ACE and Atherosclerosis

pieces of the puzzle

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ACE en atherosclerose
stukjes van de puzzel

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بنام او
تقدیم به پدرم
و به یاد مادرم و برادرم مجدالدین.

To my father;
and in loving memory of my mother,
and my brother Majdedin.

"What is a scientist after all?
It is a curious man looking through a keyhole,
the keyhole of nature, trying to know what's going on."

Jacques-Yves Cousteau
(oceanographer, 1910-1997)

Chapters 2, 3, and 4 are based on the following papers and manuscripts

Chapter 2
Sayed-Tabatabaei FA, van Rijn MJE, Schut AF, Aulchenko YS, Croes EA, Zillikens MC, Pols HAP, Witteman JC, Oostra BA, van Duijn CM. Heritability of the function and structure of the arterial wall: findings in the Erasmus Rucphen Family (ERF) study. Submitted.

Chapter 3.1
Sayed-Tabatabaei FA, Houwing-Duistermaat JJ, van Duijn CM, Witteman JC. Angiotensin converting enzyme gene polymorphism and carotid artery wall thickness: a meta-analysis. *Stroke*. 2003;34:1634-1639.

Chapter 3.2
Sayed-Tabatabaei FA, Schut AF, Hofman A, Bertoli-Avella AM, Vergeer J, Witteman JC, van Duijn CM. A study of gene-environment interaction on the gene for angiotensin converting enzyme: a combined functional and population based approach. *J Med Genet*. 2004;41:99-103.

Chapter 3.3
Mattace-Raso FU, van der Cammen TJ, Sayed-Tabatabaei FA, van Popele NM, Asmar R, Schalekamp MA, Hofman A, van Duijn CM, Witteman JC. Angiotensin converting enzyme gene polymorphism and common carotid stiffness: the Rotterdam Study. *Atherosclerosis*. 2004;174:121-126.

Chapter 4.1
Sayed-Tabatabaei FA, Schut AF, Arias Vásquez A, Bertoli-Avella AM, Hofman A, Witteman JC, van Duijn CM. Angiotensin converting enzyme gene polymorphism and cardiovascular morbidity and mortality: the Rotterdam Study. *J Med Genet*. 2005;42:26-30.

Chapter 4.2
Arias Vásquez A, Sayed-Tabatabaei FA, Schut AF, Hofman A, Bertoli-Avella AM, Vergeer J, Aulchenko YS, Witteman JC, van Duijn CM. Angiotensin converting enzyme gene, smoking and mortality in a population based study. Submitted.

Chapter 4.3
Sayed-Tabatabaei FA, van Dijck WED, Breteler MM, Hofman A, van Duijn CM, Stijnen T, Witteman JC. Adjusting for co-morbidity by inverse probability of censoring weighted analyses: the Rotterdam Study. Submitted.

Contents

Chapter 1

Introduction

Atherosclerosis literally means "hardening of blood vessels". It is a form of progressive pathology of large arteries, characterised by the accumulation of lipids and fibrous tissue in vessel walls. These changes alter both the structure (the thickness of the wall) and the function (elasticity) of the arteries. Atherosclerotic lesions can grow over time, and, in the long run, may lead to blocks in blood flow. Another clinical complication is an acute occlusion due to the formation of a thrombus or blood clot, resulting in myocardial infarction, stroke, or other end organ damages. These diseases are the most important causes of morbidity and are responsible for 50% of all mortality in the United States, Europe and much of Asia.[1]

Alterations in the structure and function of arterial walls can be measured in different ways. Non-invasive techniques are the most frequently applied methods to studies in populations at large. In the studies presented in this thesis, we used carotid artery intima media thickness (IMT) and plaque score as measurements of atherosclerosis. In addition, we also investigated arterial stiffness using carotid distensibility and carotid-femoral pulse wave velocity (PWV). Carotid IMT and plaque score mainly present changes in arterial wall thickness, while distensibility coefficient and PWV are measures of arterial stiffness.

Epidemiological studies over the past 50 years have revealed numerous risk factors for atherosclerosis.[2] Some of traditional risk factors are age, male gender, hyperlipidaemia, hypertension, diabetes, obesity, inflammation, a high-fat diet, smoking, and a lack of exercise. These factors can be grouped into those with important genetic components, and ones that are largely environmental in origin.

Genetics of atherosclerosis

The general, contribution of genetic factors to a disorder in a population can be assessed by heritability estimates, indicating what proportion of the variability of the disorder can be explained by genetic factors in a given population. Using different kinds of atherosclerosis measurements, many scientists investigated the heritability of atherosclerosis[3-5] and showed that a substantial part of this disorder is under genetic influence.

Discovering the specific genetic components of atherosclerosis, however, proved to be a challenging task. Numerous physiological pathways are involved in the establishment of vascular homeostasis. Genetic or environmental factors involved in one or more of those physiological pathways may contribute to the creation and progress of pathological status in the vascular

system. This makes atherosclerosis a typical complex trait. In this thesis, we focused on a single pathway, the renin angiotensin system.

Angiotensin converting enzyme gene and atherosclerosis

The renin angiotensin system is one of the important systems involved in cardiovascular homeostasis in which angiotensin converting enzyme (ACE) plays an important role by converting angiotensin I to angiotensin II.[6] The effects of angiotensin II on the vascular system include systemic vasoconstriction, mediating cell growth and the proliferation of vascular smooth muscle cells by stimulating various cytokines and growth factors,[7] and inducing endothelial dysfunction by reducing nitric oxide bioavailability.[8] ACE, therefore, plays a central role in cardiovascular homeostasis and is considered to be important in the pathogenesis of atherosclerosis.

ACE levels in plasma and tissue are under genetic control.[9,10] The *ACE* gene is located on chromosome 17q23 and contains numerous polymorphisms. The identification, however, of which of these variants directly influence ACE concentrations is ambiguous because of the almost complete linkage disequilibrium present in the Caucasian population.[11] The most frequently studied polymorphism is the presence (insertion, I) or absence (deletion, D) of a 287 bp *alu* repeat. Although located in the non-coding region of the gene (intron 16), it was one of the first available polymorphisms on this gene and received the attention of many researchers when it was reported that higher ACE plasma and tissue levels are present in subjects with the D allele compared with the ones carrying the I allele.[9,10]

Scope of the thesis

We investigated the genetics of atherosclerosis utilizing the *ACE* gene as one of the candidate genes that influence vascular homeostasis and may be responsible for development of atherosclerosis and possibly later clinical cardiovascular outcomes. Chapter 2 provides an overview of the contribution of genetic factors to the function and structure of the arterial wall. This study is based on the ongoing Erasmus Rucphen Family (ERF) study, based in an isolated population in The Netherlands. Using a single pedigree, we estimated the heritability of carotid-femoral PWV, common carotid IMT, and carotid plaques.

In chapter 3, we examined the association between the *ACE* I/D polymorphism and atherosclerosis measurements. We first evaluated the relation between this polymorphism and carotid IMT by means of a meta-analysis (chapter 3.1). Then, we investigated the same association in the Rotterdam Study, a population based cohort study among subjects aged 55 years or older (chapter 3.2). Finally, we examined the relationship between the I/D polymorphism and arterial stiffness using carotid distensibility and carotid-femoral PWV (chapter 3.3).

In chapter 4, the association between the *ACE* I/D polymorphism and clinical outcomes was investigated. We first looked at cardiovascular morbidity and mortality as the main outcomes (chapter 4.1). Next, we evaluated the same association considering total mortality as the outcome of interest (chapter 4.2). Finally, we tested the hypothesis of the co-morbidity effect of a single risk factor in relation to multiple outcomes by using the *ACE* I/D and risk of Alzheimer's disease as an example (chapter 4.3). In chapter 5, a general discussion is presented to give a broader perspective of other studies on the *ACE* I/D polymorphism and to provide suggestions for future research.

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Chapter 2

Genetic contributions to atherosclerosis

Heritability of the function and structure of the arterial wall: the Erasmus Rucphen Family (ERF) study

Abstract

Using 930 individuals connected in a single pedigree from an isolated population, participants of the Erasmus Rucphen Family (ERF) study, we investigated the heritability of carotid-femoral pulse wave velocity (PWV), carotid intima media thickness (IMT), and carotid plaque score. PWV was measured between the carotid and femoral arteries as an indicator of aortic stiffness. Common carotid IMT and plaque score, quantifying alterations in arterial wall structure, were measured by ultrasonography. All three traits were significantly associated with classical cardiovascular risk factors. Age and gender adjusted heritability estimates were 0.36 for PWV, 0.41 for carotid IMT, and 0.28 for plaque score. After adjustment for appropriate risk factors, the heritabilities were 0.26, 0.35, and 0.21 for PWV, IMT, and plaque score, respectively. All heritability estimates were statistically significant ($p < 0.001$). Taking into account different proportions of variance associated with covariates for each trait, genetic factors explained approximately 12% of the total variability for each of the phenotypes. To our knowledge, this is the first report on the heritability of PWV. The heritability estimates of IMT and plaque score were similar to those in previous reports. We conclude that genetic factors significantly contribute to arterial structure and function in this isolated population, presenting the opportunity to locate susceptibility genes related to cardiovascular disorders.

Introduction

Arterial stiffness and atherosclerosis are major factors in pathophysiological pathways leading to various cardiovascular diseases. In western society, these diseases are the underlying cause of about 50% of all deaths.[1] Stiffness of the arteries can be assessed by pulse wave velocity (PWV), a non-invasive measurement of arterial wall function. Aortic stiffness, as measured by carotid-femoral PWV, has been shown to be an independent predictor of cardiovascular morbidity and mortality in patients with essential hypertension[2,3] and end-stage renal disease,[4,5] and has been shown to be strongly associated with atherosclerosis at various sites in the vascular tree.[6]

Carotid intima media thickness (IMT) has been used as a proxy for atherosclerosis. It has been shown to be correlated with atherosclerosis in other arterial sites[7] and a strong predictor of myocardial infarction.[8] In addition, quantitative assessment of plaques in carotid arterial walls can also be used as an indicator of atherosclerosis.[9]

Genetic factors play a major role in alterations of arterial wall function and structure.[10] Prior findings, however, on the extent to which genetic factors may explain the variance of these traits (heritability) are highly variable and limited in number. Heritability estimates (h^2) of carotid IMT published thus far range widely from 0.21 to 0.92,[11,12] but most of the studies reported heritabilities around 0.30 and 0.40.[13-17] To our knowledge, only one study on the heritability of carotid plaques was performed,[18] yielding an estimate of 0.23, and no heritability estimate for PWV has been published to date.

We studied the heritability of carotid-femoral PWV, carotid IMT, and carotid plaques in an extended pedigree from an isolated population in The Netherlands.

Materials and methods

Study population

Subjects were participants of the Erasmus Rucphen Family (ERF) study. This is an ongoing large family based study, which began in June 2002 in a genetically isolated population located in the southwest of The Netherlands. This population was founded in the middle of the 18th century by approximately 150 individuals and was isolated until the last few decades. It is characterised by rapid growth and minimal immigration and has now expanded to roughly 20,000 inhabitants.

For this population, an extensive genealogical database including over 63,000 individuals is available. For the ERF study, 20 couples were selected that had at least six children baptised in the community church between 1880 and 1900. All living descendants of the selected couples and their spouses (n ≈ 2500) were invited to participate in the study. The pedigree members were not selected based on any disease status. This study is based on the first 930 participants for whom complete phenotypic data were available at the time of the analyses. The Medical Ethics Committee of the Erasmus Medical Centre Rotterdam approved the study and informed consent was obtained from all participants.

Data collection

At the research centre, located within the community, extensive clinical examinations were done, including the collection of fasting blood samples, anthropometric measurements, cardiovascular assessments, neurological tests, ophthalmologic examinations and personal interviews. The interviews were performed by medical practitioners and included questions on education level, smoking status, current medication use, and medical history.

Glucose, lipids, creatinine, and plasma albumin levels were measured from the fasting blood sample, according to standard procedures.[19,20] Height and weight were measured with the participant in light underclothing and body mass index (kg/m^2) was computed. Blood pressure was measured twice on the right arm in a sitting position after at least five minutes rest, using an automated device (OMRON 711). The average of the two measures was used in the analyses. Mean arterial pressure (MAP) was calculated as 1/3 systolic blood pressure + 2/3 diastolic blood pressure.

During the cardiovascular assessment, carotid-femoral pulse wave velocity (PWV) was measured by means of an automatic Complior® SP device with the subjects in a supine position. The time delay between the rapid upstroke of the feet of simultaneously recorded pulse waves in the carotid and the femoral arteries were assessed and the distance between the carotid and the femoral arteries was measured over the surface of the body with a tape measure. PWV was calculated as the ratio between the distance travelled by the pulse wave and the time delay, and expressed in meters per second. The Complior® SP device recorded the pulse waves during a 10 second time frame and automatically calculated the mean PWV of the recorded pulses.

A duplex scan ultrasonographic investigation of the carotid arteries was made using a 7.5 MHz linear array transducer (ATL, Ultramark IV).

A careful search was performed for the lumen-intima interface and the media-adventitia interface. Three optimal still images were recorded on the videotapes from each artery site (common carotid, bifurcation and internal carotid arteries at the right and left sides). Measurements of intima media thickness (IMT) were performed offline using the still images. The interfaces of the far and near wall of the distal common carotid artery are marked over a length of 10 mm. We used measurements of common carotid IMT in this study, which is calculated as the mean of the maximum IMT of the near and far wall measurements from both the left and right arteries. When a plaque was present at the 10 mm measurement site, IMT was measured at the region closest to the plaque.

During the ultrasound, the common carotid artery, carotid bifurcation and internal carotid artery were also visualised over the longest segment possible in both the left and right sides for the presence of plaques. Plaques were defined as local widening of the arterial wall relative to the adjacent segment, with protrusion into the lumen. The total plaque score reflected the total number of sites with plaques and ranged from 0 to 12 (considering the far and near walls of the artery in each of the three different arterial sites on both the left and right sides).

Statistical analyses

General characteristics were compared between men and women, using student *t*-test for continuous variables and chi-square test for dichotomous variables, with SPSS 11.0 for Windows. Inbreeding coefficients were calculated using the PEDIG software[21] based on a pedigree of the total population. The inbreeding coefficient equals the probability that two identical alleles at a given locus in an individual are identical by descent (are copies of the same ancestral allele). This coefficient represents the degree of consanguinity between the parents of the individual.

For the heritability analyses, we first performed univariable and multivariable regression analyses using SPSS. All covariates that were significant at the 0.10 level in the multivariable analysis were retained in the final model for heritability estimation. In order to obtain a normal distribution of the regression residuals for the traits under study, we used the natural logarithm transformation of (PWV – 3) and (IMT – 3). Since MAP is shown to strongly influence arterial stiffness,[22] it was used instead of systolic and diastolic blood pressures in the analyses related to PWV. In the multivariable regression models for IMT and plaque score, the beta coefficient for diastolic blood pressure is reported from a model without systolic blood

Table 1. Demographic characteristics of the study population

Variable	Men (n = 376)	Women (n = 554)
Age (years)	53.85 ± 13.71	51.05 ± 14.36*
Body mass index (kg/m^2)	27.50 ± 4.06	26.70 ± 4.81*
Systolic BP (mmHg)	145.14 ± 20.24	138.93 ± 22.86*
Diastolic BP (mmHg)	82.25 ± 10.42	78.82 ± 9.66*
Mean arterial pressure (mmHg)	103.21 ± 12.34	98.86 ± 12.61*
LDL cholesterol (mmol/L)	3.73 ± 0.97	3.74 ± 1.04
HDL cholesterol (mmol/L)	1.13 ± 0.32	1.37 ± 0.36*
Triglycerides (mmol/L)	1.59 ± 1.02	1.29 ± 0.63*
Fasting glucose (mmol/L)	4.94 ± 1.05	4.63 ± 1.04*
Current smoking (%)	33.69	48.36*
No college education (%)	90.37	90.73
Inbreeding (%)	78.72	77.08
Pulse wave velocity (m/s)	10.55 ± 2.49	9.48 ± 2.06*
Common carotid IMT (mm×10^{-1})	9.15 ± 2.18	8.31 ± 1.92*

Continuous values are mean ± standard deviation.
BP: blood pressure; HDL: high density lipoprotein; IMT: intima media thickness; LDL: low density lipoprotein.
* $p < 0.05$ compared with men.

pressure to avoid the multicolinearity effect. Both variables were used in the final analyses.

A variance component maximum likelihood method implemented in the SOLAR 2.1.2 software package[23] was used to partition the phenotypic variance of PWV, IMT, and plaque score into their additive genetic and environmental elements. The contribution of genetic factors to these traits was then estimated as the heritability, defined as the proportion of variance (after correction for covariates) explained by additive genetic components. Heritability estimates were calculated using a model with only age and gender and a full model with all significant covariates from the regression analyses to determine the extent genetic factors contribute to the variation in PWV, IMT, and plaque score, independent of the measured risk factors. We also presented the contribution of the genetic factors to the total variance of each trait in different models, calculated as: [(1 – proportion of variance explained by covariates) × heritability estimate]. Significance was determined by likelihood ratio tests.

Table 2. Univariable and multivariable regression analyses of PWV*

Variable	Univariable		Multivariable	
	Beta ± SE	*p*	Beta ± SE	*p*
Age (years)	0.014 ± 0.001	< 0.001	0.011 ± 0.001	< 0.001
Male gender	0.114 ± 0.017	< 0.001	0.079 ± 0.017	< 0.001
Body mass index (kg/m^2)	0.004 ± 0.002	0.044	–0.001 ± 0.002	0.911
Mean arterial pressure (mmHg)	0.008 ± 0.001	< 0.001	0.007 ± 0.001	< 0.001
LDL cholesterol (mmol/L)	–0.017 ± 0.008	0.035	–0.015 ± 0.008	0.049
HDL cholesterol (mmol/L)	–0.037 ± 0.024	0.117	–0.025 ± 0.024	0.289
Triglycerides (mmol/L)	0.029 ± 0.010	0.004	0.007 ± 0.012	0.534
Fasting glucose (mmol/L)	0.027 ± 0.009	0.002	0.011 ± 0.008	0.176
Current smoking	–0.010 ± 0.017	0.503	–0.003 ± 0.016	0.865
No college education	–0.001 ± 0.028	0.989	–0.008 ± 0.027	0.776
Heart rate (beat/m)	0.001 ± 0.001	0.008	0.001 ± 0.001	0.057
Inbreeding coefficient	–1.568 ± 1.232	0.203	–0.236 ± 1.181	0.842

In the univariable analyses all associations are adjusted for age and gender. The association between gender and PWV is adjusted for age, and the association between age and PWV is adjusted for gender.
Beta: regression coefficient; HDL: high density lipoprotein; LDL: low density lipoprotein; PWV: pulse wave velocity; SE: standard error.
* Natural logarithm transformation was performed to obtain a normal distribution of the residuals.

