications.[13] The results of amplification were 319-bp and 597-bp amplicons for the D and I alleles respectively. Because the D allele in heterozygous samples is preferentially amplified, there is a tendency of misclassification for about 4 to 5 percent of ID genotypes to DD. In order to avoid this misclassification, a second independent PCR has been performed with a primer pair that recognizes an insertion specific sequence. The reaction yielded a 335-bp amplicon only if the I allele was present. In the post PCR analyses, 10 µl of PCR product was loaded on 3% Agarose gel. Two independent investigators have read pictures from each gel and all ambiguous samples were analysed a second time.

Population for Analysis

Blood pressure measurements were available in 7009 subjects participating at baseline examination and in 4170 subjects who participated the third examination phase. In total, blood pressure measurements and information on the ACE genotype were available in 3966 subjects. Missing information on measures of blood pressures or ACE genotype was almost entirely due to logistic reasons.

Statistical Analyses

The ACE genotype was tested as three class variables (presence of 0, 1, or 2 D alleles). The Hardy-Weinberg equilibrium was tested by a Chi-square test. The association of the ACE genotype with changes of blood pressure levels was investigated by analysis of variance after adjustment for age, gender, body mass index, diabetes mellitus, smoking, total cholesterol, high- density lipoprotein cholesterol and use of antihypertensive medication. We calculatd the difference between mean levels of SBP, DBP and PP obtained during the two observations as follows: BP mean levels obtained at third phase- BP mean levels at baseline, and subsequently we investigated the association of the ACE I/D polymorphism and the mean

changes of SBP, DBP and PP levels. Next, the association between blood pressure changes and the ACE genotype was investigated in categories of gender, age (subjects younger and older than 70 years) and hypertension.

RESULTS

Characteristics of the study subjects are presented in Table 1. ACE genotype and allele proportion were in Hardy-Weinberg equilibrium. At baseline, no difference was seen between mean levels of pulse pressure or the prevalence of hypertension and the I/D polymorphism of the ACE gene. Subjects with the ID and DD genotype had a statistically significantly higher increase of mean levels of SBP and PP when compared with subjects with the II genotype. (Table 2). In analyses adjusted for potential confounders, mean changes and corresponding

Table 1. Characteristics of the Population at Baseline (n= 3966)

	II (878)	ID (2018)	DD (1070)
Age (years)	65.2	66.2	66.1
Men (%)	42.7	42.2	40.7
Body mass index (kg/m^2)	26.3	26.2	26.3
Smokers (%)	22.9	22.2	18.3
Diabetes mellitus (%)	5.7	8.8	5.2
Total cholesterol (mmol/l)	6.6	6.6	6.6
HDL-Cholesterol (mmol/l)	1.3	1.3	1.3
Use of antihypertensive drugs (%)	23.9	27.8	26.3
Hypertension (%)	36.1	40.1	36.6
Systolic blood pressure (mmHg)	141.1	144.1	143.9
Diastolic blood pressure (mmHg)	74.9	75.4	75.1
Pulse pressure (mmHg)	61.6	62.7	62.9

HDL- cholesterol: high- density lipoprotein cholesterol

Table 2. Mean Change of Systolic, Diastolic and Pulse Pressure Levels According to the ACE Gene I/D Genotype

	II (878)	ID (2018)	DD (1070)
Mean change of SBP (mmHg)	6.1 (4.7-7.5)	8.2 (7.5-9.3)*	7.4 (5.9-8.5)
Mean change of DBP (mmHg)	1.5 (0.6-2.1)	2.0 (1.6-2.5)	1.2 (0.5-1.9)
Mean change of PP (mmHg)	4.3 (3.3-5.4)	6.0 (5.3-6.7) †	5.9 (4.9-6.9) ‡

Models adjusted for age, gender, use of antihypertensive medications, body mass index, diabetes mellitus, smoking, total cholesterol, high- density lipoprotein cholesterol
SBP: systolic blood pressure. DBP: diastolic blood pressure. PP: pulse pressure.
* Statistically significantly different from II genotype (p = 0.005)
† Statistically significantly different from II genotype (p = 0.01)
‡ Statistically significantly different from II genotype (p = 0.03)

Table 3. Mean Change of Systolic, Diastolic and Pulse Pressure Levels According to the ACE Gene I/D Genotype in Categories of Gender, Age and Hypertension

		Men			**Women**	
	II (375)	***ID (852)***	***DD (436)***	***II (509)***	***ID (1166)***	***DD (634)***
Mean change of SBP (mmHg)	4.7 (2.6-6.8)	8.4 (7.0-9.8)*	6.9 (4.9-8.8)	6.7 (4.9-8.5)	7.9 (6.7-9.1)	7.3 (5.6-8.9)
Mean change of DBP (mmHg)	0.6 (-0.5-1.8)	2.2 (1.4-3.0)*	1.4 (0.3-2.5)	2.1 (1.1-3.1)	1.9 (1.2-2.6)	1.1(0.2-1.9)
Mean change of PP (mmHg)	4.1 (2.5-5.7)	6.1 (5.1-7.2)*	5.4 (3.9-6.9)	4.6 (3.1-6.0)	5.9 (4.9-6.9)	6.2 (4.9-7.5)
		<70 years			**≥70 years**	
	II (663)	***ID (1437)***	***DD (758)***	***II (215)***	***ID (580)***	***DD (313)***
Mean change of SBP (mmHg)	7.4 (5.8-8.9)	9.1 (8.1-10.2)	7.4 (6.0-8.8)	1.4 (-1.6-4.4)	5.4 (3.5-7.2)*	6.3 (3.8-8.8)*
Mean change of DBP (mmHg)	1.8 (0.9-2.6)	2.1 (1.5-2.7)	1.4 (0.6-2.2)	0.6 (-0.9-2.3)	1.9 (0.9-2.9)	0.6 (-0.7-2.0)
Mean change of PP (mmHg)	5.6 (4.5-6.8)	7.0 (6.2-7.8)	5.9 (4.8-7.0)	0.8 (-1.6-3.3)	3.4 (1.9-4.9)	5.7(3.7-7.8) *
	No Hypertension			**Hypertension**		
	II (531)	***ID (1208)***	***DD (678)***	***II (317)***	***ID (810)***	***DD (392)***
Mean change of SBP (mmHg)	8.9 (7.4-10.5)	11.3 (10.2-12.3)*	8.6 (7.2-10)	0.4 (-2 3.1)	3.4 (1.8-5.1)*	4.5 (2.2-6.7)*
Mean change of DBP (mmHg)	3.2 (2.3-4.1)	3.9 (3.3-4.5)	2.5 (1.4-3.1)	-1.4 (-2.7-0.02)	-0.6 (-1.5-0.1)	-0.6 (-1.8-0.6)
Mean change of PP (mmHg)	5.8 (4.5-7.1)	7.3 (6.5-8.2)	6.3 (5.2-7.5)	1.9 (0.3-3.9)	4.0 (2.8-5.2)	5.1 (3.3-6.8)*

Models adjusted for age, gender, use of antihypertensive medications, body mass index, diabetes mellitus, smoking, total cholesterol, high- density lipoprotein cholesterol.
SBP: systolic blood pressure. DBP: diastolic blood pressure. PP: pulse pressure.
*Statistically significantly different from II genotype ($p < 0.05$)

95% confidence intervals (CI) of SBP were 6.1 (4.7-7.5), 8.2 (7.5-9.3) and 7.4 (5.9-8.5) mmHg in subjects with the II, ID and DD genotype, respectively. Corresponding changes of DBP levels were 1.5 (0.6-2.1), 2.0 (1.6-2.5) and 1.2 (0.5-1.9) mmHg. Finally, corresponding mean changes of PP through genotypes were 4.3 (3.3-5.4), 6.0 (5.3-6.7) and 5.9 (4.9-6.9) mmHg, respectively. Results of analyses performed in strata of age are presented in Table 3. Overall in men we observed the most pronounced differences in blood pressure, the change in SBP in heterozygotes almost being doubled compared to I-allele homozygotes. These heterozygotes also had a high DBP and PP. In women we generally observed the same trend, although not significant. When comparing those under and over 70 years of age, the effects on SBP and PP were only observed in the latter group. SBP was increased for D-allele carriers in those with and without hypertension, whereas a significant association with PP was only observed in hypertensives.

DISCUSSION

In this large population-based study we have found that subjects with the ID and DD genotype of the ACE I/D gene polymorphism had a higher increase of both pulse and systolic blood pressure during follow-up when compared with subjects with the II genotype. We found no difference of diastolic blood pressure changes among genotypes.

Blood pressure levels changes with age and it has been shown that pulse pressure, which is a consequence of large artery stiffness, increases linearly with age and is the dominant hemodynamic factor in older adults.[14] Long-term follow-up studies in large populations have emphasized that the levels of pulse and systolic blood pressure, in addition to their rates of change with age, contribute substantially to cardiovascular risk, therefore it is interesting to know which variables can influence the development of wide pulse pressure.[3,4,15] Previous studies studies found the D allele of the ACE gene to be associated with mean increases of pulse pressure in subjects with hypertension.[9,10] However, these studies were cross-sectional and included relatively small number of participants. To the best of our knowledge no large population-based ongoing study has investigated the association of the ACE I/D gene polymorphism and pulse pressure changes over time. In a previous study, we found that the D allele of the ACE gene was associated with increased arterial stiffness, which is responsible for wide pulse pressure.[16] This is the first large prospective population-based study investigating whether the ACE I/D gene polymorphism is associated with changes of the pulse pressure during follow-up; in this study we found that subjects with the D allele of the ACE gene polymorphism present a higher increase of pulse and systolic blood pressure when compared with subjects with the I allele over time. The mean increase of the systolic and pulse blood pressure levels is higher in older subjects.