Results

The gender specific characteristics of the participants are shown in Table 1. All 930 participants were part of one extended pedigree. Men were, on average, three years older than women. Almost all of the established cardiovascular risk factors were higher in men, with the exception of low density lipoprotein (LDL) cholesterol, which showed no differences between genders, and current smoking, which was higher in women (Table 1).

Table 2 shows the association between risk factors and PWV in univariable and multivariable models. In the univariable model, the association between gender and the outcome variables was adjusted for age, and the association between age and the outcomes was adjusted for gender. All other associations were adjusted for both age and gender. Only high density lipoprotein (HDL) cholesterol, smoking, low education, and inbreeding coefficient were not significantly related to PWV (Table 2). In the multivariable model, the only factors that significantly determined PWV were age,

Table 3. Univariable and multivariable regression analyses of IMT*

Variable	Univariable		Multivariable	
	Beta ± SE	*p*	Beta ± SE	*p*
Age (years)	0.019 ± 0.001	< 0.001	0.016 ± 0.001	< 0.001
Male gender	0.095 ± 0.015	< 0.001	0.070 ± 0.017	< 0.001
Body mass index (kg/m^2)	0.007 ± 0.002	< 0.001	0.005 ± 0.002	0.016
Systolic BP (mmHg)	0.003 ± 0.001	< 0.001	0.004 ± 0.001	< 0.001
Diastolic BP (mmHg)	0.002 ± 0.001	0.023	0.002 ± 0.001†	0.052†
LDL cholesterol (mmol/L)	0.017 ± 0.007	0.021	0.020 ± 0.008	0.011
HDL cholesterol (mmol/L)	–0.093 ± 0.021	< 0.001	–0.062 ± 0.024	0.009
Triglycerides (mmol/L)	0.024 ± 0.009	0.009	0.004 ± 0.012	0.760
Fasting glucose (mmol/L)	0.026 ± 0.007	0.001	0.014 ± 0.008	0.091
Current smoking	0.046 ± 0.015	0.002	0.049 ± 0.016	0.002
No college education	0.066 ± 0.026	0.012	0.032 ± 0.027	0.235
Heart rate (beat/m)	–0.001 ± 0.001	0.012	–0.001 ± 0.001	0.015
Inbreeding coefficient	–0.377 ± 1.092	0.730	–1.368 ± 1.174	0.244

In the univariable analyses all associations are adjusted for age and gender. The association between gender and IMT is adjusted for age, and the association between age and IMT is adjusted for gender.

Beta: regression coefficient; BP: blood pressure; HDL: high density lipoprotein; IMT: intima media thickness; LDL: low density lipoprotein; SE: standard error.

* Natural logarithm transformation was performed to obtain a normal distribution of the residuals.

† Estimated from a multivariable model without systolic blood pressure.

gender, MAP, LDL cholesterol, and heart rate. These factors were included in the multivariable adjusted model for the heritability analyses. Since fasting glucose levels have been associated with PWV,[24] this variable was also included in the final model.

Table 3 presents the results of the univariable and multivariable regression analyses for IMT. The univariable model showed that all of the considered risk factors were significantly associated with IMT, excepting inbreeding coefficient. In the multivariable model, only triglycerides, low education, and inbreeding coefficient showed no significant association with IMT. For plaque score, diastolic blood pressure, triglycerides, glucose levels, low education, heart rate, and inbreeding coefficient were not significantly associated in the univariable model (Table 4). In the multivariable model, the significance level of the associations remained unchanged.

The estimated components of variance for PWV, IMT, and plaque

Table 4. Univariable and multivariable regression analyses of plaque score

Variable	Univariable		Multivariable	
	Beta ± SE	*p*	Beta ± SE	*p*
Age (years)	0.152 ± 0.006	< 0.001	0.130 ± 0.008	< 0.001
Male gender	0.767 ± 0.182	< 0.001	0.672 ± 0.206	0.001
Body mass index (kg/m^2)	–0.049 ± 0.020	0.015	–0.078 ± 0.023	0.001
Systolic BP (mmHg)	0.026 ± 0.005	< 0.001	0.040 ± 0.006	< 0.001
Diastolic BP (mmHg)	–0.001 ± 0.009	0.994	0.010 ± 0.010*	0.317*
LDL cholesterol (mmol/L)	0.193 ± 0.090	0.031	0.238 ± 0.094	0.011
HDL cholesterol (mmol/L)	–0.680 ± 0.261	0.009	–0.761 ± 0.287	0.008
Triglycerides (mmol/L)	0.147 ± 0.110	0.183	0.021 ± 0.140	0.882
Fasting glucose (mmol/L)	–0.008 ± 0.089	0.926	0.075 ± 0.102	0.460
Current smoking	1.013 ± 0.180	< 0.001	0.839 ± 0.195	< 0.001
No college education	0.473 ± 0.317	0.136	0.012 ± 0.324	0.971
Heart rate (beat/m)	–0.004 ± 0.005	0.390	–0.004 ± 0.005	0.346
Inbreeding coefficient	19.858 ± 13.244	0.134	15.769 ± 14.133	0.265

In the univariable analyses all associations are adjusted for age and gender. The association between gender and plaque score is adjusted for age, and the association between age and plaque score is adjusted for gender.
Beta: regression coefficient; BP: blood pressure; HDL: high density lipoprotein;
LDL: low density lipoprotein; SE: standard error.
* Estimated from a multivariable model without systolic blood pressure.

score are presented in Table 5. All estimates of heritability were statistically significant. Adjusted for age and gender, the heritability estimate was 0.36 for PWV, indicating that the additive effects of genes account for 36% of the variation in PWV that is not explained by age and gender. With 0.45 of the variance being explained by age and gender, genetic factors account for $[(1.0 – 0.45) \times 0.36] \approx 0.20$ of the total variance in the PWV trait. After further adjustment for additional covariates, the estimate of heritability was 0.26, meaning that $[(1.0 – 0.53) \times 0.26] \approx 0.12$ of the total variance of the PWV trait is explained by genetic factors. For IMT, the heritability was 0.41 in the first model and 0.35 in the second models, suggesting that genetic factors explain ≈ 0.16 and ≈ 0.12 of the total variance of IMT in the first and the second model, respectively. Heritability estimates of plaque score were 0.28 and 0.21, corresponding to ≈ 0.17 and ≈ 0.12 of the total variance explained by genetic factors.

Table 5. Components of variance for carotid-femoral PWV, carotid IMT, and plaque score

		Proportion of variance associated with covariates	Heritability (SE, *p* value)
PWV*	Model A	0.451	0.361 (0.087, < 0.001)
	Model B	0.532	0.257 (0.084, < 0.001)
IMT*	Model A	0.612	0.411 (0.073, < 0.001)
	Model C	0.657	0.348 (0.078, < 0.001)
Plaque score	Model A	0.402	0.279 (0.071, < 0.001)
	Model D	0.435	0.205 (0.072, < 0.001)

Model A includes age and gender.
Model B includes age, gender, mean arterial pressure, LDL cholesterol, fasting glucose, and heart rate.
Model C includes age, gender, body mass index, systolic and diastolic blood pressures, LDL and HDL cholesterol, fasting glucose, smoking and heart rate.
Model D includes age, gender, body mass index, systolic blood pressures, LDL and HDL cholesterol, and smoking.
HDL: high density lipoprotein; IMT: intima media thickness; LDL: low density lipoprotein; PWV: pulse wave velocity; SE: standard error.
* Natural logarithm transformation was performed to obtain a normal distribution of the residuals.

Discussion

In this large family based study in an isolated population, we investigated the contribution of genetic and environmental factors to carotid-femoral pulse wave velocity, common carotid intima media thickness, and carotid plaque score. Our study indicated that after adjusting for age, gender, blood pressure, heart rate, plasma lipids, fasting glucose, body mass index, and smoking, when appropriate, the additive effects of genes explain significant proportions of the variability in the function and structure of the arterial wall in this population.

To our knowledge, the present study is the first to report on the heritability of pulse wave velocity, which mainly measures aortic stiffness. In the Rotterdam Study, we previously showed that arterial stiffness is strongly associated with common carotid intima media thickness, severity of plaques in the carotid artery, and severity of plaques in the aorta.[6] The associations between CVD risk factors and PWV that we observed in this

isolate were in line with previous findings.[25] In this study, we investigated to what extent genetic factors contribute to the variation of PWV in this population and if this is comparable to the contribution of genetic factors to the variance of atherosclerosis measurements; carotid IMT and plaque score. For PWV, we found a heritability of 0.36. This estimate is quite similar to the heritability of IMT in our study. After adjustment for appropriate cardiovascular risk factors, the PWV heritability was reduced to 0.26. Although this reduction in the heritability estimate is greater than that observed for other traits in our study, we also observed a greater increase in the proportion of variance associated with the covariates. Some of these covariates are genetically mediated themselves, such as blood pressure (presented in this analysis as MAP).

Duggirala *et al* [12] reported a considerably higher heritability of carotid artery IMT (h^2 = 0.92) among a small sample (46 sibships of various sizes) from Mexico City; however, the authors suggested that their findings should be interpreted with caution because the characteristics of their data (small sample of sibships only) might have inflated their heritability estimate. In contrast, the findings from the present study are similar to those in previous reports, suggesting that genetic factors account for 0.30 to 0.40 of IMT variation in families, after adjustment for traditional CVD risk factors.[14-17]

IMT is a standardised measurement of atherosclerosis with relatively high precision. This measurement, however, takes into account both intima and media thickness of the arterial wall, and, in early ages, it might be influenced by arterial hypertrophy rather than atherosclerosis. In this study, we also considered the genetic basis of another marker of subclinical atherosclerosis, the carotid plaque score. Compared with IMT, plaque score is considered to be a more accurate measurement of atherosclerosis, although it is less quantitative than IMT.[26] The observed associations between CVD risk factors and plaque score in our study were comparable to previous findings.[18,27] We found a heritability estimate of 0.28, which decreased to 0.21 after adjusting for the established cardiovascular risk factors. These heritability estimates were fairly similar to an earlier published study[18] performed in the San Antonio Family Heart Study, also using a randomly ascertained study population.

It should be noted that direct comparison of the heritability estimates from the present study with those obtained from other studies is problematic. Different study designs, adjustments for covariates, and population specific environmental contributions to the phenotypic variance might result in different heritabilities even when the genetic variance estimates in the

different populations are similar. In the present study, we used a huge single pedigree, the largest pedigree used so far to study heritability estimates. Previously published studies were mainly based on sib-pair analyses or multiple families selected on the basis of disease status with relatively small sizes. In contrast, the members of our extended pedigree represent a random sample of our study population and were not ascertained through persons with a specific disease status. This allows us to make inferences about these heritabilities at the population level. At the same time, it is important to realise that the genetic contribution to a specific trait may not be constant between populations, even when they inherited the same genetic make-up. For instance, genes involved in salt sensitivity will not express in a population with low salt intake. Presence or absence of an environmental factor (diet, physical activity, life style, etc.) may indicate whether or not a certain genetic make-up plays an important role in the variability of a trait.

The inclusion of covariates that are known to aggregate in families may have affected our results. Indeed, some of the covariates that we included are themselves genetically mediated, for example blood pressures and plasma lipid levels. Including such variables in the heritability calculations could reduce the heritability estimates whenever there are pleiotropic effects of genes on the covariates and the phenotypic measures under study. The heritability estimates derived from a model adjusted for age and gender indicate to what extent the genetic factors (directly or through other covariates) contribute to the variance of the trait under study. On the other hand, estimated heritabilities from a fully adjusted model represent only the contribution of the genes that are acting independently of the considered covariates.

In summary, we report for the first time that a substantial proportion of the variability in PWV is explained by genetic factors. This heritability was quite similar to the heritability estimates of IMT and plaque score in our study. Our findings stimulate the search for genes responsible for arterial stiffness.

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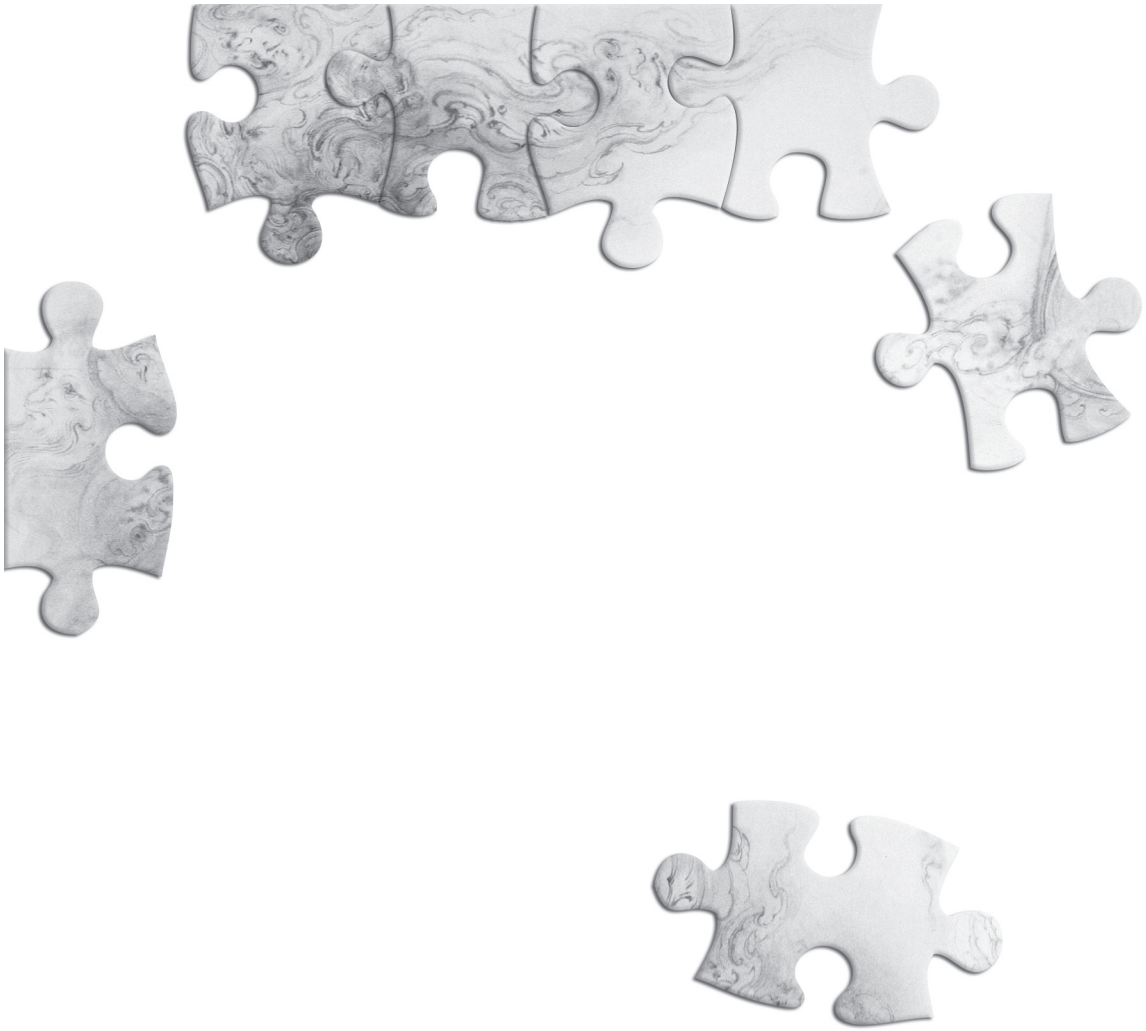


Chapter 3

The *ACE* I/D polymorphism and atherosclerosis

Chapter 3.1

The *ACE* I/D polymorphism and carotid artery wall thickness: a meta-analysis

Abstract

Many studies have investigated the association between the angiotensin converting enzyme (*ACE*) gene insertion/deletion (I/D) polymorphism and carotid artery intima media thickness (IMT); however, most studies were small and conducted in selective samples. The aim of this sudy was to evaluate this association by performing a meta-analysis on published articles.

We searched Medline for articles studying the association between the *ACE* I/D polymorphism and carotid IMT. Twenty-six studies were found from which 23 articles qualified to enter the meta-analysis containing 9833 subjects. We classified those articles on the bases of their samples into high-risk and low-risk populations and also Caucasian and Asian ethnic groups. IMT was used as a continuous variable, and data were analysed using the Cochrane Review Manager.

A significant positive association was present between the D allele and common carotid IMT (weighted mean difference between DD and II genotypes = 0.23 $mm \times 10^{-1}$, $p < 0.01$). The association was stronger among high-risk populations. The point estimates of DD versus II were higher than those of ID versus II.

Our meta-analysis showed evidence of a positive association between the D allele of the *ACE* gene and common carotid IMT. The overall results were concordant in both ethnic groups.

Introduction

The angiotensin converting enzyme (ACE), a key enzyme in the renin angiotensin system, plays an important role in vascular wall homeostasis.[1-3] Regulation of circulation and probably tissue ACE levels are under strong genetic control.[4,5] The *ACE* gene located on chromosome 17q23 has an insertion/deletion (I/D) polymorphism in the non-coding region of the gene. The insertion that gives rise to the I allele is an *alu* repeat (287 bp)[4] in intron 16 of the *ACE* gene, while the D allele results from the absence of the above insertion. It has been shown that a higher plasma ACE level is present in subjects with the D compared with the I allele.[4,5]

Numerous studies have reported a relation between the D allele and cardiovascular diseases[6-9] but findings have been controversial.[10-13] Aditionally, results from a large number of studies on the association between the *ACE* polymorphism and carotid artery intima media thickness (IMT), which was used as a measure of atherosclerosis, have been controversial. Some studies found a positive association between the D allele and IMT,[14-20] whereas others failed to find such a relation.[21-39] However, the studies were conducted in selective and heterogeneous groups from low-risk populations to hypertensive or diabetic patients. Furthermore, the sample sizes of these studies have been relatively small. The aim of our study was to evaluate the effect of this polymorphism on carotid IMT by performing a meta-analysis using all studies published until October 2002.

Materials and methods

Identification of studies

We searched Medline for all publications relating to association studies using the *ACE* I/D polymorphism and carotid IMT. References from retrieved publications were checked for any additional studies. Twenty-six articles were identified.[14-39] Two studies that used familybased design, were excluded.[24,31] One study did not report detailed statistics for IMT measurement,[37] and therefore was not included in the meta-analysis. Furthermore, two case-control studies did not report detailed results of their controls groups,[19,39] and only case groups were included.

In total, 23 articles were entered into the meta-analysis, from which 17 studies have been conducted in Caucasian populations. Most of the sample sizes were composed of less than 500 subjects. Three studies used the case-control design,[18,19,39] and six other studies included only individuals at high

risk of atherosclerosis.[15,20,23,28,32,38] In total, there were 1448 patients with hypertension, type 1 diabetes, type 2 diabetes, cerebrovascular or arterial occlusive diseases. Other studies used either a random sample of the general population or relatively healthy subjects.

Four studies used samples that included only men.[16,27-29] Among other studies that used both genders, three studies performed comparisons between men and women in regard to an association between the polymorphism and carotid IMT.[26,33,35] None of them found any evidence that the association may be different between two genders. Therefore, both genders were included in the meta-analysis.

Measurements of carotid artery IMT were done in all the studies by means of B-mode ultrasound examinations. Participants were examined in a supine position with slight extension of the neck, and measurements of the IMT were performed using a 5 to 10 MHz transducer along the segment 10 to 30 mm proximal to the origin of the carotid bulb. Some studies measured IMT in several arterial sites, and various cut points were used to convert the IMT from a continuous variable to a nominal variable. Therefore, in order to make the studies more comparable, we used IMT measurement of the far wall of the common carotid artery as a continuous variable whenever several statistics were presented in original articles.

In most of the studies the genotype frequencies were consistent with Hardy-Weinberg equilibrium (HWE). Deviations from HWE were found in some studies among high-risk populations. Because deviation may occur in diseased populations if there is an association between disease and the allele, which may have been the case in the present studies, we did not exclude them from the meta-analysis.

Statistical analyses

We entered the available data in the Cochrane Review Manager (RevMan version 4.1.1) and analysed them with Metaview 4.1. The method of moments proposed by DerSimonian and Laird[40] has been used to calculate the weighted mean differences (WMDs) in a random effects model for the pooled data. We used the funnel plot to examine publication bias of reported associations.

In addition to the total group, we analysed the studies using the high-risk populations separately from low-risk/general populations, since it has been shown that the association with the gene could be different because of risk factor profiles.[14,18,19] Furthermore, we classified the articles into studies of Asians and Caucasians because genotype frequencies as well as the prevalence of cardiovascular diseases are reported to be different among

ethnic groups.[12,13] The effect of the *ACE* gene variant was assessed by use of comparisons between DD and II, DD and ID, and ID and II genotype groups.

Whenever the standard error was reported in the original article, the value of the standard deviation has been calculated, and all descriptive data are expressed as mean ± standard deviation. The mean differences are presented in $mm \times 10^{-1}$ with the 95% confidence interval for each difference. The p values are presented with two decimal places.

Results

In total, the studies contained 9833 subjects. Details of the individual studies are shown in Table 1. Mannami *et al*[35] from Japan and Hung *et al*[30] from Australia used the largest sample sizes. Almost all studies used middle-aged samples of the population.

The total frequency of the D allele was 46.2%. There was a high range for the DD genotype frequencies: from 13.0% in Mannami *et al*[35] to 43.1% in Nergizoglu *et al*.[19] Table 2 shows the allele frequencies and distribution of genotypes in the individual studies. The lowest frequencies for the DD genotype have been observed in Asian populations.

The carotid IMTs in different studies according to the three *ACE* genotype groups are presented in Table 3. There was only one study that reported a positive effect of the I allele on carotid IMT.[39] This study introduced a highly significant heterogeneity ($p = 0.002$) and was excluded from the rest of the analyses.

Overall, there was a significant positive association between the D allele and carotid IMT (WMD = 0.23 $mm \times 10^{-1}$ between DD and II, $p < 0.01$) (Table 4). Among high-risk populations, this weighted mean difference was significant in both Caucasians (WMD = 0.49 $mm \times 10^{-1}$, $p < 0.01$) and Asians (WMD = 1.74 $mm \times 10^{-1}$, $p < 0.01$). In low-risk/general populations, however, the association was positively significant only among Caucasians (WMD = 0.14 $mm \times 10^{-1}$, $p = 0.02$). Table 4 shows the weighted mean differences of carotid IMT and corresponding 95% confidence intervals between the genotypes. The point estimates of the difference between DD and II were generally higher than the point estimates of ID versus II. The mean differences between DD and II in the individual studies and the weighted pooled data are shown in Figure 1.

Figure 2 presents the funnel plot for two different ethnic subgroups. The plot for Caucasians is quite symmetric, but this is not the case for Asians, suggesting a publication bias in these populations.

Table 1. Characteristics of the association studies between the *ACE* I/D polymorphism and carotid IMT

Study	Year	Country	Sample size, n	Age (years)*
Low-risk/general populations†				
Arnett *et al*[25]	1998	United States	495	59.0 ± 6.1
Balkestein *et al*[21]	2002	Belgium	380	39.8 ± 15.7
Castellano *et al*[14]	1995	Italy	187	57.8 ± 3.4
Dessì-Fulgheri *et al*[22]	1995	Italy	240	53.4 ± 7.0
Ferrières *et al*[27]	1999	France	355	53.7 ± 7.0
Girerd *et al*[26]	1998	France	340	49.0 ± 12.0
Huang *et al*[29]	1999	Finland	219	54.1 ± 2.9
Hung *et al*[30]	1999	Australia	1106	53.3 ± 12.5
Kauma *et al*[17]	1996	Finland	515	51.4 ± 6.0
Pujia *et al*[16]	1996	Italy	132	50.4 ± 10.3
Sass *et al*[33]	1998	France	150	41.2 ± 4.2
Kogawa *et al*[18] (controls)	1997	Japan	235	50.9 ± 10.6
Mannami *et al*[35]	2001	Japan	3657	59.7 ± 11.8
Tabara *et al*[34]	2001	Japan	205	70.0 ± 9.0
Watanabe *et al*[36]	1997	Japan	169	59.2 ± 10.4
High-risk populations‡				
Diamantopoulos *et al*[38]	2002	Greece	184	61.4 ± 7.7
Frost *et al*[23]	1998	Germany	148	30.1 ± 6.6
Markus *et al*[39] (cases)	1995	United Kingdom	101	64.8 ± 9.0
Nergizoglu *et al*[19] (cases)	1999	Turkey	51	36.2 ± 9.3
Pit'ha *et al*[28]	1999	Czech Republic	47	61.9 ± 2.5
Taute *et al*[32]	2000	Germany	98	60.5 ± 8.9
Hosoi *et al*[15]	1996	Japan	288	59.5 ± 10.6
Jeng[20]	2000	China	175	56.8 ± 10.3
Kogawa *et al*[18] (cases)	1997	Japan	356	59.6 ± 10.6

ACE: angiotensin converting enzyme gene; I/D; insertion/deletion; IMT: intima media thickness.
* Age is presented as mean ± standard deviation.
† Low-risk populations included subjects without chronic medication, non-smokers, low-risk outpatients, healthy subjects and controls (definition was given in original articles).
‡ High-risk populations included symptomatic cerebrovascular disease, type 1 and 2 diabetes, non diabetic haemodialysis, and hypertensive patients.

Table 2. Distribution of genotype and allele frequencies in individual studies

Study	Frequency of the D allele (%)*	Genotype frequency, n (%)			*p* (HWE)
		II	ID	DD	
Low-risk/general populations					
Arnett *et al*[25]	56.4	88 (17.8)	256 (51.7)	151 (30.5)	0.25
Balkestein *et al*[21]	45.8	116 (30.5)	180 (47.4)	84 (22.1)	0.37
Castellano *et al*[14]	64.2	23 (12.3)	88 (47.1)	76 (40.6)	0.75
Dessì-Fulgheri *et al*[22]	64.6	23 (9.6)	124 (51.7)	93 (38.8)	0.05
Ferrières *et al*[27]	59.2	70 (19.7)	150 (42.3)	135 (38.0)	0.02
Girerd *et al*[26]	59.0	57 (16.8)	165 (48.5)	118 (34.7)	0.96
Huang *et al*[29]	58.0	42 (19.2)	100 (45.7)	77 (35.2)	0.35
Hung *et al*[30]	55.2	228 (20.6)	535 (48.4)	343 (31.0)	0.46
Kauma *et al*[17]	54.4	103 (20.0)	264 (51.3)	148 (28.7)	0.45
Pujia *et al*[16]	61.4	16 (12.1)	70 (53.0)	46 (34.8)	0.17
Sass *et al*[33]	51.3	33 (22.0)	80 (53.3)	37 (24.7)	0.41
Kogawa *et al*[18] (controls)	38.3	87 (37.0)	116 (49.4)	32 (13.6)	0.50
Mannami *et al*[35]	35.5	1540 (42.1)	1640 (44.8)	477 (13.0)	0.22
Tabara *et al*[34]	36.3	83 (40.5)	95 (46.3)	27 (13.2)	0.98
Watanabe *et al*[36]	40.8	62 (36.7)	76 (45.0)	31 (18.3)	0.37
High-risk populations					
Diamantopoulos *et al*[38]	60.9	29 (15.8)	86 (46.7)	69 (37.5)	0.80
Frost *et al*[23]	60.5	31 (20.9)	55 (37.2)	62 (41.9)	0.01
Markus *et al*[39] (cases)	58.9	18 (17.8)	47 (46.5)	36 (35.6)	0.70
Nergizoglu *et al*[19] (cases)	64.7	7 (13.7)	22 (43.1)	22 (43.1)	0.69
Pit'ha *et al*[28]	56.4	8 (17.0)	25 (53.2)	14 (29.8)	0.58
Taute *et al*[32]	57.1	19 (19.4)	46 (46.9)	33 (33.7)	0.68
Hosoi *et al*[15]	37.2	124 (43.1)	114 (39.6)	50 (17.4)	0.01
Jeng[20]	43.1	65 (37.1)	69 (39.4)	41 (23.4)	0.01
Kogawa *et al*[18] (cases)	37.8	147 (41.3)	149 (41.9)	60 (16.9)	0.04

HWE: Hardy-Weinberg equilibrium.

* Allele frequencies are recalculated using the genotype frequencies.

Table 3. Comparison of IMT in the three genotype groups in individual studies

Study	Genotype group			*p* value*
	II	ID	DD	
Low-risk/general populations				
Arnett *et al*[25]	7.2 ± 1.5	7.3 ± 1.6	7.3 ± 1.5	NS
Balkestein *et al*[21]	5.6 ± 1.6	5.9 ± 2.3	5.8 ± 1.7	NS
Castellano *et al*[14]	7.5 ± 1.9	6.8 ± 1.9	7.4 ± 1.7	0.06
Dessì-Fulgheri *et al*[22]	10.2 ± 2.0	10.6 ± 3.0	10.5 ± 4.0	NS
Ferrières *et al*[27]	6.3 ± 1.2	6.2 ± 1.0	6.4 ± 1.2	NS
Girerd *et al*[26]	5.4 ± 1.0	5.4 ± 1.3	5.5 ± 1.1	NS
Huang *et al*[29]	10.4 ± 2.3	10.8 ± 3.3	10.1 ± 1.9	NS
Hung *et al*[30]	7.1 ± 1.4	7.1 ± 1.4	7.1 ± 1.5	NS
Kauma *et al*[17]	8.1 ± 1.8	8.0 ± 1.5	8.3 ± 1.9	NS
Pujia *et al*[16]	7.0 ± 0.8	7.6 ± 0.8	7.8 ± 0.7	0.02
Sass *et al*[33]	5.2 ± 0.4	5.4 ± 0.6	5.3 ± 0.4	NS
Kogawa *et al*[18] (controls)	6.3 ± 1.6	6.3 ± 1.7	6.4 ± 1.7	NS
Mannami *et al*[35]	8.7 ± 1.4	8.7 ± 1.4	8.7 ± 1.2	NS
Tabara *et al*[34]	7.9 ± 1.2	8.0 ± 1.4	8.1 ± 1.4	NS
Watanabe *et al*[36]	10.2 ± 3.1	11.0 ± 3.1	11.0 ± 3.1	NS
High-risk populations				
Diamantopoulos *et al*[38]	9.4 ± 2.0	9.7 ± 2.0	9.8 ± 2.1	NS
Frost *et al*[23]	6.2 ± 1.5	6.3 ± 1.8	6.3 ± 1.3	NS
Markus *et al*[39] (cases)	11.4 ± 4.0	9.4 ± 2.8	8.1 ± 2.8	< 0.05†
Nergizoglu *et al*[19] (cases)	7.1 ± 0.5	7.6 ± 0.9	8.0 ± 1.0	< 0.05
Pit'ha *et al*[28]	7.2 ± 1.3	7.1 ± 1.2	7.5 ± 1.6	< 0.10
Taute *et al*[32]	10.1 ± 2.9	11.0 ± 2.5	10.6 ± 2.6	NS
Hosoi *et al*[15]	10.0 ± 4.1	11.1 ± 6.1	12.0 ± 6.2	0.04
Jeng[20]	7.4 ± 2.7	7.6 ± 3.1	8.8 ± 3.5	0.06
Kogawa *et al*[18] (cases)	9.9 ± 3.6	10.6 ± 5.4	12.0 ± 5.9	< 0.05

NS indicates not significant at $p = 0.10$ level. IMTs are presented as mean ± standard deviation ($mm \times 10^{-1}$).

IMT: intima media thickness.

* *p* values as reported in the original articles, rounded to two decimal places.

† Association with the I allele is present.

Table 4. Weighted mean difference of carotid IMT between genotype groups

Study group	WMD (95% CI), mm×10^{-1}		
	DD versus II	DD versus ID	ID versus II
	Low-risk/general populations		
Caucasians	0.14 (0.02, 0.26)	0.08 (–0.03, 0.19)	0.09 (–0.04, 0.21)
Asians	–0.02 (–0.14, 0.11)	–0.06 (–0.18, 0.07)	0.04 (–0.05, 0.13)
Total	0.10 (0.00, 0.20)	0.04 (–0.05, 0.12)	0.07 (–0.01, 0.16)
	High-risk populations		
Caucasians*	0.49 (0.14, 0.84)	0.15 (–0.16, 0.46)	0.32 (–0.02, 0.67)
Asians	1.74 (0.87, 2.62)	1.20 (0.27, 2.12)	0.59 (–0.05, 1.22)
Total	0.74 (0.28, 1.19)	0.26 (–0.04, 0.55)	0.38 (0.08, 0.69)
Overall	0.23 (0.08, 0.37)	0.08 (–0.02, 0.17)	0.09 (0.01, 0.17)

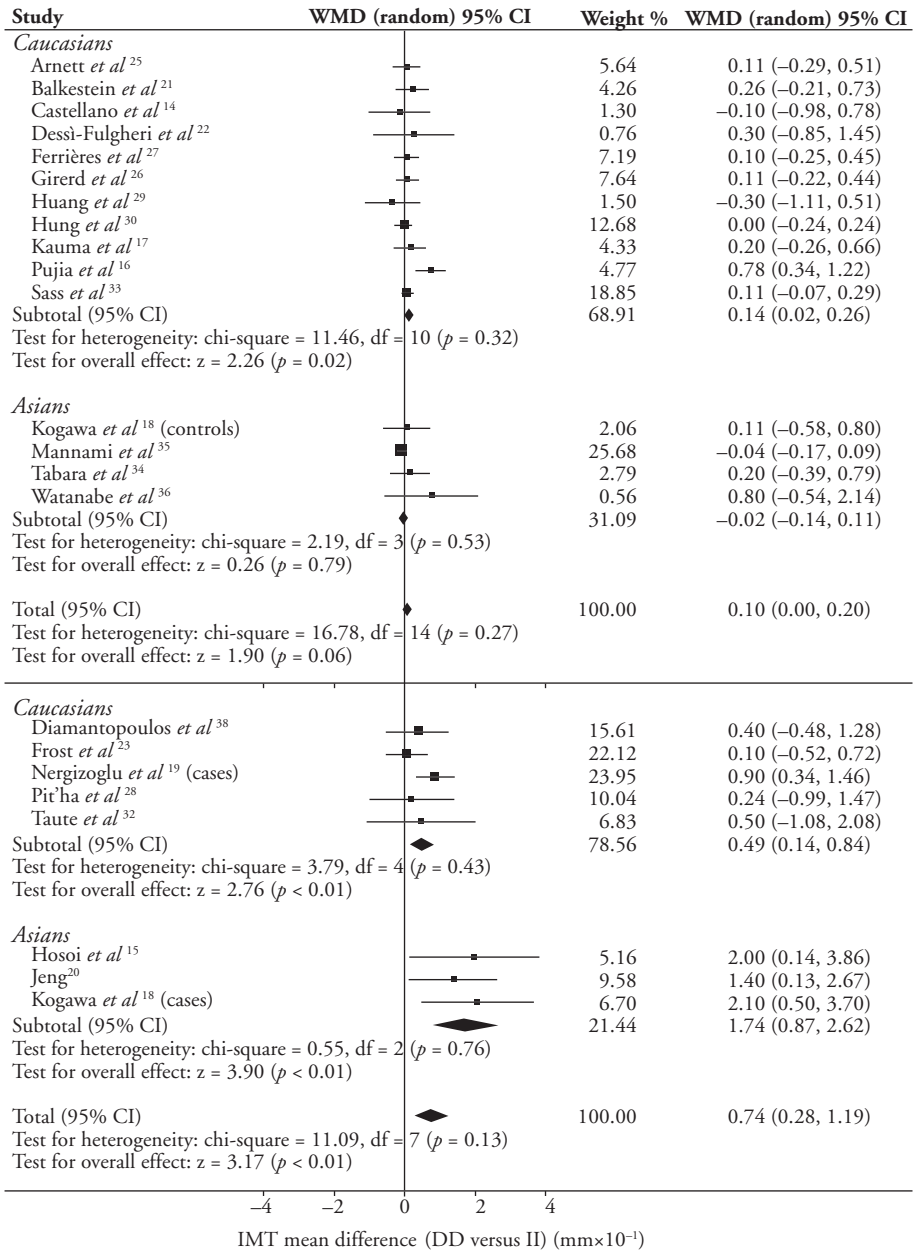
CI: confidence interval; IMT: intima media thickness; WMD: weighted mean difference.
* Excluding the study of Markus *et al.*[39]

Discussion

In this study, we pooled the data of more than 9800 subjects from available published studies to compute an estimate of the association between the insertion/deletion polymorphism of the *ACE* gene locus and the common carotid intima media thickness. We calculated the weighted mean difference between every two genotypes and presented the differences among Caucasians and Asians in low-risk and high-risk groups separately. The significant weighted mean differences between DD and II among both low-risk and high-risk populations support the positive association between the D allele and carotid IMT. Although we could not show the difference between DD and ID, the point estimates for the mean differences between DD and II were generally higher than those of the difference between ID and II, suggesting an allele-dose effect of the allele.

In a few studies the genotype frequencies were not consistent with HWE. Almost all those studies used high-risk populations, and deviation from HWE is expected among such populations. Laboratory error is not very likely because genotyping has been performed using standard protocols in all the studies and overdetection of the D allele has been corrected by performing a second polymerase chain reaction. The most likely explanation is a higher selection of the DD genotype because of the background diseased

Figure 1. Weighted mean difference and 95% confidence intervals of the carotid IMT between DD and II genotypes in low-risk (top) and high-risk (bottom) populations



CI: confidence interval; IMT: intima media thickness; WMD: weighted mean difference.

populations. There was still one study among low-risk populations that did not follow the HWE.[27] However, we considered excluding these studies from our meta-analysis and the results did not materially change.