The mechanisms that may modulate the relation between ACE gene and wide pulse pressure levels are not completely clear. Higher circulating levels and tissue ACE activity are present in subjects with the D compared to the I allele.[17-19] ACE catalyzes the conversion of angiotensin I to angiotensin II and the breakdown of bradykinin to kinin degradation products. Both angiotensin II and bradykinin are potent peptide hormones that play a role in vascular wall homeostasis reducing vascular tone, vascular smooth muscle cell growth and production of extracellular matrix.[20-23] These processes may then lead to progressive degeneration of arterial media with fractures and fragmentation of elastic lamellae, increased collagen and calcium content and dilation and hypertrophy of the large arteries with subsequent increased arterial stiffness. Hence, chronic exposure to high levels of circulating and tissue ACE may predispose to increased arterial stiffness and eventually to higher pulse pressure.

We found the highest mean increases of the systolic and pulse blood pressure in subjects with the ID polymorphism, the differences in systolic and pulse blood pressure between subjects with the ID and the DD polymorphism were minimal and were not statistically significant. One possible explanation for these results could be a higher morbidity and mortality

rate in subjects with the DD polymorphism and stiff arteries; in this case, these subjects were not able to be included in the study. Conversely, in stratified analyses, we found that older subjects (>70 years) and hypertensive subjects with the DD polymorphism had the highest mean increase of the pulse pressure.

Some aspects of this study need to be discussed. Information on blood pressure levels was not available for all participants which was primarily due to logistic reasons, therefore, we believe that this will not have biased the results. Secondly, information on blood pressure levels were obtained on a single occasion and the use of multiple blood pressure measurements or the use of ambulatory monitoring would improve accuracy and precision. Thirdly, we did not exclude subjects who used anti-hypertensive medication and this could bias our results. However, we performed multivariate analyses adjusted also for the use of antihypertensive medication we think that in this way we could avoid bias. Finally, since the population of the Rotterdam study consisted mostly of Caucasians, results may not apply to other ethnic groups.

In conclusion, in a large population-based study we found that subjects with the ID and DD genotype of the ACE I/D gene polymorphism had a higher increase of both pulse and systolic blood pressure during follow-up when compared with subjects with the II genotype. However, determining the relative contribution of genetic and environmental factors in the development of complex diseases is difficult. As regulation of blood pressure levels most probably results from a complex of interactions between gene–gene and gene–environment factors, the findings of the present study on candidate genes for blood pressure levels should be carefully interpreted.

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Chapter 4.3

Variation in the Matrix Metalloproteinase 3 Gene and Arterial Stiffness

Background and purpose - Arterial stiffness increases with age and has been found to predict cardiovascular disease. Matrix metalloproteinase (MMP)-3 is a member of a group of endopeptidases and plays an important role in the turnover of the extra-cellular matrix and composition of the vascular wall. We studied the total common genetic variation in the MMP-3 gene by means of three polymorphisms, MMP-3 1187 5A/6A, 2092 A/G and 9775 T/A and resultant haplotypes in relation to arterial stiffness.

Methods - This study (n=3892) was embedded in the Rotterdam Study, a prospective, population-based study among subjects aged 55 years and older. The associations of genotypes with pulse wave velocity, the carotid distensibility coefficient and pulse pressure were examined using analyses of variance. The associations between haplotypes and measures of arterial stiffness were assessed using the Haplo.Stats programme. All analyses were adjusted for age, sex, known cardiovascular risk factors and measures of atherosclerosis, and where appropriate also for mean arterial pressure and heart rate.

Results - A lower pulse wave velocity was found for those homozygous for the 6A-allele in comparison to 5A/6A heterozygotes (p=0.02), and also for 2092-G homozygotes in comparison with wildtype 2092 AA homozygotes (p=0.04). No associations were found between genetic variation in MMP-3 genes and the other measures of arterial stiffness. The haplotype-analyses yielded no relations with measures of stiffness.

Conclusions - The 6A6A genotype of the MMP-3 5A/6A and the GG genotype of the 2092 A/G polymorphisms were associated with a lower pulse wave velocity. No associations were observed of genetic variation in MMP-3 with carotid distensibility and pulse pressure. Further studies are needed to elucidate the influence of genetic variation in the MMP-3 gene on arterial stiffness.

INTRODUCTION

Generally, arterial stiffness increases with age and has been associated with hypertension, diabetes mellitus (DM), end-stage renal disease and atherosclerosis.[1-6] Arterial stiffness has been found to predict cardiovascular events in various populations.[7-10]

Matrix metalloproteinase-3 (MMP-3), or stromelysin-1, was first described by Chin et al.. It is an important member of a family of endopeptidases involved in the turnover of extracellular matrix in a variety of tissues and is also involved in the remodeling of the vascular wall.[11-13] MMP-3 affects several substrates, such as elastin, laminin and several collagens, and it is also involved in the activation of other MMP's (e.g. MMP-8, -9 and -13).[12,14] Variations in bioavailability of MMP-3 may influence arterial stiffness and the MMP-3 gene is a candidate gene for further study of determinants of arterial stiffness.

The MMP-3 gene is located on chromosome 11 in regions 11q22.2-22.3. The functional 5A/6A promoter polymorphism was first identified by Ye et al. and has been described to influence MMP-3 levels, blood pressure, carotid artery stenosis and arterial stiffness.[13,15-17] However, results so far have been ambiguous, as both alleles have been associated with adverse outcomes and increased expression of MMP-3.[12,14] The 5A-allele has been associated with myocardial infarction, aneurysms and arterial stiffness.[15,18-20] On the other hand, the 6A-allele has been found to be associated with increased carotid intima-media thickness (IMT), vascular restenosis and also with arterial stiffness.[15,21-24]

We studied genetic variation in the MMP-3 gene in relation to arterial stiffness within a population-based cohort study.

METHODS

The Rotterdam Study is an ongoing prospective cohort study including 7983 participants of 55 years and older. Its general aims are to investigate determinants of chronic diseases, including cardiovascular disease, dementia and osteoporosis.[25] During the first phase of this study (1990-1993), all inhabitants of a Rotterdam suburban area (Ommoord) aged 55 years and over were invited to participate in this study. The response rate was 78%. The third examination phase took place from 1997-1999, during which measurements of arterial stiffness were performed. Approval of the Medical Ethics Committee of the Erasmus University Rotterdam was obtained for the Rotterdam Study. From all participants written informed consent was acquired. A more in depth description of the design of the Rotterdam Study has been published previously.[25]

Genotyping

Genomic DNA was isolated from peripheral venous blood, previously obtained at baseline during the first examination session, according to standard protocols. We used the TaqmanTM assay from Applied Biosystems Inc. (ABI) for genotyping of the polymorphisms. The polymorphisms have been described at http://www.ncbi.nlm.nih.gov/SNP under identification numbers rs3025058 (1187 5A/6A), rs522616 (2092 A/G), and rs563096 (9775 T/A). Details on these methods have been described previously.[26]

Haplotyping

The Seattle SNPs Program for Genomic Applications (http://pga.gs.washington.edu) has, based on 23 unrelated individuals of European descent from the CEPH pedigrees, identified 41 SNPs in the gene encoding MMP-3. By genotyping three tagging SNPs with overall frequencies above 4%, we inferred four haplotypes representing the complete genetic variation in the MMP-3 gene in populations of European descent. Haplotype alleles were inferred by means of the haplo.em function of the program Haplo.stats package (http://cran.r-project.org/src/contrib/ Descriptions/haplo.stats.html) that functions as a component of the statistical program R (http://www.r-project.org/). Haplotype alleles were coded as haplotype numbers 1 through 4 in order of decreasing frequency in the population. Coding from 1187 5A/6A, 2092 A/G to 9775 T/A, we denoted the four most frequent haplotypes: haplotype 1 as 5A-A-T, haplotype 2 as 6A-G-T, haplotype 3 as 6A-A-T, and haplotype 4 as 6A-A-A (Table 1). The other haplotypes had a frequency of < 1% and were not considered in the analyses.

Table 1. Haplotypes based on MMP-3

	5A/6A	*2092 A/G*	*9775 T/A*		
Haplotype	*rs3025058*	*rs522616*	*rs563096*	n	Frequency
1	5A	A	T	3069	51.1%
2	**6A**	**G**	T	1207	20.1%
3	**6A**	A	T	919	15.3%
4	**6A**	A	**A**	811	13.5%
other				1	< 1%

The structure of each haplotype is depicted here, by way of displaying the alleles of each polymorphism, that are included in the haplotype
Based on n=6007 (number of participants successfully genotyped and haplotyped in total Rotterdam Study population)

Arterial Stiffness

Arterial stiffness was measured by two methods, i.e. the carotid-femoral pulse wave velocity (PWV) as a measure of aortic stiffness and the distensibility coefficient (DC) of the common carotid artery as a measure of common carotid arterial stiffness. In addition, pulse pressure (PP) was assessed as an indicator of arterial stiffness. All measures were obtained on the same day, during the same session during the third examination phase.

Carotid-femoral PWV (m/s) was measured using an automatic device (Complior, Colson) and was calculated as the ratio between the distance travelled by the pulse wave and the foot-to-foot time delay.

Common carotid artery distensibility was assessed by measuring the vessel wall motion of the right common carotid artery using a duplex scanner (ATL Ultramark IV, operating frequency 7.5 MHz) connected to a vessel wall movement detector system.[27,28] The cross-sectional arterial wall distensibility coefficient (DC) (1/MPa) was calculated as a measure of arterial stiffness.[29] A decreased distensibility coefficient implies increased carotid stiffness.

Pulse pressure (PP) (mmHg) was defined as the difference between systolic and diastolic blood pressure, using the mean systolic and diastolic blood pressure of two measurements obtained by measuring blood pressure on the right arm using a random-zero sphygmomanometer.

Details on all measures of stiffness, have been extensively described previously.[6,9]

Clinical Characteristics

Information on cardiovascular risk factors was collected during the third follow-up examination. Data on drug use and smoking habits were obtained during the home interview.