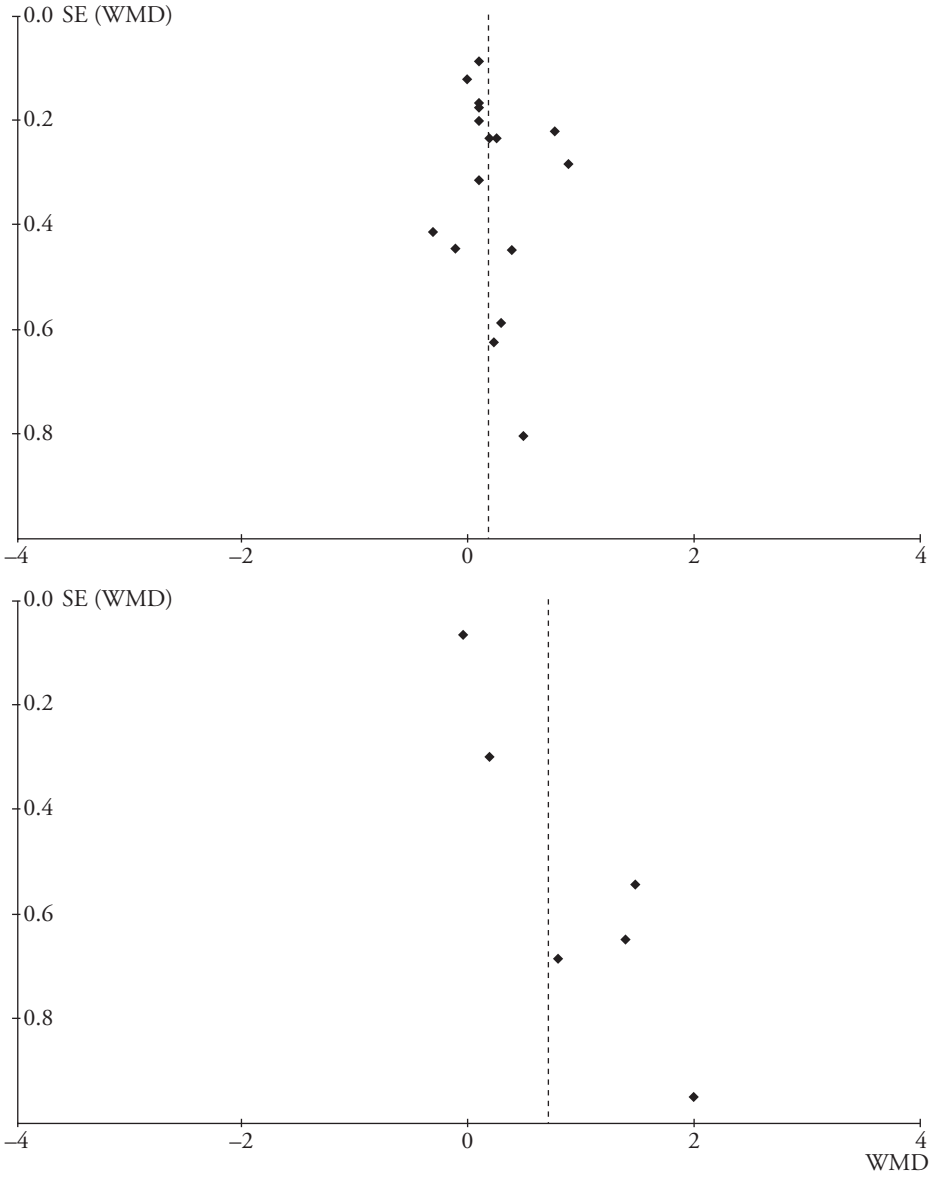
Most of the studies used similar transducers (7.5 MHz) to measure IMT at the same segment of the common carotid artery (10 mm proximal to the carotid bulb). On the other hand, any possible measurement error will be presented equally in all three genotypes and because we used weighted mean differences in our analyses, it is not likely to induce an error in our results. The study by Sass *et al*[33] had the smallest standard deviation of IMT measurements and consequently gained a relatively large weight in the meta-analysis, whereas it comprised only 2% of the total sample size. Excluding this study did not materially change the results, ruling out the possibility of overweighting effect of this study on final findings.

It is important to mention that there is a large variability of case selection among the high-risk group. However, the fact that all of those study populations are at high risk of atherosclerosis means that they share some genetic-environmental backgrounds that make them susceptible for the disorder; in this sense, they may be considered homogenous. Possible explanations for the greater gene effect observed in this group could be the interaction between those background factors and the *ACE* gene as well as the greater variation in IMT among them.

An important source of bias in every meta-analysis is publication bias because the likelihood of publishing a study could be related to the results of that study. However, among our meta-analysis, there have been many studies published with negative findings.[21-23,25-30,32-35] Although the funnel plot for Asians is not symmetric, the overall results of both ethnic groups are concordant, indicating that this bias cannot affect the final result.

On the other hand, funnel plot asymmetry is not always caused by publication bias. True heterogeneity may also lead to funnel plot asymmetry. For example, significant difference may be seen only in high-risk individuals, and these high-risk people are usually more likely to be included in small studies. This is particularly true in our meta-analysis because all the significant associations in Asians have been observed among the studies from high-risk populations. Language bias or citation bias also could be an important source in this group of studies, meaning that the studies without significant findings are preferentially published in languages other than English and less likely to be cited in other articles. Finally, it is possible, of course, that an asymmetrical funnel plot arises simply by chance.

Figure 2. Funnel plots (DD versus II) for Caucasians (top) and Asians (bottom)



SE: standard error; WMD: weighted mean difference.

In summary, we found evidence of a positive association between the presence of the D allele of the *ACE* gene with common carotid IMT in a meta-analysis of 23 published articles (9833 subjects) that was stronger among high-risk populations.

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Chapter 3.2

The *ACE* I/D polymorphism and carotid artery wall thickness: the Rotterdam Study

Abstract

Studies on the role of the insertion/deletion (I/D) polymorphism of the gene coding for angiotensin converting enzyme (*ACE*) in atherosclerosis have been inconsistent. In a meta-analysis, we recently showed that this relationship is stronger in high-risk populations. In this paper, we used a combined functional and population based approach to investigate the gene-environment interaction of the *ACE* I/D polymorphism in relation to carotid artery wall thickness. The study was part of the Rotterdam Study, a prospective population based cohort study. In 5321 subjects, IMT was measured in the carotid arteries by ultrasonography and the *ACE* genotypes were determined by size analysis of polymerase chain reaction products.

In multiple regression analysis, I/D polymorphism and smoking were the main determinants for plasma ACE levels ($r^2 = 0.28$). There was a positive association between the D allele of the I/D polymorphism and carotid artery wall thickness among current smokers ($p = 0.03$). Subjects carrying only one of the risk factors (smoking or the D allele) did not show significant differences in IMT compared with the non-/former smokers group carrying two I alleles, while carriers of both risk factors had significant higher IMT. The association was not present in non-/former smokers.

The results provide further evidence that genetic and environmental factors interact in the formation of the arterial lesions. This study shows that large population based studies could be extremely helpful in unravelling the genetic origin of complex diseases such as atherosclerosis.

Introduction

Angiotensin converting enzyme (ACE) is a key component in the renin angiotensin system, converting angiotensin I to angiotensin II.[1] It also inactivates vasodilator bradykinin. Both peptides play central roles in blood pressure regulation and are considered to be important in the pathogenesis of cardiovascular diseases. ACE levels in plasma and tissue are under genetic control.[2-4] There is a common insertion/deletion (I/D) polymorphism in the *ACE* gene characterised by the presence or absence of a 287 bp *alu* repeat. Subjects with the DD genotype have higher plasma ACE levels compared with those with the ID and II genotypes.[2,3] This finding predicts that carriers of this genotype may have increased blood pressure and a higher prevalence of cardiovascular diseases.

Findings on the association between the *ACE* I/D polymorphism and atherosclerosis using ultrasonographic measurements of carotid arteries have been inconsistent. Some studies showed a relation of atherosclerosis with presence of the D allele,[5-8] while others have failed to show such association.[9,10] The majority of the studies conducted until now were based on relatively small sample sizes, which may in part explain the inconsistency, particularly when interactions were studied.[11] A recent evaluation of candidate gene studies in a meta-analysis demonstrated that large studies are needed to show the effects of the genes involved in complex traits.[11] Recently, we performed a meta-analysis of the association between this polymorphism and carotid artery intima media thickness (IMT), using the studies conducted until October 2002.[12] When pooling the data of 23 articles (9833 subjects), we found that carriers of the DD genotype have an increased thickness of common carotid IMT. The correlation was most pronounced in high-risk populations, suggesting gene-environment interactions.[12]

From our meta-analysis it is not clear which factor is interacting with the *ACE* gene. As studies of gene-environment interactions are prone to false positive and negative findings, we used a combined functional and population based approach. To unravel the role of interactions of the *ACE* I/D polymorphism with other factors, we aimed to identify the non-genetic determinants of the renin angiotensin system, specifically those associated with plasma ACE levels. We studied various vascular risk factors in relation to ACE levels and found that only smoking was a determinant of ACE levels in addition to the *ACE* gene. As a second step, we investigated the *ACE* gene and its interaction with smoking in relation to carotid ultrasonographic measurements and found evidence that smoking is a factor modifying the prevalence of ACE related carotid artery lesions.

Materials and methods

Study population

This study is part of the Rotterdam Study, an ongoing population based follow up study designed to investigate the determinants of chronic diseases in the elderly; it has been described in more detail elsewhere.[13] In brief, baseline data were collected between March 1990 and July 1993 from 7983 subjects, aged 55 years or older, living in Ommoord, a suburb of Rotterdam, The Netherlands.[13] The study was approved by the Medical Ethics Committee of Erasmus University, and written informed consent was obtained from all participants.

All participants were interviewed at home by a trained research assistant using computerised questionnaires, and they subsequently visited the study centre. Smoking history was assessed during the interview at home and participants were categorised as never, former, or current smokers. At the study centre an extensive physical examination was performed on each participant, including ultrasonography of the carotid arteries. A blood sample was drawn, and serum and plasma were stored at –80 °C.

Carotid ultrasonography

Carotid atherosclerosis was assessed by duplex scan ultrasonography of the carotid arteries, using a 7.5 MHz linear array transducer (ATL, Ultramark IV). Measurements of IMT were performed offline using the still images recorded on videotape. Details about this measurement have been published previously.[14] Briefly, the interfaces of the far and near walls of the distal common carotid artery are marked over a length of 10 mm. We used the average of the measurements of three still images of both the left and right arteries. Carotid IMT was determined as the mean of the IMT of near and far wall measurements of both the left and right arteries. Results from a reproducibility study of IMT measurements have been published elsewhere.[15] The mean differences ± standard deviation in common carotid IMT between paired measurements of sonographers, readers, and visits were –0.004 ± 0.10 mm, 0.066 ± 0.07 mm, and –0.013 ± 0.13 mm, respectively.

We defined plaques as focal widening of the vessel wall with protrusion into the lumen, composed of calcified or non-calcified components. The protrusion was evaluated by eye, without measuring the thickness of the lesions or of the adjacent structure. The total plaque score reflected the total number of sites with plaques and ranged from 0 to 6 (left and right side common carotid arteries, bifurcation, and internal carotid arteries).

Laboratory assessments

Colorimetric determination of ACE activity levels was performed in the stored plasma samples (–80 °C). Because of cost considerations, ACE levels were assessed in a random group of 215 individuals. The measurements were carried out with a kit by Fujirebio Inc, which uses a *p*-hydroxy-Hip-His-Leu substrate.[16] Fluorimetric assay of ACE activity levels in plasma was performed by measuring the release of His-Leu from the substrates Hip-His-Leu and Z-Phe-His-Leu.[17,18]

DNA was isolated from blood samples using a standard procedure (the salting out method).[19] The II, ID, and DD genotypes were detected using the polymerase chain reaction (PCR) technique according to the method of Lindpaintner *et al*[20] with some modifications. The insertion and deletion alleles of the *ACE* gene were identified using a set of oligonucleotide primers flanking the polymorphic site in intron 16. The final volume of the PCR mix was 20 µl containing 50 ng DNA as template and 1×PCR buffer (Gibco), 1.3 mmol/L $MgCl_2$, 200 µmol/L dNTPs, 20 pmol primer mix, and 0.35 U *Taq* polymerase in a PE9600 PCR machine. The thermocycling procedure was completely identical to the method of Lindpaintner *et al*.[20] The result of amplification was a 319 bp and a 597 bp amplicon for the D and the I alleles, respectively.

Because the D allele in heterozygous samples is preferentially amplified, there is a tendency towards misclassification of about 4 to 5 percent of ID genotype to DD. In order to avoid this, a second independent PCR was performed with a primer pair that recognises an insertion specific sequence. To optimise the second PCR, 10% DMSO, 0.35 U Ampli*Taq* Gold DNA polymerase and 1×GeneAmp PCR Gold buffer (Applied Biosystems) was added to the PCR mix with annealing temperature of 67 °C. The reaction yielded a 335 bp amplicon only if the I allele was present. All reactions were performed in 96 well plates and handled by a robot (Beckman Biomek® 2000).

In the post-PCR analyses, 10 µl of PCR product was loaded onto a 3% agarose gel. Fragments were visualised using etidium brominde staining and UV transillumination. Two independent investigators interpreted the pictures from each gel and all ambiguous samples were analysed a second time.

Statistical analyses

Hardy-Weinberg equilibrium was tested with the chi-square test. We analysed the distribution of conventional cardiovascular risk factors among three genotype groups by chi-square test for dichotomous variables and

by one way analysis of variance for continuous variables. Multiple linear regression analysis was used to analyse the relation between vascular risk factors and ACE levels. The differences in ACE levels and IMT between the smoking and genotype groups were tested using the general linear model univariate procedure, adjusted for age and gender. In addition, subjects were divided into two groups based on the number of plaques in the carotid arteries (0-2 plaques and 3 or more plaques). Logistic regression was used to estimate the adjusted odds ratios for the different genotype groups using subjects with the II genotype as reference. No adjustments were initially made for vascular risk factors such as hypertension, as they may act as intermediate factors. Interaction of the *ACE* genotype with smoking was tested using a multiplicative model. All statistical analyses were conducted using SPSS 11.0 for Windows.

Results

We performed our analyses on 5321 subjects for whom the complete data for *ACE* genotypes, IMT measurements, and smoking status were available. Data for at least one of the variables were missing in the remaining subjects due to logistic reasons; mainly because the subjects were too old or disabled to visit the research centre for the examination. In our sample, the frequency of the D allele was 53.3% and the distribution of the genotype and allele frequencies were in Hardy-Weinberg equilibrium (II, 21.7%; ID, 49.9%; and DD, 28.3%; $p = 0.82$). Among the remaining subjects for whom the genotype frequencies were available (n = 1548), no deviation from Hardy-Weinberg equilibrium was observed. Furthermore, there was no significant difference in the genotype frequencies between the two samples ($p = 0.44$).

Table 1 shows the demographic characteristics of the participants by the *ACE* genotypes. No significant differences were found among the three genotypes with respect to classical cardiovascular risk factors. The carriers of the D allele were significantly older than the II carriers but the difference was only 1.22 years. Furthermore, carriers of the DD genotype had a significantly higher mean systolic blood pressure and a higher mean of carotid IMT compared with the II genotype. Although there was a slight increase in the percentage of subjects with three or more plaques, the frequency did not increase significantly in carriers of the D allele compared with the II genotype (Table 1).

In the sample of 212 individuals for whom both plasma ACE levels and I/D genotypes were measured, the allele and genotype proportions were

Table 1. Demographic characteristics of subjects according to the *ACE* I/D genotypes

	ACE genotype		
	II (n = 1156)	ID (n = 2657)	DD (n = 1508)
Age (years)	68.03 ± 8.53	69.08 ± 8.67*	69.25 ± 8.89*
Male gender (%)	41.70	40.80	39.66
Body mass index (kg/m^2)	26.39 ± 3.82	26.22 ± 3.65	26.31 ± 3.63
Current smoking (%)	22.88	23.60	20.82
Total cholesterol (mmol/L)	6.62 ± 1.28	6.66 ± 1.17	6.61 ± 1.21
HDL cholesterol (mmol/L)	1.34 ± 0.35	1.35 ± 0.35	1.35 ± 0.37
Systolic BP (mmHg)	137.67 ± 21.61	139.09 ± 22.66	140.01 ± 22.84*
Diastolic BP (mmHg)	73.48 ± 11.64	73.53 ± 11.63	73.73 ± 11.29
Hypertension (%)†	31.78	36.03*	35.05
Common carotid IMT (mm×10^{-1})	7.88 ± 1.53	7.99 ± 1.59	8.03 ± 1.59*
Number of carotid plaques ≥ 3 (%)	20.90	23.58	23.75

Continuous values are mean ± standard deviation.
ACE: angiotensin converting enzyme gene; BP: blood pressure; HDL: high density lipoprotein; I/D: insertion/deletion; IMT: intima media thickness.
* $p < 0.05$ compared with II genotype.
† Hypertension, defined as systolic BP ≥ 160 mmHg or diastolic BP ≥ 95 mmHg or medication use.

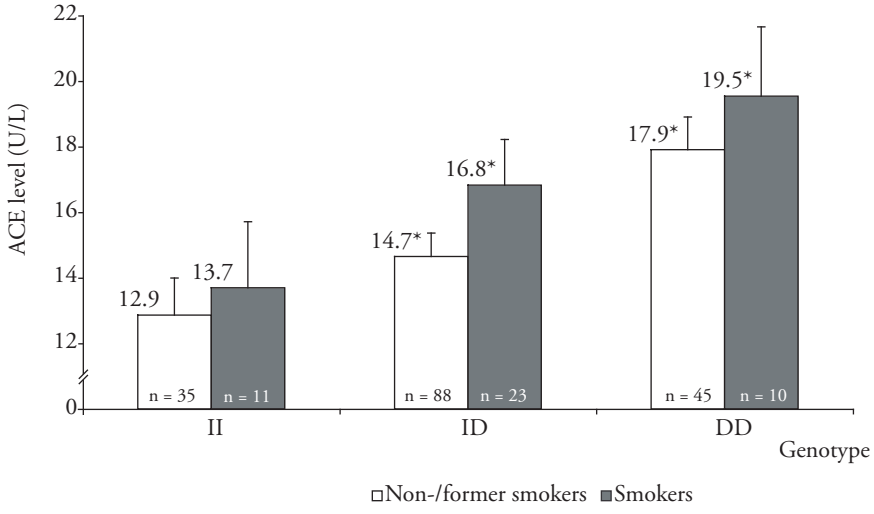
Table 2. Vascular risk factors and their association with plasma ACE levels

Variable	Beta	SE	*p* value
ACE I/D polymorphism*	2.71	0.35	< 0.01
Current smoking	1.92	0.61	< 0.01
Age (years)	–0.07	0.06	0.20
Male gender	0.63	0.54	0.24
Body mass index (kg/m^2)	0.07	0.08	0.37
Total cholesterol (mmol/L)	–0.19	0.23	0.42
HDL cholesterol (mmol/L)	0.95	0.76	0.22
Systolic BP (mmHg)	–0.01	0.02	0.84
Diastolic BP (mmHg)	–0.02	0.04	0.56

ACE (*ACE)*: angiotensin converting enzyme (gene); Beta: regression coefficient; BP: blood pressure; HDL: high density lipoprotein; I/D: insertion/deletion; SE: standard error of the coefficient.
* Number of the D alleles was used in the equation (II = 0, ID = 1, and DD = 2).

consistent with Hardy-Weinberg equilibrium (p = 0.48). In multiple linear regression analysis, the *ACE* I/D polymorphism and smoking were the only determinants of plasma ACE levels (Table 2). These two factors together explained 28% of the variation in enzyme levels in plasma (r = 0.53, p < 0.01). The levels of ACE were not significantly different between former smokers and non-smokers (14.83 ± 3.57 and 15.58 ± 3.87 U/L, respectively) therefore we combined these two groups. Overall, the ACE level was 1.8 U/L higher in current smokers compared with non-/former smokers (p < 0.01). Although the difference in ACE levels between smokers and non-/former smokers was largest in the ID and DD group, it was not significantly different from the difference in the II group (Figure 1).

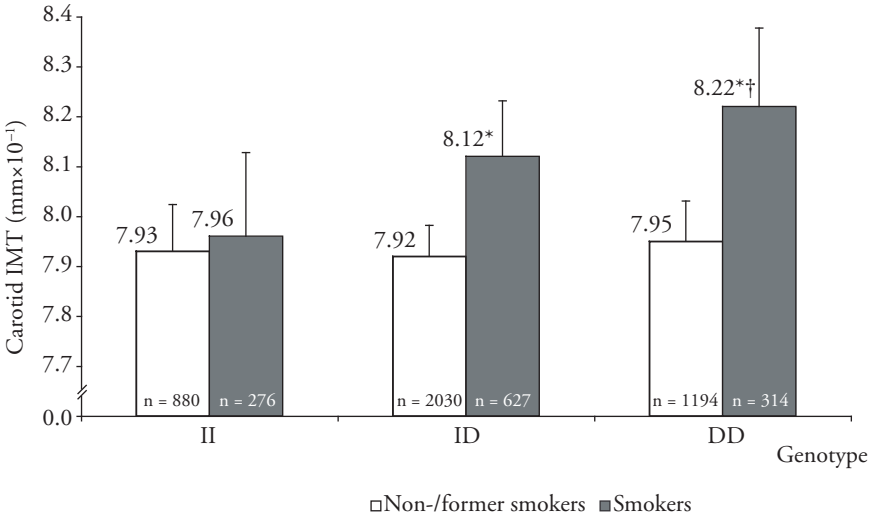
Figure 1. Plasma ACE levels in different genotypes stratified by smoking status



Data are adjusted for age and gender.
ACE: angiotensin converting enzyme.
* Significantly different from the genotype II non-/former smokers group.

When studying the joint effect of smoking and the *ACE* gene on IMT, there was a significant increase in mean carotid IMT in those carrying the D allele in current smokers (p value for trend was 0.04). The IMT difference (mean ± standard error) between DD and II genotypes in smokers was 0.26 ± 0.12 mm×10^{-1}, (p = 0.03). In contrast, IMT was not significantly associated with the D allele among non-/former smokers (Figure 2). The p value for interaction between the *ACE* genotype and

Figure 2. Carotid IMT in different genotypes stratified by smoking status



Data are adjusted for age and gender.
IMT: intima media thickness.
* Significantly different from the genotype II non-/former smokers group.
† Significantly different from the genotype II smokers group.

smoking status was 0.08. The differences between genotype groups in smokers reduced but did not disappear when adjusted for systolic blood pressure. Subjects carrying only one of the risk factors (smoking or the D allele) did not show significant differences in IMT compared with the genotype II non-/former smokers group, while carriers of both risk factors had significantly higher IMT ($p < 0.01$).

The odds ratios of having three or more plaques in carotid arteries in current smokers were 1.14 (95% confidence interval (CI): 0.81, 1.59) and 1.40 (95% CI: 0.96, 2.04) for ID and DD genotypes, respectively. This increase of odds ratios with the number of the D alleles was borderline significant ($p = 0.07$) while in non-/former smokers the odds ratios remained the same among genotype groups.

Stratified analyses concerning the combined effect of the *ACE* polymorphism and other cardiovascular risk factors (hypertension, hyperlipidaemia, diabetes, obesity, age and gender) on carotid IMT did not show any significant differences among and between the stratified groups. In all the analyses the mean IMT difference between DD and II genotypes was less than 0.20 mm×10^{-1} and did not reach significance level ($p > 0.10$).

Discussion

In this study, we found a modest but significant association between the *ACE* I/D polymorphism and carotid IMT in the presence of smoking. In non-/former smokers, no significant association between the *ACE* genotype and IMT was observed.

This study is the largest population based study performed on the *ACE* I/D polymorphism and carotid artery lesions. The next largest study, which used a sample size of 3657 individuals in Japan[21] failed to find a relation between the *ACE* and IMT. Difference in ethnicity could be a possible explanation; however, the problem in comparing findings is that the interaction between the *ACE* polymorphism and smoking was not studied by Mannami *et al*.[21] Findings of other studies on the interaction between the *ACE* gene and smoking have not been consistent. An interaction between smoking and the *ACE* I/D genotype on atherosclerosis was reported in 1997 by Hibi *et al*,[22] who showed a smoking associated effect of the *ACE* genotype on the severity of coronary atherosclerosis.[22] Another study[23] found an association between the *ACE* I/D and carotid IMT only among non-/former smokers, particularly those on chronic cardiovascular medication. Yet others failed to find evidence for such an interaction.[24]

Genetic studies aiming to uncover gene interactions are extremely prone to false positive and negative findings. To obtain internal consistency in our study, we studied not only the relation of the *ACE* gene to atherosclerosis but also to ACE levels, which have been studied extensively with regard to polymorphisms in the *ACE* gene.[25-28] Since Rigat *et al*[2] reported in 1990 that the *ACE* I/D polymorphism determines the plasma levels of the enzyme, many studies have replicated this finding.[3,8,10] Our data also confirm that the presence of the deletion allele is significantly associated with the plasma ACE levels. The ACE levels in our study were in the same range as those from other population studies, which used the same method of measurement.[8,29] Although ACE levels were determined in stored sera and laboratory drift may have occurred, such drift is unlikely to be associated with the *ACE* genotype.

To unravel the role of interactions of the *ACE* I/D polymorphism with other vascular risk factors, we focused on identifying the determinants in the renin angiotensin system, specifically those associated with plasma ACE levels. In our population based study, we found that smoking is the only other factor related to plasma ACE levels, suggesting that they use the same pathways. The vascular risk factors that might be related to plasma ACE levels are not well known. In one report smoking and blood pressure[30]

and in another, male gender and history of hypertension[31] correlated with the plasma ACE levels. In our sample, the I/D polymorphism and smoking together explained 28% of the variance in ACE levels. On average, current smokers had 1.8 U/L higher ACE level in plasma. Although the differences were not significant, in those carrying the ID and DD genotypes the effect of smoking on ACE levels was larger than in the II genotype. A possible effect of smoking on cleavage secretion of ACE from the endothelial cells may explain this finding.[32] Additionally, there are indications that nicotine increases expression of a number of genes in the endothelium including *ACE*.[33]

The values for IMT in our study strongly concurred with those reported by Tabara *et al,* [34] using a sample with the same age. In order to test if the observed association between the I/D polymorphism and carotid IMT in the presence of smoking was through blood pressure, we adjusted our analysis for systolic blood pressure. Our result suggests that blood pressure does not fully explain the association. Recently, it has been shown that the DD genotype is associated with a significant blunting in vasodilation through nitric oxide (NO) pathways (due to increased angiotensin II induced NO breakdown and/or reduced bradykinin mediated NO release).[35] This finding is followed by other studies showing that smoking decreases plasma NO level[36] that may lead to impaired endothelium dependent vasodilation.[37,38] In addition, NO has important antioxidative capacities, and smoking induces oxidative stress by reducing concentrations of NO and other antioxidants in plasma.[39] Concurrently, carriers of the DD genotype show a lower antioxidant response compared with the II and ID genotypes.[40] The above observations suggest that carriers of the DD genotype who smoke are likely to be at higher risk of atherosclerosis.

Using a combined functional and population based approach provides us with an a priori hypothesis for the environmental factor(s) that may interact with the gene. However, the question remains if other environmental factors show the same pattern of interaction. The results of the stratified analyses showed no significant evidence for joint effect of the *ACE* polymorphism and other factors, prompting smoking as the best candidate for this interaction.

In summary, we found a positive association between the D allele of the I/D polymorphism and carotid IMT in the presence of smoking. This association provides further evidence that genetic and environmental factors interact in the formation of the arterial lesions. There may be various pathways underlying the observation of an effect of the *ACE* gene on IMT in smokers only, but on the basis of the present results it is not possible to fully explain the underlying mechanism. Our findings remain to be confirmed in future studies.

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Chapter 3.3

The *ACE* I/D polymorphism and arterial stiffness: the Rotterdam Study

Abstract

The insertion/deletion (I/D) polymorphism of the angiotensin converting enzyme (*ACE)* gene may be involved in structural arterial changes. The aim of the present study was to assess the relationship between the *ACE* I/D polymorphism and arterial stiffness among older adults.

The study was conducted within the Rotterdam Study, a population based cohort study including subjects aged 55 years or older. The II, ID, and DD genotypes of the *ACE* gene were determined by size analysis of polymerase chain reaction products. The distensibility coefficient of the carotid artery, and the carotid-femoral pulse wave velocity were measured during the third phase of the Rotterdam Study (1997-1999) and were used as measures of arterial stiffness. Data on both carotid stiffness and the *ACE* genotypes were available for 3001 participants. After adjustment for age and gender, subjects with the ID and DD genotypes had higher carotid stiffness compared with subjects with the II genotype. Distensibility coefficient (10^{-3}/kPa) was 10.65 (95% confidence interval (CI): 10.37, 10.93), 10.24 (95% CI: 10.06, 10.43), and 10.27 (95% CI: 10.02, 10.52) for II, ID, and DD genotypes, respectively ($p = 0.02$ for ID versus II, $p = 0.04$ for DD versus II). In stratified analyses, the association was stronger in subjects younger than 70 years. No differences were found for pulse wave velocity between the genotypes.

In conclusion, the results of this population based study show that the *ACE* ID and DD genotypes are associated with higher common carotid stiffness.

Introduction

One of the characteristics of the aging cardiovascular system is stiffening of the vessel wall. Arterial stiffness has been shown to be an independent predictor of cardiovascular morbidity and mortality in patients with essential hypertension[1,2] and end-stage renal disease.[3,4] This association may be explained by increased cardiac afterload,[4,5] decreased coronary artery perfusion,[6] and promotion of plaque rupture.[7] Arterial stiffness increases with age,[8] and is positively associated with hypertension,[9,10] atherosclerosis,[11,12] diabetes mellitus,[8] and end-stage renal disease.[13]

Genetic factors may be involved in development of arterial stiffness. The angiotensin converting enzyme (*ACE*) gene has been implicated in structural changes of the vessel wall.[14,15] The *ACE* gene has an insertion/deletion (I/D) polymorphism in intron 16, which has been previously found to be associated with cardiovascular diseases and atherosclerosis.[16-19]

Two previous studies[20,21] reported a relationship between the *ACE* I allele and increased arterial stiffness in patients with hypertension and type 2 diabetes, whereas no association was found in healthy controls. To our knowledge, only one study investigated the association between the *ACE* I/D polymorphism and arterial stiffness in a general population.[22] The results of that study, which was conducted among young adults, suggest that the *ACE* D allele predisposes to a decreased compliance of elastic arteries. The aim of the present study is to assess the role of the *ACE* I/D polymorphism in determining arterial stiffness in a population based study among older adults.

Materials and methods

Study population

This study was conducted within the framework of the Rotterdam Study, an ongoing prospective population based cohort study among subjects aged 55 years or older, living in Ommoord, a suburb of Rotterdam, The Netherlands. The rationale and design of the Rotterdam Study have been described elsewhere.[23] Baseline data were collected from 1990 to 1993. The third examination phase took place from 1997 to 1999. The Medical Ethics Committee of Erasmus University approved the study and written informed consent was obtained from all participants.

Cardiovascular risk factors

Information on cardiovascular risk factors was collected during the third follow up examination. Data on medication use and smoking habits were obtained during the home interview. Participants were categorised as never, former, or current smokers.

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 a systolic blood pressure ≥ 160 mmHg and/or a diastolic blood pressure ≥ 100 mmHg and/or the use of antihypertensive medication. Height and weight of the participants were measured and body mass index (kg/m^2) was computed. Plasma total cholesterol and high density lipoprotein (HDL) cholesterol values were determined by an automated enzymatic procedure (Boehringer Mannheim System). Diabetes mellitus was defined as use of antidiabetic medication and/or a non-fasting plasma glucose level ≥ 11.1 mmol/L.

Arterial stiffness

Arterial stiffness was measured by two different methods, i.e. the distensibility coefficient of the common carotid artery as a measure of common carotid arterial stiffness and the carotid-femoral pulse wave velocity (PWV) as a measure of aortic stiffness. Both measures were obtained on the same day, in the same room. Subjects were instructed to refrain from smoking and from taking coffee, tea, or pain-related medications on the day of measurement, and from taking alcohol on the day of measurements and the day before.

Common carotid distensibility was assessed with the participant in a supine position and the head tilted slightly to the left. The vessel wall motion of the right common carotid artery was measured by means of a duplex scanner (ATL Ultramark IV, operating frequency 7.5 MHz) connected to a vessel wall movement detector system. The details of this technique have been described elsewhere.[24,25] After five minutes of rest, a region at 1.5 cm proximal to the origin of the bulb of the carotid artery was identified using B-mode ultrasound. The displacement of the arterial walls was obtained by processing the radio frequency signals originating from two selected sample volumes positioned over the anterior and posterior walls. The end-diastolic diameter (D), the absolute stroke change in diameter during systole (ΔD), and the relative stroke change in diameter (ΔD/D) were computed as the mean of four cardiac cycles of three successive recordings. Blood pressure was measured twice on the right arm with a Dinamap

automatic blood pressure recorder during the measurement session. The mean was taken as the subject's reading. Pulse pressure (ΔP) was defined as the difference between systolic and diastolic blood pressure. Mean arterial pressure was calculated by the following formula: 1/3 systolic blood pressure + 2/3 diastolic blood pressure. The cross-sectional arterial wall distensibility coefficient was calculated according to the following equation:

$$\text{distensibility coefficient} = 2\frac{\Delta D}{D}\Big/\Delta P \ (10^{-3}/\text{kPa}).$$[26]

In the present study, measurements were restricted to the right side to save time. In previous studies no differences could be detected between arterial wall properties of the right and left common carotid arteries (Samijo SK, unpublished results, 1997).

Carotid-femoral PWV was measured with the subject in a supine position. Blood pressure was measured twice with a sphygmomanometer after five minutes of rest, and the mean was taken as the subject's reading. Mean arterial pressure was calculated by the following formula: 1/3 systolic blood pressure + 2/3 diastolic blood pressure. Carotid-femoral PWV was assessed with an automatic device (Complior®, Colson)[27] that measured the time delay between the rapid upstroke of the feet of simultaneously recorded pulse waves in the carotid and the femoral arteries. The distance between the carotid and the femoral arteries was measured over the surface of the body with a tape measure. PWV was calculated as the ratio between the distance travelled by the pulse wave and the foot-to-foot time delay and expressed in meters per second. The average of at least 10 successive measurements, to cover a complete respiratory cycle, was used in the analyses. In a reproducibility study in 47 subjects the intra-class correlation coefficient was 0.80 for both distensibility coefficient and carotid-femoral PWV.

Data description

Of the 4024 subjects who underwent the physical examination of the third phase of the Rotterdam Study, PWV was measured in 3447 subjects whereas common carotid distensibility was measured in 3098 subjects. Missing information on measures of arterial stiffness was almost entirely due to logistic reasons. Information on PWV measurements and the *ACE* gene polymorphism were available for 3406 subjects, and information for both common carotid distensibility and the *ACE* gene polymorphism were available for 3001 participants.

Laboratory assessments

The II, ID, and DD genotypes were determined at baseline, with blood samples, by the polymerase chain reaction technique (PCR) using a PE9600 PCR machine according to the method of Lindpaintner *et al*[28] with some modifications. The result of amplification was a 319 bp and a 597 bp amplicon for the D and the I alleles, respectively. Because the D allele in heterozygous samples is preferentially amplified, there is a tendency towards misclassification of about 4 to 5 percent of ID genotype to DD. In order to avoid this, a second independent PCR was performed with a primer pair that recognises 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 onto a 3% agarose gel. Two independent investigators interpreted the pictures from each gel and all ambiguous samples were analysed a second time.

Statistical analyses

ACE I/D polymorphism was tested as a three class variable (presence of 0, 1, or 2 D alleles). The Hardy-Weinberg equilibrium was tested by a chi-square test. General characteristics were compared between men and women using student *t*-test for continuous variables and chi-square test for dichotomous variables. The association between the *ACE* genotype and arterial stiffness was next investigated by analysis of variance after adjustment for potential confounding variables. Subsequently, analyses were conducted in strata of age, gender, hypertension, body mass index, diabetes mellitus, and smoking status. Two categories of age were defined; subjects younger than 70 years, and subjects aged 70 years or older. The median value of body mass index (26.7 kg/m^2) was used to define two strata. Linear regression analysis was used to test interaction between age and the *ACE* genotype.

Results

Characteristics of the study subjects (3001 subjects with common carotid distensibility measurement) are presented in Table 1. The distribution of the *ACE* genotypes was consistent with Hardy-Weinberg equilibrium. None of the traits differed significantly between those with and without a successful genotype.