At the research center, blood pressure was measured twice on the right arm using a random-zero sphygmomanometer. The average of the two blood pressure values was used in the analyses. Length and weight were measured and body mass index (weight/height^2) (BMI) was calculated. Serum total cholesterol and high-density lipoprotein (HDL) cholesterol values were determined by an automated enzymatic procedure (Boehringer Mannheim System). Diabetes mellitus (DM) was defined as use of anti-diabetic medication and/or a fasting serum glucose level of equal to or above 7.0 mmol/L.[30] Evaluation of the atherosclerotic status of participants was accomplished using ultrasonography (carotid intima-media thickness [IMT]) and radiography (aorta calcification); these methods have been extensively described previously.[31,32]

Population for Analysis

The Rotterdam Study comprises 7983 subjects. Of these, 6007 were successfully and completely genotyped. A total of 4797 subjects participated in the third phase of the Rotterdam Study. Of these, 4024 subjects underwent the full physical examination of the third phase. PWV was measured in 3550 subjects; 69 subjects (1.9%) were excluded from the analyses because of poor quality of the PWV recordings, leaving 3481 subjects. Common carotid distensibility was measured in 3098 subjects. Data on successful genotyping of at least one polymorphism and on at least one measure of arterial stiffness was available for 3892 participants. Missing information on measures of arterial stiffness was almost entirely due to logistic reasons.

Statistical Analyses

Chi-square tests were performed to test for deviations from Hardy-Weinberg equilibrium. Missing data on clinical characteristics were imputed using Expectation-Maximization algorithms available in SPSS. The associations between genotypes and arterial stiffness were investigated using analyses of variance (SPSS). The associations between haplotypes and arterial stiffness were analyzed using the function haplo.glm available in the program Haplo Stats.[33-35] The haplo.glm analysis is based on a generalized linear model and computes the regression of a trait on haplotypes and other covariates. All analyses were adjusted for age and sex and, when analyzing PWV and DC, also for mean arterial pressure (MAP) and heart rate. Additionally, the analyses were adjusted for body mass index, HDL and total cholesterol levels, smoking and diabetes mellitus, and in the full model also for measures of atherosclerosis. A p-value of 0.05 and smaller was considered significant in all analyses. The statistical analyses were performed using SPSS version 11.0.1 for MS-Windows.

RESULTS

General characteristics of the study population are described in Table 2. All genotype and allele proportions were in Hardy Weinberg equilibrium.

Table 2. General Characteristics of Study Population

Characteristic	
Total number	3892
Age – yrs	72.4 ± 7.1
Male sex – %	43
Body mass index – kg/m^2	27 ± 7
Systolic blood pressure – mm Hg	143 ± 21
Diastolic blood pressure – mm Hg	75 ± 11
Mean Arterial Pressure – mm Hg	98 ± 13
Total cholesterol – mmol/l	5.8 ± 1.0
HDL-cholesterol – mmol/l	1.4 ± 0.4
Smoking Current – %	30
Former – %	38
Never – %	32
Diabetes – %	9
Pulse Wave Velocity – m/s	13.6 ± 3.0
Distensibility Coefficient – 1/MPa	10.5 ± 4.3
Pulse Pressure – mm Hg	68 ± 18
Intima Media Thickness – mm	0.78 ± 0.13
Aortic calcifications* – %	22

Values depicted as mean ± SD

* % Aortic calcifications of ≥ 2.5 cm length

Table 3. Arterial Stiffness by MMP-3 Genotype

MMP-3 5A/6A	*n*	*Pulse Wave Velocity (m/s)*	*n*	*Distensibility Coefficient (1/MPa)*	*n*	*Pulse Pressure (mmHg)*
5A/5A	851	13.5 (13.4-13.7)	752	10.4 (10.2-10.7)	996	67.9 (66.9-69.0)
5A/6A	1515	13.7 (13.5-13.8)	1356	10.5 (10.3-10.7)	1833	68.2 (67.4-68.9)
6A/6A	757	13.4 (13.2-13.6)*	663	10.4 (10.2-10.7)	912	68.4 (67.3-69.5)
MMP-3 2092 A/G						
AA	2041	13.6 (13.5-13.7)	1799	10.5 (10.3-10.6)	2420	68.3 (67.7-69.0)
AG	987	13.6 (13.4-13.7)	876	10.4 (10.2-10.6)	1193	67.8 (66.8-68.7)
GG	136	13.1 (12.7-13.6)**	124	10.1 (9.5-10.7)	170	69.3 (66.8-71.9)
MMP-3 9775 T/A						
TT	2354	13.6 (13.5-13.7)	2086	10.4 (10.3-10.6)	2812	68.1 (67.4-68.7)
AT	746	13.6 (13.4-13.8)	659	10.4 (10.1-10.6)	894	68.6 (67.5-69.7)
AA	50	14.0 (13.3-14.6)	40	11.2 (10.1-12.2)	58	70.0 (65.7-74.4)

Results show mean (95% confidence interval)
Analyses were adjusted for age and sex, and for pulse wave velocity and the distensibility coefficient also for mean arterial pressure and heart rate
*6A/6A < 5A/6A p=0.02
**GG < AA p=0.04

5A/6A polymorphism

Pulse wave velocity was 0.3 m/s lower for 6A-homozygotes in comparison with heterozygotes (5A/6A) (p=0.02), also after full adjustment (Table 3). However, no significant trend was found across genotypes. No differences in the distensibility coefficient or pulse pressure were found between the genotypes (Table 3).

2092 A/G polymorphism

GG homozygotes had a significantly lower pulse wave velocity (0.5 m/s) than wildtype homozygotes (AA) (p=0.04) (Table 3). The difference remained significant after full adjustment. After adjustment for traditional cardiovascular risk factors and, additionally, for measures of atherosclerosis, GG-homozygotes also had a significantly lower pulse wave velocity in comparison with AG-heterozygotes (p=0.04), but no significant trend was found. For the distensibility coefficient and for pulse pressure no significant differences between genotypes were observed (Table 3).

9775 T/A polymorphism

Analyses showed no significant differences in pulse wave velocity, the distensibility coefficient and pulse pressure between genotypes (Table 3).

MMP-3 haploptypes

Haplotype reconstruction resulted in six haplotypes. Four of these haplotypes had frequencies of >1%: haplotype 1 (5A-A-T, 51%), haplotype 2 (6A-G-T, 20%), haplotype 3 (6A-A-T, 15%),

Table 4. Measures of Arterial Stiffness by MMP-3 Haplotypes

	Pulse Wave Velocity (m/s)		***Distensibility coefficient (1/MPa)***		***Pulse pressure (mmHg)***	
	(n=2969)		***(n=2635)***		***(n=3561)***	
Haplotype	*β-coefficient (SE)*	*P-value*	*β-coefficient (SE)*	*P-value*	*β-coefficient (SE)*	*P-value*
1 (5A-A-T)*						
2 (6A-G-T)	-0.11 (0.08)	0.16	-0.09 (0.11)	0.41	-1.03 (0.55)	0.06
3 (6A-A-T)	-0.07 (0.09)	0.44	0.08 (0.13)	0.53	0.16 (0.64)	0.80
4 (6A-A-A)	0.04 (0.10)	0.64	0.06 (0.14)	0.68	-0.10 (0.66)	0.88

Analyses adjusted for age and sex, and, for pulse wave velocity and the distensibility coefficient, also for mean arterial pressure and heart rate

Results are based on the haplo.glm analysis; the β-coefficient reflects the regression coefficient

* Haplotype 1 is used as the reference

and haplotype 4 (6A-A-A, 14%) (Table 1). Analyses yielded no evidence for any other associations of the haplotypes with either pulse wave velocity, the distensibility coefficient or pulse pressure (Table 4). Additional adjustment did not alter these findings.

Gender-stratified analyses yielded no results essentially different from the sex-combined analyses.

DISCUSSION

We studied genetic variation in the MMP-3 gene, using three polymorphisms 5A/6A, 2092 A/G and 9775 T/A and resultant haplotypes in relation to arterial stiffness. Subjects homozygous for the 6A-allele had a lower pulse wave velocity than heterozygotes. Furthermore, subjects homozygous for the 2092-G allele had a lower pulse wave velocity than wildtype homozygotes. No significant associations of haplotypes with either pulse wave velocity, the distensibility coefficient or pulse pressure were found.

The 5A/6A polymorphism is located in the promoter of the MMP-3 gene and may influence transcription. The 5A-allele is generally associated with increased expression of the gene.[14,15,36] Consequently, one may expect increased matrix and elastin degradation associated with the 5A-allele and decreased degradation with the 6A-allele. Indeed, the 5A-allele has been associated with pathology related to increased matrix degradation, e.g. with coronary and aortic aneurysms and plaque rupture-induced myocardial infarction.[20,37,38] In turn, the 6A-allele has been associated with pathology associated with matrix accumulation and decreased degradation, such as carotid stenosis and increased IMT.[21,22,24] Literature on this polymorphism and its relation with arterial stiffness is very limited. Medley et al. found a higher stiffness in both the 5A and 6A homozygotes in 77 elderly Australians.[15] In 213 healthy Australians the 5A-allele was associated with increased large artery stiffness. Also, 5A-homozygotes had a higher systolic blood pressure than 6A-allele carriers in 1111 Australians.[17]

We found 6A-homozygotes to have a lower pulse wave velocity than 5A/6A heterozygotes. This may be explained by the fact that elastic properties are mostly mediated by the matrix, therefore, decreased degradation of elastic components may result in decreased stiffening of the vascular wall.[15] This finding, however, was not supported by findings for the distensibility coefficient and pulse pressure.

Both MMP-3 2092 A/G and 9775 T/A have not, to our knowledge, been studied before in relation to arterial stiffness or described in relation to other conditions in other studies. We found the 2092 G-allele to be associated with decreased pulse wave velocity. However, the effects of 2092 A/G and 9775 T/A polymorphisms on gene expression and matrix degradation are unknown and remain to be elucidated in further studies. This finding was also not supported by concordant findings for the distensibility coefficient and pulse pressure.

We found no significant association of the haplotypes with any of the measures of arterial stiffness. However, in the genotype analyses there is evidence for a recessive effect. We did not specifically test for a recessive model in the haplotype analyses. This does not exclude the possibility that individual polymorphisms affect arterial stiffness. The number of haplotype groups was larger than the number of genotypes. Any effects of an individual polymorphism, such as the functional 5A/6A polymorphism, may therefore have gone undetected in the haplotype analyses due to the decrease in power.

To interpret the findings correctly, several methodological aspects of the measures of arterial stiffness need to be discussed. First, pulse waves in the carotid artery and the femoral artery travel in opposite directions, while measurements of carotid-femoral pulse wave velocity are based on the assumption that the pulse wave travels from the carotid artery to the femoral artery. In this way, measuring the distance between the carotid and the femoral artery lead to an overestimation of the distance between the sites of the pulse waves resulting in overestimation of the velocity of the pulse waves. However, variations in anatomy are limited and this error may be considered similar for all subjects examined, therefore we do not think it has seriously biased our results. Second, the distance between the carotid and the femoral artery may be overestimated in (especially obese) subjects when this distance is measured by tape. To avoid this error we adjusted the analyses for body mass index. Third, in computing the carotid distensibility coefficient, we used the brachial pulse pressure rather than the carotid pulse pressure. Information on comparisons between the carotid and the brachial pulse pressure indicates that the carotid pulse pressure is lower than the brachial pulse pressure but differences are relatively small.[39]

Finally, some other methodological issues need to be discussed. Our study is based on a large ongoing population-based study. We adjusted for established risk factors and atherosclerosis. Data on measures of stiffness were not available for all subjects who visited the research center. Missing information was primarily due to logistic reasons, which is likely to be random and thus will not have biased our results. Furthermore, one has to appreciate that any effects of genetic variation on a trait with a complex and multifactorial pathogenesis,

such as arterial stiffness, are generally modest. Finally, because our study was performed in a population of predominantly elderly Caucasian subjects, the generalizability of our findings to younger individuals or other ethnicities remains uncertain.

In summary, we studied genetic variation in the MMP-3 gene by means of three polymorphisms (5A/6A, 2092 A/G and 9775 T/A) in relation to arterial stiffness. We found that the 6A allele of the MMP-3 5A/6A and the G-allele 2092 A/G polymorphisms have a moderate inverse effect on pulse wave velocity. No associations were found between genetic variation in MMP-3 and other measures of arterial stiffness. Additional and larger studies are needed to confirm our findings and to further elucidate the influence of genetic variation in the MMP-3 gene on arterial stiffness.

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Chapter 5
General Discussion

The aim of the studies described in this thesis was to study the influence of genetic variation in genes that may affect arterial stiffness and cardiovascular events. Of each study the merits and limitations have been described in detail in the previous chapters. The current chapter will provide a more general discussion and includes the background of this thesis, the main findings and suggestions for future research.

BACKGROUND

Arterial Stiffness

An important function of the arterial system, is that the (elastic) arteries store volume energy in order to limit the rise in the systolic blood pressure. Generally in the arterial system the pulsatility of the blood flow, generated by the heart, is decreased, which eventually results in a continuous flow at the level of the capillaries. The function of the arterial system is mainly achieved by the structure of the wall of the blood vessels, especially that of the arterial wall. The stiffness of a blood vessel is dependent on several structural components of the arterial wall, such as collagen and elastin. At low pressures, especially the elastin fibers which are very stretchable but less rigid, determine stiffness.[1] In high pressure situations, collagen plays a larger part in determining stiffness, as this can withstand greater stress, but is not as stretchable as elastin fibers.[2,3] The combination of these structural elements allow for a cushioning effect, the so-called "Windkessel effect": normally, during systole about 40% of the cardiac stroke volume is forwarded to the peripheral arteries. The remainder is stored in the capacitive arteries (large arteries), whereby the arterial wall is distended. During diastole, the arteries recoil and thus propel the blood into the peripheral circulation. Changes in arterial stiffness will therefore have haemodynamic consequences. With increasing age, arterial stiffness increases due to changes in the structure of the arterial wall: elastin will thin and fracture, and in addition, collagen deposition will increase.[4-6] This increased arterial stiffness is the dominant haemodynamic factor, responsible for the increase in systolic blood pressure, decrease in diastolic blood pressure and increase in pulse pressure later in life.[7]

The Rotterdam Study

The studies presented in this thesis are based on the Rotterdam Study (ERGO Onderzoek, an ongoing prospective population-based cohort study, which started in 1990. The original cohort consists of 7983 subjects, aged 55 years and over, who live in Ommoord, a suburb of Rotterdam. Recently, also subjects aged 45 years and over, became eligible for inclusion in this study. Its overall aim is to investigate determinants of chronic disabling diseases, such as cardiovascular disease, dementia and osteoporosis.[8] Baseline data were obtained during the first phase of this study (1990-1993). This phase included a home interview and two visits to the research center. Extensive information was obtained, including information on cardio-

vascular risk factors, measurements of atherosclerosis and blood samples. These procedures were repeated at consecutive phases of the study.

Measures of arterial stiffness were obtained during the third examination phase (1997-1999). These measurements included aortic pulse wave velocity (PWV), the carotid distensibility coefficient and pulse pressure. A total of 4797 subjects participated in the third phase of the Rotterdam Study. Of these, 4024 subjects underwent the full physical examination of the third phase. PWV was measured in 3550 subjects; 69 subjects (1.9%) were excluded from the analyses because of poor quality of the PWV recordings, leaving 3481 subjects. Common carotid distensibility was measured in 3098 subjects. Pulse pressure was available for all subjects participating in the third phase.

General practitioners (GPs) in the research district, with whom 85% of the participants of the Rotterdam Study were enlisted, reported fatal and non-fatal cardiovascular events. Research assistants verified all information by checking medical records at the GPs' offices. All medical records of the participants under the care of general practitioners outside the study area 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, the cause and circumstances of death were established by a questionnaire from the GPs. Two research physicians independently coded all reported events according to the International Classification of Diseases, 10th edition (ICD-10).[9] Codes on which the research physicians disagreed were discussed in order to reach consensus. Finally, a medical expert in cardiovascular disease, whose judgement was considered final, reviewed all events. CHD was defined (based upon ICD-10) as the occurrence of an MI (I21), revascularization procedure (percutaneous transluminal coronary angioplasty or coronary artery bypass graft) (Z95.5 & Z95.1), ischaemic heart disease (I20, I22-25), sudden cardiac death (I46), ventricular fibrillation or tachycardia (I49), congestive heart failure (I50) or sudden death undefined (R96) during follow-up. Sudden death was defined as death occurring instantaneously or within 1 hour after onset of symptoms. Incident MI was defined as the occurrence of a fatal or non-fatal MI after the baseline examination. Information on all potential strokes and transient ischaemic attacks were reviewed by both a research physician and an experienced stroke neurologist (P.J.K.) to verify all diagnoses. Subarachnoid hemorrhages and retinal strokes were excluded. Stroke was subclassified as ischaemic when a CT or MRI scan, made within 4 weeks after the stroke occurred, ruled out other diagnoses, or when indirect evidence (deficit limited to 1 limb or completely resolved within 72 hours, atrial fibrillation in absence of anticoagulants) pointed at an ischaemic nature of the stroke. A stroke was subclassified as hemorrhagic when a relevant hemorrhage was shown on CT or MRI scan, or when the subject lost consciousness permanently or died within hours after onset of focal signs. If a stroke could not be subclassified, it was called unspecified.

Genotyping was performed on blood samples obtained at baseline and stored at -80 degrees Centigrade.

MAIN FINDINGS

Interleukin 6 -174 G/C Promoter Polymorphism and Risk of Myocardial Infarction and Coronary Heart Disease

Inflammatory processes play a pivotal role in the pathogenesis of atherosclerosis.[10-13] C-reactive protein (CRP) and IL-6 levels have been found to be associated with risk of cardiovascular disease.[14-23] The IL-6 -174 G/C polymorphism is a functional polymorphism. However, findings of previous studies on the relation between this polymorphism and risk of cardiovascular diseases are inconsistent. We found this polymorphism not to be associated with coronary heart disease, both in the Rotterdam Study, as well as in a meta-analysis of nearly 20,000 subjects. We did find, however, the C-allele to be associated with increased plasma levels of CRP. No significant association with IL-6 plasma levels was found. These levels, however, were only determined in a small subgroup.

Transforming Growth Factor β1 and Risk of Myocardial Infarction and Stroke

Transforming growth factor β1 (TGF-β1) is a pleiotropic cytokine with a central role in inflammation. However, TGF-β has many different functions, both pro- and anti-atherogenic. Some consider the overall effect of TGF-β to be protective, reducing the risk of cardio- and cerebrovascular disease.[24-30] Others describe TGF-β as inducing or facilitating cardio- and cerebrovascular pathology such as vascular stenosis and thrombogenesis.[31-36] Little is known about the relation of variations within the gene and risk of cardio- and cerebrovascular disease. We investigated five polymorphisms in the TGF-β1 gene (-800 G/A, -509 C/T, codon 10 Leu/Pro, codon 25 Arg/Pro and codon 263 Thr/Ile) in relation to risk of myocardial infarction and stroke. No association with risk of myocardial infarction was found. A significantly increased risk of stroke, both overall and ischaemic, was found, associated with the risk alleles of the -509 C/T polymorphism (relative risk of 1.24) and the codon 10 polymorphism (relative risk of 1.26). Both the -509 C/T and codon 10 polymorphism are potentially functional and the associations between the polymorphisms and (ischaemic) stroke may very well be due to changes in expression or activity of TGF-β. It remains unclear why similar effects on risk of myocardial infarction were not observed.

Interleukin 6 -174 G/C Promoter Polymorphisms and Arterial Stiffness

Interleukin 6 (IL-6) is a pleiotropic cytokine with many different functions. It plays an important role in the acute-phase response and inflammatory cascade, such as up-regulation of acute-phase proteins as CRP.[37-40] An association between IL-6 levels and increased arterial stiffness has been described.[41-43] Also elevated CRP levels have been shown to be associated with increasing pulse pressure and increased arterial stiffness.[43-46] Fishman et al. described the functional -174 G/C promoter polymorphism in the IL-6 gene.[47] This polymorphism appears to influence the IL-6 gene transcription and is associated with elevated CRP levels.[47-51] We studied the association of the -174 G/C polymorphism, arterial stiffness and plasma levels

of interleukin 6 and C-reactive protein within the Rotterdam study. We found the C-allele of the -174 G/C polymorphism to be associated with increased pulse wave velocity. Although not significant, results on pulse pressure suggested a similar pattern. Concordantly, CRP plasma levels were associated with increased arterial stiffness. IL-6 plasma levels were not significantly associated with arterial stiffness.