Subjects with the ID and DD genotypes had a higher stiffness of the

Table 1. Demographic characteristics of the study population

	Men (n = 1263)	Women (n = 1738)
Age (years)	71.71 ± 6.50	72.18 ± 6.93*
Body mass index (kg/m^2)	26.17 ± 3.20	27.12 ± 4.38*
Systolic BP (mmHg)	134.14 ± 19.19	132.14 ± 19.66*
Diastolic BP (mmHg)	73.99 ± 9.42	67.76 ± 9.38*
Mean arterial pressure (mmHg)	94.03 ± 11.44	89.22 ± 11.51*
Heart rate (beat/m)	71.69 ± 12.68	74.62 ± 11.56*
Hypertension (%)†	33.80	36.36
Total cholesterol (mmol/L)	5.54 ± 0.95	6.06 ± 0.96*
HDL cholesterol (mmol/L)	1.25 ± 0.33	1.51 ± 0.41*
Current smoking (%)	18.13	14.27*
Diabetes mellitus (%)	7.81	6.85
Distensibility coefficient (10^{-3}/kPa)	10.94 ± 4.21	9.91 ± 4.01*
Pulse wave velocity (m/s)	13.91 ± 3.11	13.06 ± 2.88*
ACE D allele (%)	52.10	53.89

Continuous values are mean ± standard deviation.
ACE: angiotensin converting enzyme gene; BP: blood pressure; HDL: high density lipoprotein.
* $p < 0.05$ compared with men.
† Hypertension, defined as systolic BP ≥ 160 mmHg or diastolic BP ≥ 100 mmHg or medication use.

common carotid artery compared with subjects with the II genotype, while no difference was found between the ID and DD genotypes (Table 2). Pulse wave velocity was not different between the three genotype groups (Table 2).

In analyses stratified for age (Figure 1), 1308 subjects were younger than 70 years and 1693 subjects were aged ≥ 70 years. Subjects younger than 70 years with the ID and DD genotypes had higher stiffness of the common carotid artery compared with subjects with the II genotype after adjustment for age, gender, mean arterial pressure and heart rate. The distensibility coefficients of II, ID, and DD genotypes were 12.48 (95% confidence interval (CI): 12.11, 12.84), 11.90 (95% CI: 11.64, 12.16) and 11.96 (95% CI: 11.62, 12.31), respectively. The interaction between age and the *ACE* I/D polymorphism was statistically significant (p for interaction was 0.04). When we adjusted for total and HDL cholesterol, smoking, body mass index, and diabetes mellitus, results maintained statistically significant.

Table 2. Distensibility coefficient of common carotid artery and carotid-femoral PWV according to the *ACE* genotypes

	ACE genotype			*p* (ANOVA)
	Distensibility coefficient, mean (95% CI)			
	II (n = 675)	ID (n = 1508)	DD (n = 818)	
Model A	10.65 (10.37, 10.93)	10.24 (10.06, 10.43)*	10.27 (10.02, 10.52)*	0.05
Model B	10.58 (10.34, 10.82)	10.24 (10.08, 10.40)*	10.33 (10.11, 10.54)	0.06
Model C	10.62 (10.38, 10.86)	10.28 (10.12, 10.44)*	10.35 (10.13, 10.57)	0.03
	PWV, mean (95% CI)†			
	II (n = 777)	ID (n = 1704)	DD (n = 925)	
Model A	13.45 (13.26, 13.65)	13.53 (13.40, 13.66)	13.57 (13.39, 13.74)	0.73
Model B	13.50 (13.33, 13.68)	13.56 (13.44, 13.67)	13.49 (13.33, 13.65)	0.99
Model C	13.51 (13.34, 13.69)	13.48 (13.37, 13.60)	13.50 (13.34, 13.66)	0.69

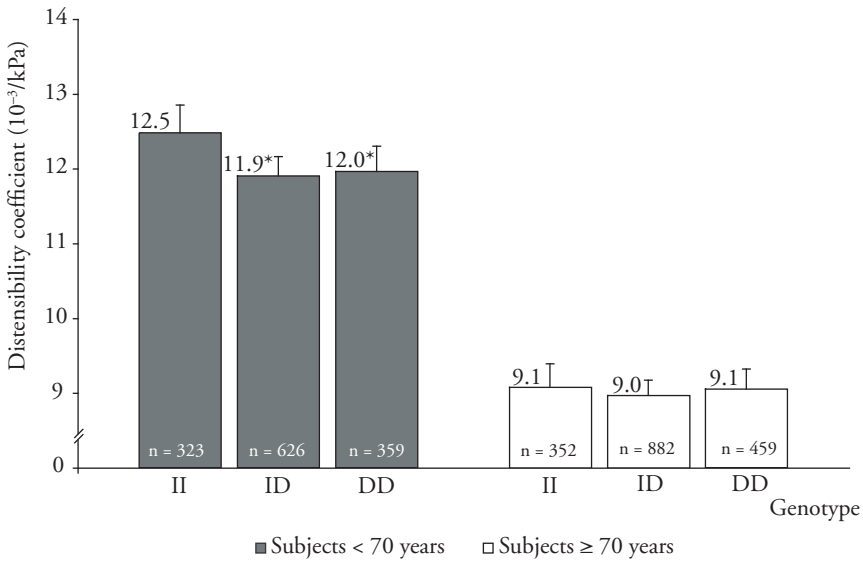
Model A includes age and gender.
Model B includes age, gender, heart rate, and mean arterial pressure.
Model C includes age, gender, heart rate, mean arterial pressure, body mass index, diabetes mellitus, total and HDL cholesterol, and smoking.
ACE: angiotensin converting enzyme gene; ANOVA: analysis of variance; CI: confidence interval; HDL: high density lipoprotein; PWV: pulse wave velocity.
* $p < 0.05$ compared with II genotype.
† PWV measurements were available for 3406 subjects.

We did not find any difference in carotid stiffness between genotypes in the subjects aged ≥ 70 years. In analyses stratified for gender, hypertension, body mass index, diabetes mellitus, and smoking habits, no differences in the effect of the genotype on stiffness were seen between the strata (data not shown).

Discussion

In this population based study we found that the presence of the *ACE* ID and DD genotypes was associated with higher stiffness of the common carotid artery. The association was stronger in subjects younger than 70 years old and no longer present in the older age category. No relation was found between the *ACE* genotype and arterial stiffness measured by carotid-femoral pulse wave velocity.

Figure 1. Distensibility coefficient of the common carotid artery in different genotypes stratified by age categories



Data are adjusted for age, gender, mean arterial pressure, and heart rate.
* Significantly different from the genotype II < 70 years group.

Some aspects of this study need to be discussed. Information on arterial stiffness was not available for all participants which was primarily due to logistic reasons. Therefore, we conclude that this will not have biased the results. Secondly, by calculating the distensibility coefficient, distension of the common carotid artery is adjusted for pulse pressure measured in the brachial artery. We thereby assume that pulse pressure measured in the brachial artery is representative of pulse pressure in the carotid arteries. In dogs it has been demonstrated that pulse pressure in the brachial artery is linearly related to pulse pressure in the carotid artery over a wide range of blood pressure. However, it is known that the arterial pressure waves undergo transformation in the arterial tree and therefore the pulse pressure is higher in the brachial artery than in more central vessels like the carotid artery. On the other hand, non-invasive cuff-based measurement of blood pressure underestimates pulse pressure. Several investigators compared non-invasively measured elastic arterial properties and showed the validity of brachial pressure to measure pulse pressure.[29,30] Thirdly, the distensibility coefficient 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. Consequently, we repeated the analysis after adjustment for mean arterial pressure.

Our results are in agreement with the results of a previous study, which showed that higher stiffness of the common carotid artery was associated with the *ACE* D allele in a small group of young adults.[22] Two other studies assessed the involvement of the *ACE* I/D polymorphism in arterial stiffness.[20,21] Benetos *et al*[20] reported that aortic stiffness, assessed by measuring aortic pulse wave velocity, was similar among the three *ACE* I/D genotypes in normotensive subjects, whereas it was slightly higher among hypertensive subjects with the II genotype. Taniwaki *et al*[21] reported that the I allele of the *ACE* gene was associated with stiffness of the large arteries, such as the carotid artery and the aorta in patients with type 2 diabetes. In our study, the sample was definitely too small to see significant differences among strata of blood pressure and diabetes mellitus. We found that the association was stronger in subjects younger than 70 years and no longer present in the older age category.

We found no association between the *ACE* genotype and carotid-femoral pulse wave velocity. The measure of carotid distensibility is a local measure of stiffness that gives information on an elastic artery, while carotid-femoral pulse wave velocity reflects arterial stiffness of several territories providing information on both elastic and muscular arteries. There may be differences between various types of arteries with respect to the contribution of each of these components, and genetic determinants may have a different outcome according to the type of artery studied. It has been shown that in elastic arteries, hypertrophy of the wall is predominantly due to intima thickening, whereas in muscular arteries this phenomenon mainly reflects remodelling of the media,[31] which might consequently induce primarily an increased distensibility of the muscular arteries. We cannot exclude that the difference in findings for the distensibility coefficient and pulse wave velocity is due to differences in the validity or reproducibility of the measurement. The validity of both the distensibility coefficient and pulse wave velocity has been shown in studies that examined associations of these measures with cardiovascular risk factors and cardiovascular diseases.[1,2,8,10,12] We found that the reproducibility of both measures was adequate. Therefore, we do not think that this is a likely explanation for our results.

The mechanisms that may modulate the relation between the *ACE* gene and arterial stiffness are not completely clear. Higher circulating and tissue ACE levels are present in subjects with the D compared with the I allele.[32-34] ACE catalyses 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, vascular tone, vascular smooth muscle cell growth, and production of extracellular matrix.[35-38] 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. Finally, it has been shown that treatment with ACE inhibitors may increase vascular compliance and thereby reduce arterial stiffness, independently from blood pressure levels. The results confirm the role of ACE in the development of arterial stiffness.

In summary, the results of our population based study show that the presence of the *ACE* ID and DD genotypes was associated with higher stiffness of the common carotid artery. The association was stronger in subjects younger than 70 years old.

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Chapter 4

The *ACE* I/D polymorphism and clinical outcomes

Chapter 4.1

The *ACE* I/D polymorphism and cardiovascular morbidity and mortality: the Rotterdam Study

Abstract

Findings on the association between the insertion/deletion (I/D) polymorphism of the angiotensin converting enzyme (*ACE*) gene and cardiovascular morbidity and mortality have been inconsistent. Considering the possible interaction between this polymorphism and smoking, we evaluated the association between the *ACE* I/D polymorphism and myocardial infarction (MI), and mortality due to coronary heart disease (CHD) and cardiovascular diseases (CVD).

The study was performed within the Rotterdam Study, a population based cohort study that started in 1990. The *ACE* I/D polymorphism was determined for 6714 participants, and smoking status recorded at baseline. Fatal and non-fatal MIs and mortality events were regularly recorded. Cox proportional hazards analysis was performed separately for current smokers and non-/former smokers. We used age as the follow up time in the model, presenting age specific survivals.

During follow up 248 MIs, and 301 and 482 deaths, respectively, due to CHD and CVD occurred. There were no significant differences between the genotypes in relation to incidence of MI. Among smokers, there was an increased risk of CHD and CVD mortality in carriers of the DD genotype compared with the II genotype, which diminished at later ages (p value < 0.01 for gene-age interaction). Subgroup analyses in younger and older groups (based on the median age of 68.2 years) showed a significantly increased risk of CVD mortality in the younger group (hazard ratio = 5.19; 95% confidence interval: 1.15, 23.42).

This study showed that the *ACE* I/D polymorphism is not a strong risk factor for MI, but its interaction with smoking might play a role in mortality from cardiovascular diseases, especially at younger ages.

Introduction

The insertion/deletion (I/D) polymorphism in intron 16 of the angiotensin converting enzyme (*ACE*) gene has been associated with myocardial infarction (MI)[1,2] and coronary heart disease (CHD).[3,4] However, the findings of various large studies have been inconsistent. This situation is typical for genetic research of complex diseases, where genes may be involved in a disease through complex interactions with other genes and environmental factors. Using all the data we had available in the Rotterdam Study, we searched for modifiers of the relation between the *ACE* gene and plasma ACE levels and found that smoking is the only other factor significantly influencing plasma ACE activity level.[5] Further, we found interaction between the *ACE* polymorphism and smoking in relation to carotid intima media thickness (IMT),[5] as well as systolic blood pressure.[6] These findings prompted us to evaluate the effect of the *ACE* gene on MI as the main morbidity outcome of CHD, taking into account the putative interaction with smoking. We also investigated the association between the gene and mortality due to CHD, as well as mortality from cardiovascular diseases (CVD). We conducted our study in a large cohort, which has been characterised for CVD and has been followed for over 8 years, allowing us to assess the genotype associations at different ages.

Materials and methods

Study population

This study is embedded in the Rotterdam Study, a prospective population based cohort study of 7983 men and women aged 55 years or older, living in Ommoord, a suburb of Rotterdam, The Netherlands. The study is designed to investigate the determinants of chronic diseases in the elderly and has been described in more detail elsewhere.[7] Baseline data were collected between March 1990 and July 1993. The study was approved by the Medical Ethics Committee of Erasmus University, and written informed consent was obtained from all participants. A trained research assistant interviewed all participants at home using computerised questionnaires. The information obtained included current health status, medical history, medication use, and smoking behaviour. In addition, during two visits to the research centre, established cardiovascular risk factors were measured.

Follow up procedures

The present analyses on MI and CHD mortality are based on follow up data collected from baseline (1990-1993) until January 1, 2002. The information on cause specific mortality was obtained until January 1, 2000, and was the source of data on CVD mortality outcome. Information on the vital status of the participants was obtained at regular intervals from the municipal population registry. Fatal and non-fatal MIs were reported by general practitioners in the research area by means of a computerised system. An MI was considered fatal if death occurred within 28 days after the onset of symptoms. In the case of recurrent MI during follow up, the first event was used in the analyses. All reported events were verified by research physicians who collected information from the patients' medical records. This information also included copies of discharge letters for hospital admissions. All events were coded independently by two research physicians according to the International Statistical Classification of Diseases and Related Health Problems, 10th revision (ICD-10).[8] CHD mortality is defined as ICD codes I20-I25, I46, I50, and R96. CVD mortality includes all CHD deaths plus deaths due to stroke (I60-I64).

Laboratory assessments

Blood samples were drawn and serum and plasma were stored at –80 °C. DNA was isolated from blood samples using a standard procedure (the salting out method)[9] and the II, ID, and DD genotypes were detected using the polymerase chain reaction technique according to the method of Lindpaintner *et al*[10] with some modifications. Details of the genotyping method were published previously.[5]

Statistical analyses

Data were analysed using SPSS version 11.0 and S-PLUS version 6.0 for Windows. Hardy-Weinberg equilibrium was tested with the chi-square test. We used smoking as a dichotomous variable, grouping subjects in current smokers or non-/former smokers. Survival time was calculated as the number of years from entry into the study until the time of the first event or until the end of follow up, whichever came first. We used Kaplan-Meier curves with age as the follow up time in the model, presenting age specific survivals. Cox proportional hazards analysis was used to calculate the hazard ratios between the genotype groups using the II genotype as the reference group. Data were analysed separately for current and non-/former smokers

to examine effect modification by smoking. We also examined our hypothesis in gender subgroups, as one may expect differences between genders regarding the causal chain leading to CVD. Possible interactions were tested using multiplicative models.

Results

The complete data of the *ACE* genotypes and smoking status were available for 6714 subjects. Missing data were mainly due to logistic reasons. The distribution of the genotype and allele frequencies were in Hardy-Weinberg equilibrium ($p = 0.74$). Table 1 shows the demographic characteristics of the participants by the *ACE* genotypes. The carriers of the D allele were somewhat older than the II carriers. Carriers of the DD genotype had a significantly higher mean systolic blood pressure as well as

Table 1. Demographic characteristics of subjects according to the *ACE* I/D genotypes

	ACE genotype		
	II (n = 1473)	ID (n = 3358)	DD (n = 1883)
Age (years)	68.79 ± 9.15	69.46 ± 8.97*	69.50 ± 9.23*
Male gender (%)	41.07	40.59	39.56
Body mass index (kg/m^2)	26.34 ± 3.84	26.26 ± 3.72	26.35 ± 3.66
Current smoking (%)	22.81	23.17	20.92
Total cholesterol (mmol/L)	6.58 ± 1.26	6.62 ± 1.20	6.60 ± 1.22
HDL cholesterol (mmol/L)	1.34 ± 0.36	1.35 ± 0.35	1.35 ± 0.37
Systolic BP (mmHg)	138.33 ± 22.08	139.32 ± 22.66	140.17 ± 22.14*
Diastolic BP (mmHg)	73.60 ± 11.78	73.69 ± 11.69	73.96 ± 11.29
Hypertension (%)†	31.75	35.24*	34.71
Prevalence MI (%)‡	11.52	13.24	12.27
Common carotid IMT (mm×10^{-1})	7.89 ± 1.53	7.99 ± 1.59	8.03 ± 1.59*

Continuous values are mean ± standard deviation.

ACE: angiotensin converting enzyme gene; BP: blood pressure; HDL: high density lipoprotein; I/D: insertion/deletion; IMT: intima media thickness; MI: myocardial infarction.

* $p < 0.05$ compared with II genotype.

† Hypertension, defined as systolic BP ≥ 160 mmHg or diastolic BP ≥ 100 mmHg or medication use.

‡ Myocardial infarction, verified by cardiologist, general practitioner or electrocardiogram.

Table 2. Incidence rates of MI and mortality due to CHD and CVD according to the *ACE* I/D genotypes

a) in smoking groups*

	ACE genotype								
	All			Non-/former smokers			Smokers		
	II (n =1221)	ID (n = 2758)	DD (n = 1558)	II (n = 937)	ID (n = 2113)	DD (n = 1242)	II (n = 284)	ID (n = 645)	DD (n = 316)
Fatal and non-fatal MI	60 (56)	69 (131)	49 (61)	54 (45)	69 (100)	47 (45)	76 (11)	54 (31)	60 (16)
CHD mortality	63 (67)	58 (151)	57 (83)	61 (54)	57 (120)	52 (66)	49 (13)	55 (31)	68 (17)
CVD mortality	126 (102)	122 (243)	121 (137)	125 (86)	117 (192)	110 (109)	108 (16)	130 (51)	160 (28)

b) in gender groups†

	ACE genotype					
	Men			Women		
	II (n =478)	ID (n = 1068)	DD (n = 581)	II (n = 743)	ID (n = 1690)	DD (n = 977)
Fatal and non-fatal MI	82 (33)	85 (80)	65 (34)	33 (23)	32 (51)	29 (27)
CHD mortality	71 (29)	66 (64)	59 (32)	58 (38)	52 (87)	56 (51)
CVD mortality	124 (39)	126 (93)	120 (50)	127 (63)	119 (150)	122 (87)

Values are incidence rates per 10,000 person-years (number of events).

ACE: angiotensin converting enzyme gene; CHD: coronary heart disease; CVD: cardiovascular diseases; I/D: insertion/deletion; MI: myocardial infarction.

* Adjusted for age and gender.

† Adjusted for age.

an increased mean of carotid IMT compared with the II carriers. Although the prevalence of hypertension was increased in both homozygote and heterozygote subjects for the D allele, this increase was significant only in the largest group, the heterozygotes. There was a tendency of higher prevalence of MI in carriers of the D allele, but the difference was not statistically significant (Table 1). We excluded the subjects with prevalent MI in our further analyses as differential mortality may have occurred in this subgroup before the start of the study.