Variation in the CRP Gene and Arterial Stiffness

CRP is an inflammatory marker and was found to be associated with arterial stiffness. Genetic factors account for part of the variance in CRP levels. We studied the association of the CRP polymorphisms 1184 C/T, 2042 C/T, 2911 C/G and haplotypes with arterial stiffness within the Rotterdam study. CRP serum levels were significantly associated with pulse wave velocity and pulse pressure. Also, CRP serum levels were associated with the individual polymorphisms: 1184 C/T (positively), 2042 C/T (inversely) and 2911 C/G (positively). Genotype and haplotype analyses showed no consistent significant associations between pulse wave velocity, the distensibility coefficient or pulse pressure. These findings might suggest that CRP itself is not causally related to arterial stiffness. However, genetic variation in the CRP gene has only a small effect on CRP level. Therefore, a small effect of genetic variation in CRP on arterial stiffness may have gone undetected and a judgment about causality cannot be given with certainty.

Variation in the TGF-β1 Gene and Arterial Stiffness

Transforming growth factor β1 (TGF-β1) is a pleiotropic cytokine. Its many effects include influence on T-cells, on the release of (other) cytokines and fibroblast growth factor, but it also exerts influence on the vascular wall, e.g. on angiogenesis, on endothelial cells and on modulation of the extra-cellular matrix.[14,24,28,52-55] We investigated five potentially functional polymorphisms in the TGF-β1 gene (-800 G/A, -509 C/T, codon 10 Leu/Pro, codon 25 Arg/Pro and codon 263 Thr/Ile) in relation to arterial stiffness, both as separate genotypes, as well as in haplotypes.[56-58] No associations with pulse wave velocity, distensibility coefficient or pulse pressure were found. We hypothesize that any effects that these TGF-β polymorphisms might have on the vascular wall, do not lead to significant changes in arterial stiffness.

Variation in the Fibrinogen α and γ Genes and Arterial Stiffness

Fibrinogen is an acute phase protein and has been associated with arterial stiffness. We studied genetic variation in the fibrinogen α (FGA) and γ gene (FGG), by means of seven SNPs (FGA: -58 G/A, 1374 G/A, 1526 T/C and 312 Thr/Ala, and FGG: 4288 G/A, 6326 G/A and 7792 T/C) and concordant haplotypes in relation to arterial stiffness and pulse pressure. Fibrinogen plasma levels were positively associated with aortic stiffness (pulse wave velocity) and pulse pressure. Analyses showed fibrinogen plasma levels to be positively associated with the FGA 1526 T/C SNP, and haplotype 4 (containing the 1526 C-allele), and inversely with the FGA 312

Thr/Ala SNP and FGG 6326 G/A SNP. Aortic stiffness was inversely associated with fibrinogen α haplotype 3. Pulse pressure was positively associated with fibrinogen α haplotype 4, and inversely with haplotype 3 and fibrinogen γ haplotype 4. These findings indicate that fibrinogen levels and genetic variation in the fibrinogen α and γ gene are associated with arterial stiffness. However, we cannot conclude a causal relation between fibrinogen plasma level and stiffness with certainty, because genetic variation in these genes may (also) also affect structure and thereby function of fibrinogen.

Variation in Renin Angiotensin System Genes and Arterial Stiffness

The renin-angiotensin system (RAS) influences the vascular system in many ways. Renin converts angiotensinogen (AGT) into the inactive angiotensin I (AT-I); angiotensin-converting enzyme (ACE) converts the AT-I into the active angiotensin II (AT-II), which is the main product of the RAS.[59] This system is involved in regulation of blood pressure and circulating volume. In addition the RAS plays an important role in vascular regulation and atherogenesis, it influences cell growth and proliferation and endothelial dysfunction.[60-62] Genetic variation in components of this system influences the vascular wall and arterial stiffness.[63] We studied polymorphisms of angiotensin II (ACE I/D), angiotensinogen (AGT 235 M/T) and the angiotensin II receptor type I (AT1R 573 C/T) in relation to arterial stiffness. The ACE I/D polymorphism was not associated with pulse wave velocity, but the D-allele was shown to be associated with decreased distensibility coefficient (in a previous study) and with increased pulse pressure (current study).[64] For the angiotensinogen 235 M/T polymorphism no significant associations with either pulse wave velocity, the distensibility coefficient or pulse pressure were found. Also, no associations with the angiotensin II receptor type 1 573C/T polymorphism were observed. Our findings suggest genetic variation in genes of the renin-angiotensin system, especially ACE I/D, to be moderately associated with carotid stiffness and pulse pressure.

Insertion/Deletion Gene Polymorphism of the ACE and Blood Pressure Changes

The insertion/deletion (I/D) polymorphism of the ACE gene may also be involved in blood pressure changes. Aim of the present study was to assess the relationship between the ACE I/D gene and the change of blood pressure levels during follow up. The association of the ACE I/D polymorphism and mean changes in SBP, DBP and pulse pressure levels between the first and third examination phase of the Rotterdam Study was studied. In adjusted models, subjects with the D allele had higher mean changes of systolic and pulse pressure than subjects with the I allele. No difference was found for mean change of diastolic blood pressure among genotypes. These results suggest the ACE ID/DD genotypes to be associated with increased mean changes of systolic and pulse pressure.

Variation in the Matrix Metalloproteinase 3 Gene and Arterial Stiffness

Matrix metalloproteinase (MMP)-3 is a member of a group of endopeptidases and plays an important role in the turnover of the extra-cellular matrix and composition of the vascular wall. We studied genetic variation in the MMP-3 gene by means of three polymorphisms, MMP-3 5A/6A, 2092 A/G and 9775 T/A and concordant haplotypes, in relation to arterial stiffness. We found a lower pulse wave velocity for those homozygous for the 6A-allele in comparison to 5A6A heterozygotes, and also for 2092-G homozygotes in comparison with 2092 A/G heterozygotes and wildtype 2092 AA homozygotes. No associations were found for the 9775 T/A polymorphism and for the other measures of arterial stiffness. The haplotype-analyses yielded no significant findings. Our findings suggest the risk alleles of the MMP-3 5A/6A and 2092 A/G polymorphisms to have an inverse effect on pulse wave velocity. The findings were not supported by the results on other measures of stiffness. Haplotype analyses showed no associations with arterial stiffness.

METHODOLOGICAL ASPECTS

Study Design

In this thesis several study designs were used: the studies on arterial stiffness were generally cross-sectional in design. The two studies on coronary heart disease and stroke were cohort studies.

When studying determinants of a certain outcome in a longitudinal design, these are generally obtained or measured before the outcome occurs. This is feasible in case the end-points are well-described in time, such as myocardial infarction or stroke. With respect to genetic variation as a determinant, time of "measurement" is generally less relevant, because this variation is constant throughout life and does not change. However, also for genetic determinants there may be 'selective survival'. Risk allele carriers may have died before onset of the study due to other diseases, also associated with the genetic variation.

Also for non-genetic determinants (e.g. fibrinogen levels) timing of measurements is sometimes difficult. Often one is interested in lifelong exposure. However, it is not always clear which measurements best reflect this lifelong exposure. In the Rotterdam Study, for example, measurements may have been performed at baseline (e.g. fibrinogen levels), but sometimes also at later moments during the study (e.g. CRP). When studying an outcome as arterial stiffness, which lacks a clear onset, it may be even more difficult to assess which of the measurements of the determinant is the best measure of exposure.

Measures of Stiffness

Large topographic/anatomical differences in structure and function of the arterial system exist. As a consequence, arterial stiffness cannot be measured at any random site within

the arterial system. To present a representative assessment of arterial stiffness, we used three diverse measures of arterial stiffness in the Rotterdam Study: pulse wave velocity as a measure of aortic stiffness (a more general measure of arterial stiffness), the distensibility coefficient as a measure of carotid stiffness (a local measure) and additionally pulse pressure, as an indicator of stiffness.

Pulse wave velocity is generally considered and accepted as the most robust and non-invasive method to assess arterial stiffness in general.[65] It is easy to perform and is reproducible. It is a direct measurement and is has been shown to be an independent predictor of cardiovascular disease.[65-67] Carotid-femoral PWV is a measure for aortic stiffness. It primarily involves the aorta, of which the supradiaphragmatic part plays a major role in the buffering function of the arterial system (together with the carotid arteries). In addition, "local" PWV (i.e. non-aortic PWV), such as brachial PWV was shown to have no predictive value.[68] Recording of the pressure waveforms can be done with various devices and techniques, but generally all methods using mechanotransducers (such as the Complior, Colson, that was used in the Rotterdam Study) or applanation tonometers are well accepted for PWV measurements.

In addition to the regional measurement PWV, we also used a local measure of stiffness (the distensibility coefficient). Local measurements of stiffness (i.e. measured at a single site, which does not reflect a larger area, such as the carotid-femoral PWV) may also be of prognostic importance, depending on the site of measurement. Carotid stiffness is one of the most important of the local measurements, since the carotid arteries are a frequent site of atherosclerosis and atheroma formation. Local measurements are mostly obtained using ultrasound systems, as was also the case in the Rotterdam Study. Ultrasounds are also, at the moment, the only means to determine arterial distensibility non-invasively. Several methods are used, such as the distensibility coefficient, the compliance coefficient, and the stroke change in diameter and lumen. There appears to be no preference for a specific index, however, the distensibility coefficient is among the indices recommended as part of the suggested standard in a recent consensus paper on measurement of arterial stiffness.[65] Generally, all superficial arteries can be used, i.e., those arteries accessible for ultrasonographic evaluation. Although both PWV and the distensibility coefficient offer information on stiffness, in hypertensives/diabetics aortic stiffness increases more with age and other cardiovascular risk factors than carotid stiffness.[69] Therefore, the addition of the distensibility coefficient is both useful and important, especially in patients with increased cardiovascular risk.