Mean follow up time for CVD was 8.4 years. During this period 248 persons had a first MI and 301 deaths due to CHD occurred. For the period of follow up for CVD mortality outcome (mean = 7.1 years) 482 deaths caused by CVD occurred. Table 2a presents the age and gender adjusted incidence rates by genotypes. No obvious pattern was observed in the association between the genotypes and the risk of MI. Among smokers, the incidence rates for CHD and CVD mortality were higher in carriers of the DD genotype, while no such differences were seen in non-/former smokers. However, the differences in incidence rates between the genotypes were not statistically significant (Table 2a). Further analyses, stratified by gender, did not reveal any evidence for increasing incidence rates associated with genotype in men or women (Table 2b)

Table 3. Test of interaction between the *ACE* I/D genotypes, smoking status, and age

	CHD mortality		CVD mortality	
	ID	DD	ID	DD
		Gene-age interaction*		
p (smokers)	0.01	< 0.01	< 0.01	< 0.01
p (non-/former smokers)	0.75	0.58	0.63	0.15
p (total group)	0.24	0.20	0.39	0.32
		Gene-smoking interaction†		
p (below median of age)‡	0.13	0.06	0.24	0.05
p (above median of age)‡	0.46	0.83	0.65	0.98
p (total group)	0.27	0.42	0.50	0.39

The II genotype is used as the reference group.

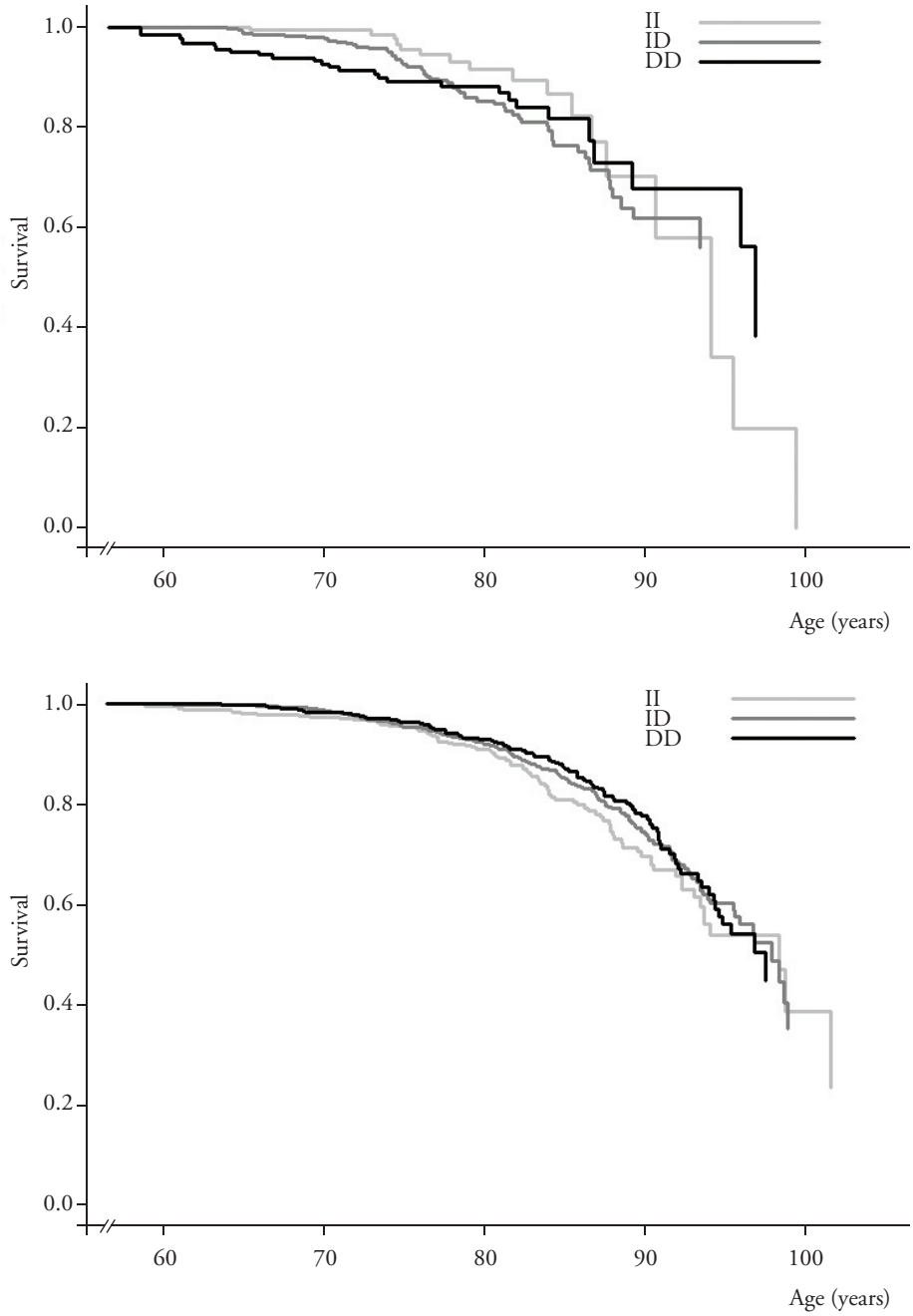
ACE: angiotensin converting enzyme gene; CHD: coronary heart disease; CVD: cardiovascular diseases; I/D: insertion/deletion.

* Adjusted for gender.

† Adjusted for age and gender.

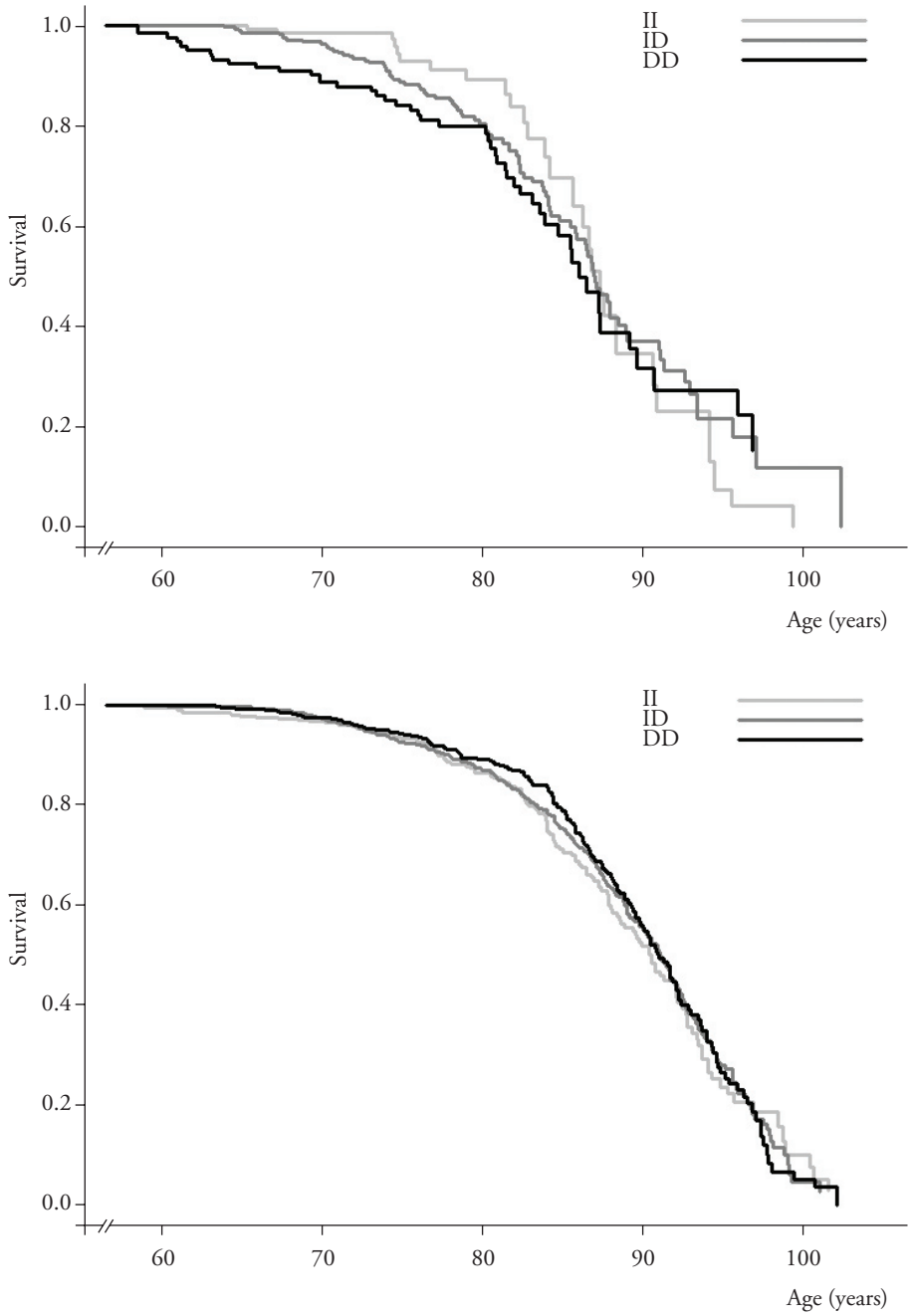
‡ Median age = 68.2 years

Figure 1. Age specific CHD mortality for different genotype groups in smokers (top) and non-/former smokers (bottom)



Data are adjusted for gender.
CHD: coronary heart disease.

Figure 2. Age specific CVD mortality for different genotype groups in smokers (top) and non-/former smokers (bottom)



Data are adjusted for gender.
CVD: cardiovascular diseases.

Table 4. Hazard ratios (95% CI) of mortality due to CHD and CVD according to the *ACE* I/D genotypes

	CHD mortality			CVD mortality		
	ID	DD	*p* (Wald test)	ID	DD	*p* (Wald test)
			Below median of age*			
Smokers	2.78 (0.33, 23.10)	7.33 (0.92, 58.65)	0.06	1.91 (0.41, 9.02)	5.19 (1.15, 23.42)	0.03
Non-/former smokers	0.43 (0.15, 1.29)	0.74 (0.25, 2.22)	0.32	0.63 (0.25, 1.60)	0.89 (0.33, 2.36)	0.60
Men	0.98 (0.29, 3.25)	2.02 (0.62, 6.57)	0.26	0.98 (0.37, 2.61)	1.83 (0.69, 4.88)	0.25
Women	0.50 (0.12, 1.98)	1.06 (0.29, 3.97)	0.47	0.73 (0.21, 2.59)	1.47 (0.43, 5.03)	0.45
			Above median of age*			
Smokers	1.21 (0.69, 2.13)	0.81 (0.41, 1.61)	0.33	1.14 (0.72, 1.78)	0.99 (0.59, 1.65)	0.73
Non-/former smokers	0.97 (0.74, 1.25)	0.88 (0.65, 1.17)	0.63	1.03 (0.83, 1.27)	0.98 (0.77, 1.23)	0.87
Men	1.04 (0.74, 1.44)	0.85 (0.58, 1.25)	0.49	1.13 (0.85, 1.51)	1.06 (0.77, 1.45)	0.67
Women	0.93 (0.68, 1.28)	0.81 (0.56, 1.16)	0.48	0.94 (0.74, 1.19)	0.89 (0.69, 1.16)	0.69

Data are adjusted for age and gender in smoking categories, and adjusted for age in gender categories.
The II genotype is used as the reference group.
ACE: angiotensin converting enzyme gene; CHD: coronary heart disease; CI: confidence interval; CVD: cardiovascular diseases; I/D: insertion/deletion.
* Median age = 68.2 years.

When considering age specific mortality (Figures 1 and 2), an increased risk of CHD and CVD mortality in the DD genotype group was observed only in smokers. The effect of the gene diminished with age. No such effect was seen in non-/former smokers (Figures 1 and 2). The interaction between the *ACE* genotype and age was statistically significant in smokers ($p < 0.01$), but not in non-/former smokers or the total group (Table 3). The same pattern of genotype-age interaction was observed in both men and women. No interaction between the genotype and gender was observed in any of the smoking categories (data not shown).

With this observation, we retested our hypothesis separately for the groups younger and older than the median age of the population (68.2 years) using the age as a time dependent variable, that is, when a participant in the younger group reached the age of 68.2 he was counted in the older group. Table 4 presents the hazard ratios of CHD and CVD mortality for the ID and DD genotypes in comparison to the II genotype in different subgroups. In the younger group, the risk of CVD mortality showed a significant difference between the genotypes in smokers ($p = 0.03$) but not in non-/former smokers ($p = 0.60$), men ($p = 0.25$) or women ($p = 0.45$). Higher risk of mortality was observed in those with the ID and DD genotypes compared with the II genotype among smokers in this group (Table 4). A multiplicative model for gene-smoking interaction showed borderline significance ($p = 0.06$) in CHD mortality and significance ($p = 0.05$) in CVD mortality among the younger group (Table 3). There was no evidence of association between the genotypes and the mortality risks in the older group in any of the smoking or gender categories (Table 4).

Discussion

In this large population based cohort study, we observed an increased risk of cardiovascular mortality for carriers of the D allele of the *ACE* I/D polymorphism among smokers. This difference was only observed in younger people and diminished at later ages. No association was observed between the *ACE* genotype and MI.

The *ACE* gene became one of the most studied candidate genes in relation to CVD after Rigat *et al*[11] reported that more than half of the variance of plasma ACE concentration is under the influence of the I/D polymorphism in this gene. Although several studies showed a positive association between the D allele and cardiovascular outcomes, findings have been inconsistent. A meta-analysis showed that smaller studies tend

to show a stronger association and concluded that in general the association is weak.[12] However, interactions between genetic and environmental factors are expected in all complex diseases and may partly explain the previous inconsistencies. A possible interaction between smoking and the I/D polymorphism in relation to carotid IMT[5] and systolic blood pressure[6] prompted the present study to test the same interaction effect on the risk of MI as the main morbidity outcome of CHD, as well as mortality due to CHD and CVD.

Our findings in this cohort study were in line with the findings from other large studies,[10,13,14] showing no strong association between the *ACE* I/D and MI. Hibi *et al*[15] showed a smoking associated effect of the *ACE* genotype on the severity of coronary atherosclerosis. Although we confirmed the same interaction in our previous findings,[5,6] we did not observe any evidence for such an interaction in relation to MI in this study.

Considering mortality, the main differences between the genotypes were observed at earlier ages in our population. At older ages, the effect of the gene apparently diminished. One may argue that the older individuals are the survivors from a larger cohort and have been subject to selective mortality.[16] We previously reported an interaction between age and the *ACE* I/D genotype in association with carotid stiffness.[17] An age dependent association has also been found in the other genes involved in CVD, for instance apolipoprotein E.[18,19] Although chance might play a role in our observations, the possibility remains that the interaction between the D allele and smoking in fact has a role in younger age groups, but we are not able to fully demonstrate it in our study population. In other words, if there is a real effect of the D allele and smoking at young ages, it might very well have happened before the start of our study, which only includes a cohort aged 55 years or older.

The mechanism by which the *ACE* I/D polymorphism may affect cardiovascular morbidity and mortality is unclear. It has been shown that the DD carriers convert angiotensin I at higher rates.[20,21] The vasoconstrictor effect of angiotensin II in addition to its role in vascular smooth muscle cell growth and hypertrophic changes of the vessel walls[22,23] were speculated to be the possible pathway. It is also suggested that ACE might play an important role in inflammatory responses.[24] Furthermore, smoking increases *ACE* gene expression and the activity levels of the enzyme.[5,25,26] Considering the complexity of CVD, a combination of several different pathways could be involved in the present observations and the mechanisms involved in cardiovascular events at younger ages could be different from those in later life. Similarly, differences in causal chains in different samples could be the

main reason for controversial findings, but discovery of the whole underlying system is beyond the scope of a single observational study.

In conclusion, our study showed that the I/D polymorphism of the *ACE* gene is not a strong risk factor for myocardial infarction but its interaction with smoking might play a role in mortality from the cardiovascular diseases, especially at an earlier age.

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Chapter 4.2

The *ACE* I/D polymorphism and total mortality: the Rotterdam Study

Abstract

Genetic and environmental risk factors that may influence longevity and mortality have received considerable attention over the last few decades. One of the major risks for mortality is cardiovascular diseases and the renin angiotensin system plays a major role in maintaining blood pressure homeostasis. In this system, the angiotensin converting enzyme (ACE) is one of the key regulators and has been studied with respect to cardiovascular diseases and mortality. In order to evaluate the *ACE* insertion/deletion (I/D) polymorphism and its relationship to overall mortality, 6968 elderly individuals from the Rotterdam Study were genotyped for the this polymorphism. Smoking was studied as a possible effect modifier. To examine the effect of the *ACE* genotype on mortality, a Cox proportional hazards model was fitted. Our results show an increased risk of overall mortality (hazard ratio = 1.8; 95% confidence interval: 1.1, 2.9; $p = 0.02$) in subjects with an age at death below 65 years in carriers of the DD genotype. This association was significant only in those who smoke. Our findings suggest that the *ACE* gene is associated more with early mortality than with late. Individuals who carry the DD genotype appear to be susceptible to early mortality if they smoke, suggesting a possible interaction between smoking and the *ACE* gene.

Introduction

The insertion/deletion (I/D) polymorphism in the angiotensin converting enzyme (*ACE*) gene has been studied extensively with respect to cardiovascular diseases and mortality. The D allele has been consistently related to higher ACE levels.[1-3] Even though findings have been inconsistent, the large majority of studies suggest the D allele of the *ACE* gene is associated with atherosclerosis,[3,4] and an increased risk of cardiovascular diseases.[5,6] The contribution of the gene to mortality remains a matter of debate. Some studies found an increased risk of mortality for carriers of the D allele,[7,8] others for carriers of the I allele,[9] while a substantial number of studies found no effect.[10,11] Recently, we found that smoking and the *ACE* gene are both associated with plasma ACE levels and may interact in the process of atherosclerosis.[3,12] These findings triggered the present study to examine the role of the *ACE* gene in mortality and longevity, utilizing smoking as a potential modifier of the effect of the *ACE* gene.

Materials and methods

Study population

Our study is part of the Rotterdam Study,[13] a population based follow up study of determinants of diseases in the elderly. All inhabitants of Ommoord, a suburb of Rotterdam, aged 55 years or older, were invited to participate. The design of the study has been previously described.[13] From all subjects, informed consent was obtained and the Medical Ethics Committee of the Erasmus University approved the study. 7983 participants were examined at baseline (1990-1993). Information on age, smoking behaviour and medical history were obtained using a computerised questionnaire. Two blood pressure measurements were taken in a sitting position on the right arm using a random-zero sphygmomanometer. The average of the two measures was used in the analyses. Information on all-cause mortality was collected through February 3, 2003.