Systemic arterial stiffness can also be assessed non-invasively by using methods based on analogies with electrical models (using capacitance and resistance in series).[65] But to date, measuring systemic arterial stiffness using these methods has not found to be of independent predictive value for cardiovascular disease.[65]

Central pulse-wave analyses (central pulse pressure, central systolic pressure and the Augmentation Index AIx) are indirect measures of stiffness. PWV and DC are, by contrast, direct measurements. Central pulse pressure and AIx also have several disadvantages (e.g.

they do not represent intrinsically arterial stiffness, they are more sensitive to influence of medication and heart rate). Age, however, is of greater influence on PWV, than on AIx, which is an advantage of the latter method because other determinants can be better studied when age plays a lesser role. Despite the limitations, central pulse wave analyses provide useful information on arterial stiffness. Central pulse pressure, mentioned above, however, is not as easily obtainable as peripheral pulse pressure. Peripheral pulse pressure is also no direct measurement of arterial stiffness, but can be used as an indicator of arterial stiffness. Peripheral pulse pressure is an easily obtainable measurement. However, peripheral pulse pressure is higher than pulse pressure in central arteries and such peripheral measurements (e.g. brachial pulse pressure) overestimate central SBP and pulse pressure, especially in younger subjects (with less stiff arteries), and can therefore not be used as a proxy for central pulse pressure. We use the peripheral pulse pressure as an easy and readily available, but additional, indicator of arterial stiffness.

Although many methods of assessing arterial stiffness are related or based on similar principles, the diversity may limit possibilities to compare and evaluate studies. The diversity may complicate the choice for a "gold standard" measure of arterial stiffness. This "gold standard" does not have to be limited to a single index of arterial stiffness, but may be comprised of standards for regional and local measurements. Recently, an overview was published by the European Network for Non-invasive Investigation of Large Arteries, in which several indices were suggested as the "gold standard".[65] It is a positive development, that the need for standardization is being recognized and these processes will hopefully lead to a choice and general acceptance of a global "gold standard".[70]

Methodology of Measuring Arterial Stiffness

To interpret the findings on arterial stiffness correctly, several methodological aspects of the measures of arterial stiffness that we used in this thesis need to be discussed. First, pulse waves in the carotid artery and the femoral artery travel in opposite directions, while measurements of carotid-femoral pulse wave velocity is based on the assumption that the pulse wave travels from the carotid artery to the femoral artery. In this way, measuring the distance between the carotid and the femoral artery lead to an overestimation of the distance between the sites of the pulse waves resulting in overestimation of the velocity of the pulse waves. However, variations in anatomy are limited and this error may be considered similar for all subjects examined, therefore we do not think it has seriously biased our results. Second, the distance between the carotid and the femoral artery may be overestimated in, especially obese, subjects when this distance is measured by tape. To avoid this error we adjusted the analyses for body mass index. Third, in computing the carotid distensibility coefficient, we used the brachial pulse pressure rather than the carotid pulse pressure. Information on comparisons between the carotid and the brachial pulse pressure indicates that the carotid pulse pressure is lower than the brachial pulse pressure, but differences are relatively small.[71] Finally, brachial

pulse pressure can be used as an easy and readily available indicator of arterial stiffness, but is not a direct measurement of stiffness.

Genetic Research

The process of findings genes of complex diseases, and thereby potential targets and subjects for treatment, can involve several strategies.

The classical approach is linkage analysis, i.e., to analyse the co-segregation of two of more loci in a family. The aim is to determine whether the two chromosomes demarked by differing alleles at those loci, segregate independently according to the Laws of Mendel, or tend to be inherited together more often than not (in violation of the Laws of Mendel).[72] This is a powerful approach for studying disorders in which a single or limited number of genes are involved. However, for complex diseases or conditions such as arterial stiffness and other cardiovascular pathology, in which a large number of genetic and environmental factors are involved, it may not be the most appropriate approach.

A single genetic variation in a gene, usually a polymorphism, may only render a moderate effect on the risk of a disease. It may, however, contribute to the discovery or exploration of (molecular) pathways, underlying the disease of interest.[73] A disadvantage is that a significant association implies a correlation between (certain variation in) a gene and an outcome; this does not necessarily imply a causal relation.[74] However, an association of a gene/genetic variation with an outcome is unlikely to be confounded by other factors. Therefore, an association between a gene and outcome may provide more information on potential causality than associations of environmental factors with disease.

A considerable amount of research into the genetic background and basis of complex diseases has been performed using candidate gene association studies. This type of study offers an advantage in the ability to detect genes with small effects, assuming the study population size is large. This may play an important role in the genetics of complex diseases or conditions, such as arterial stiffness.[75,76]

Although studying a single polymorphism may sometimes yield highly significant results, many studies of this type have resulted in marginal effects. Confirmation studies often yielded conflicting results. Recently, large SNP databases have become available, containing data on polymorphisms across the entire human genome, which enable us to also study multiple genetic variations to cover genetic variation over larger areas with a relatively small number of polymorphisms, which are tagging a stretch of DNA (haplotype), thereby improving the power and reducing the problem of multiple testing when testing all SNPs in the gene. However, finding an association with a haplotype, does not pinpoint the specific (functional) polymorphism(s) in the haplotype associated with the disease.

The studies, described in this thesis, are based on the Rotterdam Study cohort. As a large population-based cohort, it is a suitable population for both traditional candidate gene association studies, as well as gene-finding genome-screen studies (i.e., testing a trait for

linkage to polymorphic markers spread regularly throughout the genome; linkage may mark genes that are associated with the trait). Although the Rotterdam Study is a large-sized study, it remains possible that true associations have remained uncovered, especially when the studied effects are small and the polymorphisms are rare. Although the overall effect may be small, possibly due to gene-gene interactions, findings may be relevant in subgroups and the genes involved may still be clinically relevant. On the other hand, using a large dataset may also yield associations that are false positive findings.

Therefore, replication of results in other studies is necessary for confirmation of the findings.

Power issues are not limited to studies using a small number of polymorphisms, such as in genotype or haplotype analyses. Especially studies in which large numbers of polymorphisms are genotyped (such as a genome screen) are affected, because in these studies, the possibility of spurious results is relatively high due to the vast number of statistical tests.[77] Correction for "multiple testing" requires large samples to reach adequate power and replication studies.

Causal Associations

The studies described in this thesis, aimed to investigate genes that may affect inflammation and the vascular wall in relation to arterial stiffness. Whenever available, also associations with levels of proteins were included.

A significant association implies only a correlation between variation in a gene and an outcome; it does not enable us to draw conclusions on potential causal relations.[76] However, if genetic variation in a gene specifically affects levels, then differences in levels have effectively been allocated at random at the moment of conception, i.e. "Mendelian randomization".[78] An association between genetic variation and an outcome would then provide convincing evidence for a causal relation between a risk factor and the outcome. However, this is only valid when the genetic variation only affects levels, and not the function of the factor, and when the differences in levels are not a mere reflection of confounding by other factors.[78]

To assess the relation between inflammation and arterial stiffness, variation in two inflammatory genes (IL-6, CRP) was investigated. Genetic variation in the IL-6 gene was associated with measures of arterial stiffness. Also levels of CRP were associated with arterial stiffness. On the other hand, variation in the CRP gene was not associated with arterial stiffness. It should be mentioned, however, that the genetic variation in the CRP gene only has a small effect on the CRP level. Therefore, a small effect of variation in the CRP gene on arterial stiffness may have gone undetected. Therefore, although our findings do suggest the possibility of causality with respect to the relation between inflammation and arterial stiffness, our results cannot support a final conclusion on causality. Further research to this end is needed.

In general, we studied several genes, all coding for proteins with multiple and different functions. In addition, we studied a trait that can be considered complex and multifactorial by means of three different measures, which cover various anatomical regions. This taken together may explain some variability in our findings

FUTURE RESEARCH

In addition to the genes that are described in this thesis, other genes may also affect arterial stiffness. These other genes are therefore potential candidates for further study of arterial stiffness.

As one of the important components of the vascular wall, collagen is an obvious candidate for further studies into arterial stiffness. There are several types of collagen, but in the human vascular wall the major types of collagen are type I and type III, and to a lesser extent also type IV, V, VI and VIII.[79] To our knowledge, the role of collagen and genetic variation in collagen genes in arterial stiffness, has not been extensively studied, yet, there is evidence for collagen to influence arterial stiffness.[80,81] Although current knowledge on genetic variation in collagen genes is still limited, they are certainly likely candidate genes for study into arterial stiffness.

The other major component of the vascular wall is elastin, and again, this is an obvious candidate for further study. Arterial stiffness is dependent on the elastin component.[82] Indeed, an association of an elastin polymorphism (422 Ser/Gly) with the distensibility of elastic arteries was found.[83] Although research on this topic so far has been limited, elastin and the elastin gene may provide another worthwhile goal in the study of arterial stiffness.

In addition to structural components of the vascular wall, such as collagen, elastin and other components, also the process of the turnover of the vascular wall may provide interesting subjects for studying arterial stiffness. In this thesis, we investigated genetic variation in the MMP-3 gene. However, not only MMP-3, but also the other MMPs, e.g. MMP-1 and 9, may influence degradation of vascular wall components and may thus influence arterial stiffness.[80,84]

Another interesting area is the vascular endothelium and its influence on arterial stiffness. The endothelium itself produces vasoactive substances, such as nitric oxide, which have been demonstrated to play a significant role in peripheral resistance, blood pressure and vascular reactivity.[85,86] Also agonists that stimulate endothelial nitric oxide release, such as acetylcholine, reduce stiffness of muscular arteries in vivo.[87,88] Basal nitric oxide production has been demonstrated to influence muscular arteries distensibility in vivo positively and the effect of acetylcholine on large arteries is also mainly nitric oxide-mediated.[89] Therefore, genetic variation in endothelial (dys)function is an interesting candidate for studying arterial stiffness. Recently, a correlation between endothelial function and arterial stiffness was

found in a small study.[90] Furthermore, as inflammatory processes also inhibit endothelium-dependent vasodilatation, the inflammatory pathway may offer further opportunities to study stiffness, such as cytokines and inflammatory markers, other than the ones described in this thesis.[44,91]

The suggestions here for further research are certainly not complete and only offer a limited number of options. The increasing amount of information from studies on the pathophysiology and determinants of arterial stiffness, both genetic and otherwise, indicates that arterial stiffness is by no means a simple unifactorial condition, but a multifactorial complex trait. Therefore, increasing knowledge will offer additional insight and more potential candidates for further study, both on associations with arterial stiffness, but also to elucidate and explore more in depth the underlying biological mechanisms by which determinants affect arterial stiffness.

As for methodology, future studies should not be limited to candidate gene studies. Genome wide association studies will be used more often, especially as ongoing technical developments allow for easy and relatively low-cost genotyping of increasing, cq. vast, numbers of markers (e.g. on so-called 'arrays'). Using a whole genome screen subjects are genotyped for polymorphisms placed across the entire genome at regular distances from each other. This enables us to localize markers highly associated with the outcome and to thus identify genes, which may play a role in the disease of interest, but were not known to be related to the disease before. Such an approach may also yield new topics and new insights for the study of arterial stiffness. The Rotterdam Study based genome screen, which is currently being performed, will provide a rich source of data to detect common variants with small effects.

An alternative for using population-based methods, is the use of a (genetically) isolated population in the search of susceptibility genes for complex diseases. Generally, one assumes affected subjects in these populations, to have a tendency to share ancestral haplotypes and thus common disease mutations.[92] The ERF (Erasmus Rucphen Family Study, a family-based study in a genetic isolate) population offers great potential to study arterial stiffness in such a setting, enabling us to search for a disease locus and potentially "new" genes involved in stiffness. Data from the ERF Study, including a genome screen in this population (to detect rare variants with large effects), will hopefully yield many interesting findings, but will also offer challenges on genetic-statistical level, as this is novel and largely uncharted terrain. The future availability of the Rotterdam Study genome screen will provide a rich source of data and many new options to study various topics in its own right. It will, however, also form a convenient and usable dataset to replicate findings from the ERF Study, in order to confirm findings. On the other hand, findings from the Rotterdam Study may be confirmed in the ERF study. However, one must bear in mind that differences may exist between the populations, as one is population-based and the other is based on a genetically isolated population.

We feel that it is important, that the number of possible arterial stiffness measures will be decreased and that the scientific community will reach global consensus on a "gold standard" for arterial stiffness. This does not necessarily imply that the standard should only consist of one measurement. Given the anatomical differences, the standard may also consist of several measures, covering both regional and local arterial stiffness.

Arterial stiffness has only recently been established as a risk factor for cardiovascular disease. Further research is needed to explore the possibilities for use of measuring stiffness in general medical practice. Previous, current and future research and increasing knowledge on the pathophysiology and underlying mechanisms of arterial stiffness will offer many possibilities for (pharmaceutical) research, which may hopefully result in development of therapeutic options. In case research results show increased levels of certain proteins to influence the structure of the vascular wall, then medication that affect these levels or sensitivity of receptors for those proteins, may offer possibilities to slow down an increase in stiffness, or even reduce stiffness.

OVERALL CONCLUSION

Arterial stiffness is, at least in part, heritable, but the genes that form the basis of the regulation and pathogenesis of arterial stiffness are still largely unknown. In this thesis we aimed to investigate variation in several genes that may influence arterial stiffness. We found variations in certain genes, such as the IL-6 and fibrinogen gene, to be associated with increased arterial stiffness, and also an association of CRP and fibrinogen levels with stiffness. Variations in the MMP-3 gene and the ACE gene were also associated with stiffness, whereas variations in the CRP and TGF-β gene were not clearly associated with arterial stiffness.

The pathogenetic background of increased arterial stiffness is still largely uncovered. Our results suggest that inflammation is associated with arterial stiffness, but we cannot conclude a causal role for inflammatory mediators. Our results further implicate that not a single, but multiple systems influence arterial stiffness. This multifactoriality emphasizes the importance of further study into (other) genes that may be involved in regulation of arterial stiffness and to further elucidate the underlying mechanisms. When it becomes clearer which systems are involved and when a "gold standard" for measuring arterial stiffness has been globally accepted, one may begin speculating on possibilities of practical risk profiling and possible (aims for) interventions. Although in this thesis only genetic variation in a few genes was described, our results demonstrate the complex nature of arterial stiffness and substantiate the need for further research in this field.

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Chapter 6
Summary - Samenvatting

SUMMARY

Arterial stiffness increases with age. It is also associated with various diseases, such as diabetes mellitus and hypertension. Recently, arterial stiffness has also been found to independently predict cardiovascular disease. The pathogenesis of arterial stiffness, however, has not been fully explored. The aim of the studies described in this thesis was to study the influence of variation in genes that may affect arterial stiffness and cardiovascular events. All studies were based on the Rotterdam Study, a population-based cohort study, originally including 7983 participants of 55 years and older.

Chapter 1 offers a general introduction to the studies and topics that are described in this thesis.

The first studies in this thesis focused on variation in inflammatory genes in relation to cardiovascular events such as myocardial infarction and stroke.

Chapter 2.1 describes the association between the well-known inflammatory interleukin 6 (IL-6) -174 G/C promoter polymorphism and risk of coronary heart disease. Inflammatory processes play an important role in the pathogenesis of coronary heart disease. However, no evidence was found for an association between the IL-6 -174 G/C polymorphism and coronary heart disease in the Rotterdam Study, nor in a meta-analysis on this topic. The risk allele of the IL-6 -174 G/C polymorphism was, however, associated with increased levels of C-reactive protein (CRP).

In chapter 2.2 several polymorphisms in the transforming growth factor β1 (TGF-β1) gene, which influences inflammatory processes, were studied in relation to risk of myocardial infarction and stroke. We found the risk alleles of the -509 C/T and codon 10 Leu/Pro polymorphisms, both functional, to be associated with an increased risk of ischaemic stroke. This supports an atherogenic role for TGF-β in the pathogenesis of stroke.

The remaining studies focused on inflammatory genes and genes that affect the structure of the vascular wall in relation to arterial stiffness (pulse wave velocity, the carotid distensibility coefficient and pulse pressure). Inflammatory processes may influence arterial stiffness, therefore we examined several genes related to inflammation.

Chapter 3.1 described the association of the IL-6 -174 G/C polymorphism, CRP and arterial stiffness. Both the risk allele of this polymorphism as well as the CRP-level were associated with increased pulse wave velocity, supporting a causal role of inflammatory processes in arterial stiffness.

In chapter 3.2, variation in the CRP gene was studied in relation to arterial stiffness. CRP is an inflammatory protein and CRP level has been associated with arterial stiffness. Genetic variation in the CRP gene was associated with CRP level. Geno- and haplotype analyses, however, yielded no evidence for an association between genetic variation in the CRP gene and arterial stiffness. This finding might suggest that CRP itself is not causally related to arterial stiffness. However, genetic variation in the CRP gene has only a small effect on CRP level. Therefore, a

small effect of genetic variation in CRP on arterial stiffness may have gone undetected and a judgment about causality cannot be given with certainty.

Chapter 3.3 focused on polymorphisms in the TGF-β1 gene in relation to arterial stiffness. We found none of the studied polymorphisms, or resultant haplotypes, to be associated with arterial stiffness. Although a possible small effect may have gone undetected, these polymorphisms appear to have no large effects on stiffness, despite their functionality.

Chapter 3.4 described the association between variation in the fibrinogen α and γ genes and arterial stiffness. Fibrinogen is an acute phase protein and may also influence the vascular wall. We found genetic variation in the fibrinogen α and γ genes to be associated with the level of fibrinogen and with pulse wave velocity and pulse pressure. However, we cannot conclude a causal relation between fibrinogen level and stiffness, because genetic variation in these genes may also affect structure and thereby function of fibrinogen. Therefore, the effect on stiffness may be through an effect on function of fibrinogen rather than through an effect on level of fibrinogen. Although fibrinogen is thought to be associated with inflammation and matrix interactions, the precise mechanisms by which fibrinogen might affect arterial stiffness remains to be elucidated.

Arterial stiffness may also be influenced by genes that structurally affect the vascular wall or the composition of the vascular wall.

Genes of the renin-angiotensin (RAS) system are an important example. In chapter 4.1, variation in the angiotensin-II, angiotensinogen and angiotensin-II receptor type I genes in relation to arterial stiffness is described. The D-allele of the angiotensin converting enzyme (ACE) I/D polymorphism was associated with carotid distensibility. We found the D-allele also to be associated with pulse pressure. The variations in the other genes of the RAS system were not associated with arterial stiffness.

Chapter 4.2 focused on the ACE I/D polymorphism in relation to changes in blood and pulse pressure during follow up. The findings suggest the D-allele to be associated with larger changes in systolic and pulse pressure over time.

Finally, chapter 4.3 described the association between variation in the matrix-metalloproteinase-3 (MMP-3) gene and arterial stiffness. MMP-3 influences the extra-cellular matrix and composition of the vascular wall. Indeed, the risk alleles of the 5A/6A and 2092 A/G polymorphisms appeared inversely associated with pulse wave velocity, although haplotype analyses yielded no significant relation between MMP-3 haplotypes and measures of arterial stiffness. Our findings on the relation of the functional 5A/6A polymorphism with arterial stiffness suggest MMP-3 to affect arterial stiffness, possibly due to its effects on matrix degradation.

In the general discussion, chapter 5, the main findings of this thesis are discussed. The results indicate that variations in some genes that affect inflammatory processes and the structure of the vascular wall, are associated with arterial stiffness. However, due to generally modest effects of genetic variation on complex traits, in addition to limited knowledge on the pathogenetic mechanisms by which arterial stiffness is influenced, additional research is

needed before one can draw more definite conclusions on questions of causality. Our findings support, nevertheless, the view that arterial stiffness can be considered a multifactorial and complex trait. For clinical and practical applications of arterial stiffness, we emphasize the need for standardization in measurement techniques.

SAMENVATTING

Arteriële vaatstijfheid neemt toe met de leeftijd. Het is ook geassocieerd met verscheidene ziektes, zoals diabetes mellitus en hypertensie. Recentelijk is ook ontdekt dat arteriële vaatstijfheid van onafhankelijke voorspellende waarde is bij cardiovasculaire ziekte. De pathogenese van arteriële vaatstijfheid is echter nog grotendeels onbekend. Het doel van de studies, die beschreven zijn in dit proefschrift, was dan ook de invloed te onderzoeken van variaties in genen die mogelijk van invloed zijn op arteriële vaatstijfheid en cardiovasculaire events. Alle studies zijn gebaseerd op de ERGO Studie (Rotterdam Study), een zgn. "population-based" cohort studie, die van origine 7983 deelnemers van 55 jaar en ouder omvat.

Hoofdstuk 1 biedt een algemene inleiding op de studies en onderwerpen die in dit proefschrift worden beschreven.

De eerste studies richten zich op variaties in ontstekingsgenen en hun relatie met cardiovasculaire events.

Hoofdstuk 2.1 beschrijft de associatie tussen het bekende inflammatoire interleukine 6 (IL-6) -174 G/C promotor polymorphisme en het risico op coronaire hartziekte. Ontstekingsprocessen spelen een belangrijke rol in de pathogenese van coronaire hartziekte. Wij vonden echter in de ERGO Studie geen bewijs voor een associatie tussen het IL-6 -174 G/C polymorphisme en coronaire hartziekte, noch in een meta-analyse over dit onderwerp. Het risico-allel van het IL-6 -174 G/C polymorphisme was echter wel geassocieerd met hogere C-reactive protein (CRP) waardes.

In hoofdstuk 2.2 worden verscheidene polymorphismen bestudeerd in het transforming growth factor β1 (TGF-β1) gen, dat ontstekingsprocessen beïnvloedt, in relatie tot het risico op een myocardinfarct en beroerte (CVA). Wij vonden de risico allelen van de -509 C/T en codon 10 Leu/Pro polymorphismen, beide functioneel, geassocieerd met een verhoogd risico op een ischaemisch CVA. Deze bevindingen ondersteunen een atherogene rol van TGF-β in de pathogenese van een CVA.

De resterende studies richten zich op genen, die ontsteking en de bouw van de vaatwand kunnen beïnvloeden, en hun relatie met arteriële vaatstijfheid (als maten hiervoor zijn gebruikt de zgn. polsgolfsnelheid [pulse wave velocity], de distensibiliteitscoëfficiënt van de carotiden [carotid distensibility coefficient] en de polsdruk [pulse pressure]). Ontstekingsprocessen kunnen mogelijk invloed uitoefenen op arteriële vaatstijfheid. Om die reden hebben wij verscheidene genen bestudeerd die gerelateerd zijn aan ontstekingsprocessen.

Hoofdstuk 3.1 beschrijft de associatie tussen het IL-6 -174 G/C polymorphisme, het CRP niveau en arteriële vaatstijfheid. Zowel het risico allel als het CRP niveau waren geassocieerd met een hogere "pulse wave velocity", hetgeen een causale rol van ontstekingsprocessen bij arteriële vaatstijfheid ondersteunt.

In hoofdstuk 3.2 is variatie in het CRP gen bestudeerd in relatie tot arteriële stijfheid. Variatie in het CRP gen bleek geassocieerd met het CRP niveau. Geno- en haplotype analyses

leverden echter geen bewijs voor een associatie tussen variatie in het CRP gen en arteriële vaatstijfheid. Deze bevinding zou kunnen suggereren dat variatie in het CRP gen geen causale relatie heeft met arteriële vaatstijfheid. Echter, genetische variatie in het CRP gen heeft echter maar een klein effect op het CRP niveau. Om die reden kan een klein effect van deze genetische variatie op arteriële vaatstijfheid onopgemerkt gebleven zijn en kan een definitief oordeel over causaliteit niet gegeven worden.

Hoofdstuk 3.4 beschrijft de associatie tussen variatie in de fibrinogeen α en γ genen en arteriële vaatstijfheid. Fibrinogeen is een acute fase eiwit en beïnvloedt mogelijk de vaatwand. Wij vonden dat variatie in de fibrinogeen α en γ genen geassocieerd is met het fibrinogeen niveau, met "pulse wave velocity" en "pulse pressure". Echter, wij kunnen niet met zekerheid een concluderen dat er een causale relatie bestaat tussen het fibrinogeen niveau en vaatstijfheid, omdat variatie in deze genen ook de structuur en daarmee de functie van fibrinogeen zou kunnen beïnvloeden. Derhalve zou het effect op vaatstijfheid mogelijk ook gemedieerd kunnen zijn door het effect op de functie van fibrinogeen, meer dan door het effect op het niveau. Hoewel fibrinogeen beschouwd wordt ontstekingsprocessen en matrix interactie te beïnvloeden, moeten de mechanismen hoe fibrinogeen vaatstijfheid zou kunnen beïnvloeden nog nader onderzocht worden.

Arteriële vaatstijfheid zou ook beïnvloed kunnen worden door genen die een effect zouden kunnen hebben op de structuur en samenstelling van de vaatwand.

Genen van het renine-angiotensine (RAS) systeem zijn hiervan een belangrijk voorbeeld. In hoofdstuk 4.1 wordt ingegaan op de relatie tussen variatie in de angiotensine-II, angiotensinogeen en angiotensine-II receptor type I genen en arteriële vaatstijfheid. Het D-allel van het "angiotensin converting enzyme" (ACE) I/D polymorphisme was geassocieerd met de distensibiliteit van de carotiden en met "pulse pressure". De variaties in de andere RAS genen bleken niet geassocieerd met arteriële vaatstijfheid.

Hoofdstuk 4.2 beschrijft de relatie tussen het ACE I/D polymorphisme en veranderingen in bloeddruk en "pulse pressure" in een vervolgonderzoek. De bevindingen suggereren dat het D-allel door de tijd heen geassocieerd is met toegenomen veranderingen in systolische bloeddruk en polsdruk.

Tot slot wordt in hoofdstuk 4.3 de associatie beschreven tussen variatie in het matrix metalloproteinase 3 (MMP-3) gen en arteriële vaatstijfheid. MMP-3 beïnvloedt de extracellulaire matrix en de samenstelling van de vaatwand. De risico-allelen van de 5A/6A en 2092 A/G polymorphismen waren (negatief) geassocieerd met "pulse wave velocity". Haplotype analyses leverden echter geen bewijs voor een associatie tussen variatie in het MMP-3 gen en arteriële vaatstijfheid. Desalniettemin suggereren onze bevindingen over het functionele 5A/6A polymorphisme en arteriële vaatstijfheid, dat MMP-3 vaatstijfheid zou kunnen beïnvloeden, mogelijk door de effecten op matrix degradatie.

In de algemene bespreking, hoofdstuk 5, worden de voornaamste bevindingen van dit proefschrift besproken. De resultaten suggereren dat variaties in een aantal genen, die

invloed hebben op ontstekingsprocessen en de structuur van de vaatwand, geassocieerd zijn met arteriële vaatstijfheid. Daar genetische variatie in het algemeen echter meestal slechts beperkte invloed heeft op complexe processen en de kennis over de pathogenese van arteriële vaatstijfheid beperkt is, is verder onderzoek nodig voor definitievere conclusies getrokken kunnen worden over causaliteitsvraagstukken. Onze bevindingen ondersteunen desalniettemin het inzicht dat arteriële vaatstijfheid als een complex en multifactorieel proces beschouwd kan worden. Voor klinische en practische toepassingen van vaatstijfheid, benadrukken wij het belang van standaardisatie in meettechnieken.

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Sie MP, Sayed-Tabatabaei FA, Oei HH, Uitterlinden AG, Pols HA, Hofman A, van Duijn CM, Witteman JC. Interleukin 6 -174 g/c promoter polymorphism and risk of coronary heart disease: results from the Rotterdam study and a meta-analysis. *Arterioscler Thromb Vasc Biol.* 2006;26:212-217.

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Sie MP, Isaacs A, de Maat MP, Mattace-Raso FU, Uitterlinden AG, Kardys I, Hofman A, Hoeks AP, Reneman RS, van Duijn CM, Witteman JC. Genetic variation in the fibrinogen α and γ genes in relation to arterial stiffness: the Rotterdam Study (submitted).

Sie MP, Yazdanpanah M, Mattace Raso FU, Uitterlinden AG, Hofman A, Hoeks AP, Reneman RS, Asmar R, Witteman JC, van Duijn CM. Genetic variation in the renin-angiotensin system and arterial stiffness; the Rotterdam Study (submitted).

Sie MP, van Rooij FJ, de Maat MP, Isaacs A, Mattace-Raso FU, Uitterlinden AG, Hofman A, Hoeks AP, Reneman RS, Asmar R, van Duijn CM, Witteman JC. Genetic Variation in the matrix metalloproteinase 3 gene in relation to arterial stiffness: the Rotterdam Study (submitted).

CURRICULUM VITAE

The author of this thesis was born in Schiedam on July 20, 1978. He attended the "Stedelijk Gymnasium Schiedam" from which he graduated in 1996. In the same year he started his medical studies at the Erasmus University in Rotterdam. He obtained his Medical Degree in 2002 at the age of 23 years. During his medical training, the author was a "Student Representative" and member of the MFVR (Medische Faculteits Vereniging Rotterdam) Educational Council for five consecutive years. After Medical School he worked as a resident in Internal Medicine at the Erasmus MC University Medical Center Rotterdam for one year, after which he started working on this thesis at the Department of Epidemiology & Biostatistics of the Erasmus MC (Head: Prof. Dr. A. Hofman). During this period he was also a member of the Board of this department, representing the PhD Students. In addition, he was a member of the Erasmus MC PhD-Committee (Head: Prof. Dr. P.J. Koudstaal). The author was also a member of the Dutch group involved in the official registration of prion-related diseases (mostly Creutzfeldt-Jacob Disease) (Head: Prof. Dr. Ir. C.M. van Duijn). In June 2005 he obtained a Master's Degree in Genetic Epidemiology from the Netherlands Institute of Health Sciences. In January 2007 he continued his training as an Internist at the Department of Internal Medicine of the Erasmus MC (Head: Prof. Dr. E.J. Kuipers and Dr. J.L.C.M. van Saase). The author is currently working at the Department of Internal Medicine of the IJsselland Ziekenhuis (Head: Dr. H.E. van der Wiel), as part of his training as an Internist.