Laboratory assessments

The *ACE* I/D polymorphism was genotyped in 6869 subjects of the Rotterdam Study (86%). DNA was isolated from blood samples using a standard procedure (the salting out method).[14] The II, ID, and DD genotypes were detected using the polymerase chain reaction technique according to the method of Lindpaintner *et al*[15] with some modifications.[3]

Statistical analyses

Hardy-Weinberg equilibrium of the I/D polymorphism was tested with the chi-square test. Frequencies of mortality and smoking status in the different genotype groups were compared using the chi-square test. Differences in age and blood pressure between genotype groups were tested using the Kruskal-Wallis non-parametric test for independent samples. To examine the effect of the *ACE* genotype on mortality, a Cox proportional hazards model was fitted, taking the II genotype as the reference category. We used age as the underlying time of the model. Analyses were subsequently stratified for age (55-64.9 years, 65-74.9 years, 75-84.9 years, and ≥ 85 years of age) and smoking status (current and non-/former smoker). The statistical analyses were carried out using SPSS version 11.0 for Windows. Survival curves were plotted using S-PLUS version 6.0 for Windows.

Results

Table 1 presents the characteristics of the DD, ID, and II genotypes. The distributions of the genotype and allele frequencies were in Hardy-Weinberg equilibrium (p = 0.70). No significant differences between the genotype groups were found in mean age at entry, smoking status, or diastolic blood pressure. Mean systolic blood pressure was higher in the DD carriers compared with the II carriers (p = 0.02). Overall, mortality was increased in the DD carriers but not significantly. Figure 1 shows the age specific survival curves by the *ACE* genotypes adjusted for gender. The DD carriers had slightly increased mortality compared with the II carriers at early age.

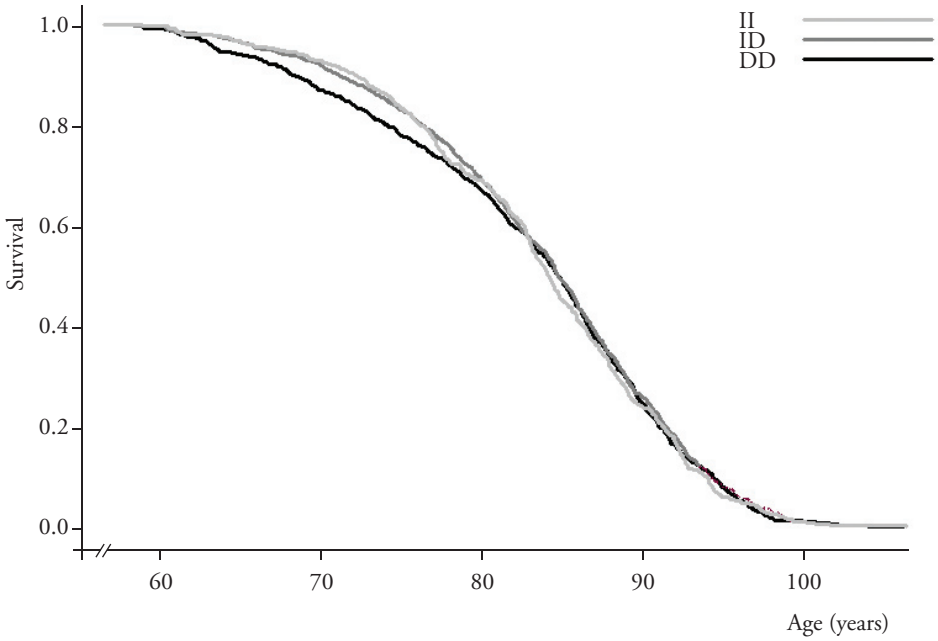
Stratified analyses by age categories showed that mortality for subjects with ages below 65 years at baseline carrying the DD genotype was 1.5-fold increased (95% confidence interval (CI): 1.1, 2.1; p = 0.01) during 10 years of follow up. In Figure 2, the survival curves by the *ACE* genotypes for smokers and non-/former smokers are shown. In the former group, the risk of mortality was significantly increased throughout life (p = 0.03). The risk of mortality for the DD compared with the II carriers was increased 1.3-fold (95% CI: 1.0, 1.6), while in non-/former smokers there was no evidence for an effect of the *ACE* (Figure 2). Among smokers, the effect of the *ACE* gene was strongest early in life; the risk for the DD carriers aged below 65 years who smoke was 1.8 (95% CI: 1.1, 2.9) times higher than in subjects carrying the II genotype in the same age and smoking categories.

Table 1. Demographic characteristics of the study population according to the *ACE* I/D genotypes

	ACE genotype		
	II (n = 1513)	ID (n = 3429)	DD (n = 1927)
Age (years)	69.07 ± 9.30	69.60 ± 9.03	69.72 ± 9.40
Male gender (%)	40.52	40.30	39.28
Current smoking (%)	22.81	23.17	20.92
Systolic BP (mmHg)	138.44 ± 21.98	139.22 ± 22.64	140.20 ± 21.16*
Diastolic BP (mmHg)	73.64 ± 11.81	73.67 ± 11.70	73.90 ± 11.29
Mortality during follow up (%)	32.45	33.13	35.13

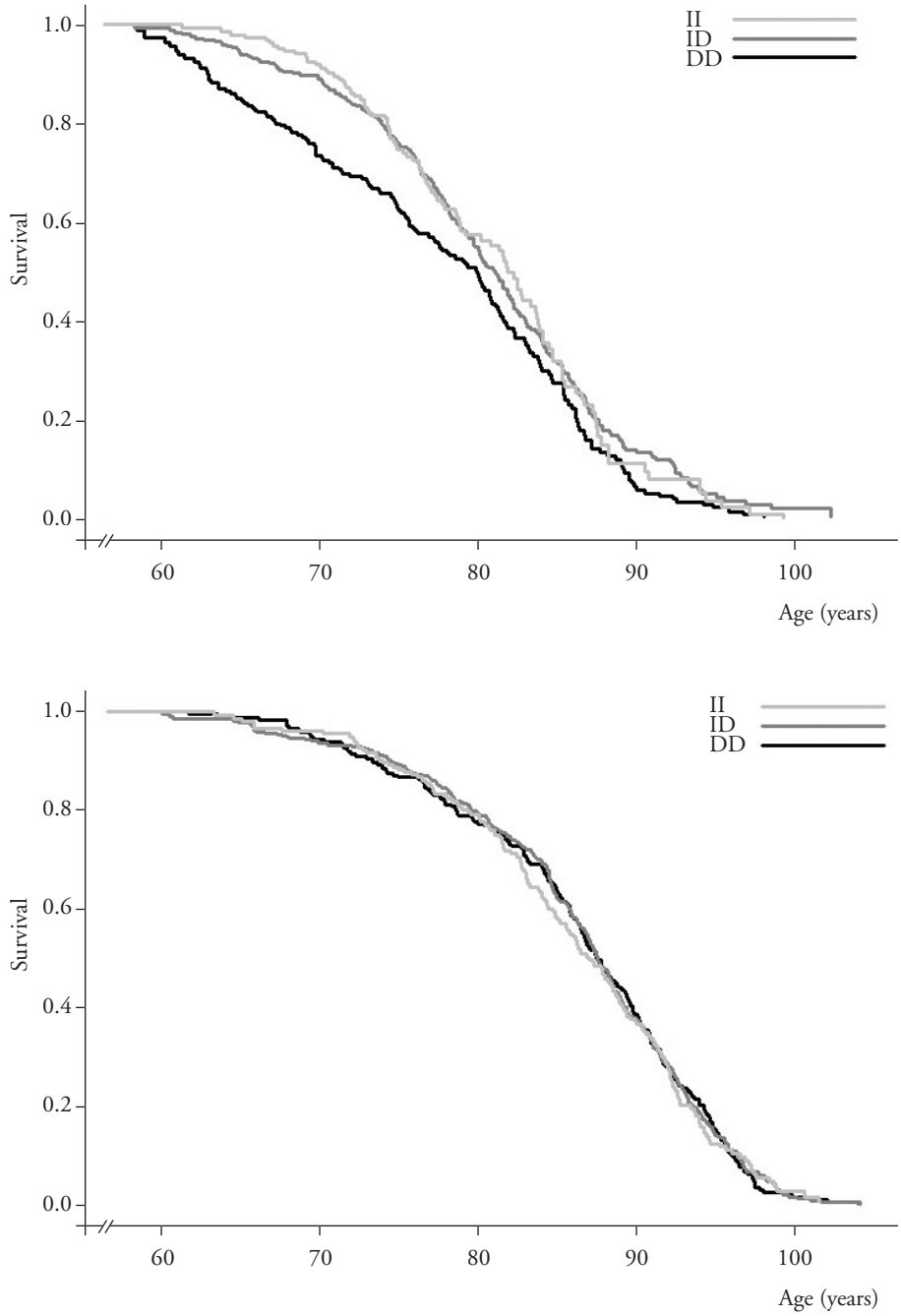
Continuous values are mean ± standard deviation.
ACE: angiotensin converting enzyme gene; BP: blood pressure; I/D: insertion/deletion.
* $p < 0.05$ compared with II genotype.

Figure 1. Survival curve for the *ACE* I/D genotype groups



Data are adjusted for gender.
ACE: angiotensin converting enzyme gene; I/D: insertion/deletion.

Figure 2. Survival curve for the *ACE* I/D genotype groups in smokers (top) and non-/former smokers (bottom)



Data are adjusted for gender.

ACE: angiotensin converting enzyme gene; I/D: insertion/deletion.

Discussion

In our study of 6896 elderly participants of the Rotterdam Study we found an increased risk of mortality at early age for carriers of the DD genotype. This association was significant only in smokers. Although the risk of mortality for the DD carriers compared with carriers of the other genotypes was highest at early age, the increased risk of mortality also persisted later in life.

Several studies have addressed the role of the *ACE* gene on mortality but findings have been inconclusive. Our findings are at odds with another large cohort study embedded within the Copenhagen City Heart Study including 10,150 individuals.[10] They could not show a relationship between the *ACE* DD genotype and cardiovascular mortality or longevity. However, they did not stratify for smoking but merely adjusted for it. In addition, a large study of French centenarians (n = 563) failed to show an association between the *ACE* genotype and longevity.[11] In contrast, another study showed that the DD variant of *ACE* was more frequent in centenarians compared with younger healthy controls.[16] Our results suggest that mortality related to the *ACE* gene is dependent on age. We found a particularly increased risk of early mortality in carriers of the D allele.

The relationship between the *ACE* gene and mortality at different ages may not be similar. The *ACE* gene might be an important risk factor in causal pathways leading to early mortality, while not playing a significant role later in life. A plausible explanation for the interaction between *ACE* and smoking in early mortality could be a smoking dependent effect of ACE on vascular homeostasis. We found the effect of the D allele on atherosclerosis was smoking dependent.[3] In that study, smoking was the only cardiovascular risk factor associated with ACE levels together with the *ACE* gene. Although chance might play a role in these observations, our findings are in line with a study which demonstrated a synergistic effect of smoking and the *ACE* DD genotype on coronary artery disease.[17] Moreover, a smoking dependant association has also been found in other genes involved in vascular pathophysiology.[18]

In conclusion, our findings support a role for the *ACE* I/D polymorphism in increasing the risk of mortality at early ages that is prominent in smokers, providing evidence of a gene-environment interaction.

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Chapter 4.3

The *ACE* I/D polymorphism and co-morbidity effect: the Rotterdam Study

Abstract

There is increasing evidence that atherosclerosis is not only involved in cardiovascular diseases (CVD) but also other diseases of the elderly such as Alzheimer's disease (AD). Since CVD are highly fatal and usually occur earlier than Alzheimer's disease, the co-morbidity by CVD might hamper studies on the association between atherosclerosis risk factors and AD. Here we used the inverse probability of censoring weighted analyses method to test this hypothesis, in a study on the relationship between the insertion/deletion polymorphism of the angiotensin converting enzyme gene (*ACE* I/D) and risk of AD.

We used data from the Rotterdam Study, a prospective population based study. We first performed a Cox proportional hazards model including all available cardiovascular risk factors to calculate the probability of death from CVD and thereby the probability of being free from fatal CVD events. These probabilities were then used to weight the subjects who were uncensored by fatal disease by the inverse of their survival probability. In a weighted analysis we estimated the association between the *ACE* I/D polymorphism and AD.

Among 2431 men that were free from Alzheimer's disease at baseline, 51 persons were diagnosed for AD and 196 individual had fatal cardiovascular events without occurrence of AD during the follow up. Among 3281 women, these numbers were 89 and 159, respectively. Survival analyses showed that the association between the I allele and AD was mainly present in women (p values for trend was 0.09). In the weighted analyses the hazard ratios did not materially change in any of the gender groups. Our results indicate that the association between the I allele of the *ACE* gene and Alzheimer's disease is not due to the co-morbidity effect of CVD.

Introduction

There is increasing evidence that atherosclerosis is not only an etiologic factor for cardiovascular diseases (CVD), but also for other complex diseases of the elderly such as Alzheimer's disease (AD).[1-3] In an observational study, those who died during follow up from CVD may have been at higher risk of developing Alzheimer's disease compared with the survivors in the population. Therefore, studies on the etiological role of atherosclerotic risk factors in AD might be hampered, since CVD is highly fatal and usually occurs at earlier ages than the former disease.

The insertion/deletion polymorphism of the angiotensin converting enzyme gene (*ACE* I/D) is a risk factor for both CVD and AD. Although individual studies were inconsistent, several meta-analyses indicated that the D allele of this polymorphism is a risk factor for atherosclerosis[4] and increases the risk of CVD.[5,6] However, the findings on the association between this polymorphism and AD showed a different pattern. It has been reported that the I allele of the I/D polymorphism might increase the risk of Alzheimer's disease[7,8] and this finding has been confirmed by subsequent meta-analyses.[9-11] In a case-control study, Kehoe *et al*[7] suggested that the low frequency of the DD genotype in AD patients might have been due to the exclusion of cases due to cardiovascular mortality before the start of the study.

In this study, we implemented a methodology to adjust for the effect of co-morbidity, which has been used previously to correct for non-compliance and dependent censoring in a clinical trial.[12] We hypothesised that the observed positive association between the I allele and AD is due to early mortality of the carriers of the D allele from CVD, and correction for that co-morbidity bias would diminish that association. We tested this hypothesis using data from the Rotterdam Study.

Materials and methods

Study population

This study is embedded in the Rotterdam Study, an ongoing population based follow up study. The study is designed to investigate the determinants of chronic diseases in the elderly and has been described in more detail elsewhere.[13] In brief, baseline data were collected between March 1990 and July 1993 from 7983 subjects, aged 55 years or older, living in Ommoord, a suburb of Rotterdam, The Netherlands.[13] The study was approved by the Medical Ethics Committee of Erasmus University, and written informed

consent was obtained from all participants. The participants were interviewed at home by a trained research assistant using computerised questionnaires, and subsequently visited the study centre.

At baseline, the height and weight of the participants were measured and body mass index (kg/m^2) was computed. An electrocardiogram was recorded and two blood pressure measurements were taken, with a random-zero sphygmomanometer after a minimum of five minutes rest with the participant in a sitting position, and averaged. Total plasma cholesterol and high density lipoprotein cholesterol levels were assessed by an automated enzymatic procedure using a non-fasting blood sample. Diabetes mellitus was defined as the use of glucose-lowering medication and/or a non-fasting plasma glucose level ≥ 11.1 mmol/L according to Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus.[14] Smoking status was defined as current smoker and non-/former smoker, in accordance with the interview at baseline.

Carotid, aortic, and lower-extremity atherosclerosis were assessed. The common carotid artery, carotid bifurcation, and internal carotid artery were visualised over a length as large as possible by use of ultrasound and examined in both the left and right sides for the presence of plaques. A plaque score was computed, ranging from 0 to 6 (the maximum number of sites). Common carotid intima media thickness (IMT) was determined as the average of the IMT of the near- and far-wall measurements over a length of 1 cm in the right and left side arteries. Aortic atherosclerosis was diagnosed by radiographic detection of calcified deposits in the abdominal aorta on a lateral abdominal X-ray. Ankle-arm index was computed as the ratio of the systolic blood pressure at the ankle to the systolic blood pressure at the right arm. For these analyses, we used the lowest value of this measurement in both legs.

Laboratory assessments

Blood samples were drawn, and serum and plasma were stored at –80 °C. DNA was isolated from blood using a standard procedure (the salting out method)[15] and the II, ID, and DD genotypes were detected using the polymerase chain reaction technique according to the method of Lindpaintner *et al*[16] with some modifications. Details of the genotyping method are previously published.[17]

Follow up procedures

Follow up examinations took place in 1993-1994 (first follow up) and in 1997-1999 (second follow up). Subjects are continuously monitored for clinical events through automated linkage of the study database of the Rotterdam Study with files from the general practitioners. When an event or death has been reported, additional information is obtained from the general practitioner and hospital discharge records. All possible coronary heart disease (CHD) events (myocardial infarctions and cardiac deaths) and strokes are reviewed by physicians and diagnoses are reached during consensus meetings. CHD and stroke were classified according to the International Classification of Diseases, 10th version (ICD-10).[18] Cardiovascular death was defined as myocardial infarction (I21), ischaemic heart disease (I20, I22-I25), sudden death (I46, R96), and death due to congestive heart failure (I50). Strokes were defined as ICD codes I60-I64.

The procedures for the baseline and follow up examinations for Alzheimer's disease diagnoses have been described in detail elsewhere.[19] The Mini-Mental State Examination[20] and the cognitive questions from the Geriatric Mental State Schedule[21] were administered during the home interview. Participants in whom dementia was suspected were examined by a neurologist and tested by a neuropsychologist, and, if possible, underwent brain nuclear magnetic resonance imaging. In addition, participants were continuously monitored for development of Alzheimer's disease through linkage between the general practitioners' medical record system and the Rotterdam Study database; this made it possible to obtain follow up diagnoses for more than 99% of the cohort. The diagnosis of Alzheimer's disease was based on criteria from the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's disease and Related Disorders Association.[22]

Data description

In this study we included subjects who screened negative for Alzheimer's disease at baseline. We limited our sample to participants who were 80 years old or younger at baseline. This selection was in line with our hypothesis that the present association is influenced by cardiovascular mortality related to the D allele, because the association between the D allele and CVD mortality is mainly present among younger age groups.[23]

There was, generally, less than 5% missing data for the determinants of CVD in this sample. Yet, for some variables like alcohol consumption, ankle-arm index, carotid IMT, and plaque scores, there were between 10%

and 18% missing values. The missing data were imputed by a multiple imputation technique,[24] using Multivariate Imputation by Chained Equations (MICE) software library written for S-PLUS. Five complete data sets were generated (n = 5712) and each analysis was the pooled result of the analyses done on each of those five data sets.

Method of adjustment for competing risks

We defined disease D_C (co-morbidity disease) to represent fatal CVD, and D_I (disease of interest) to represent Alzheimer's disease in our study. We used the inverse probability of censoring weighted analyses method to adjust for dependent censoring.[12] We assumed that we have data on a sufficient number of covariates at baseline so that, conditional on these covariates, censoring due to death from D_C is independent of the risk of D_I. We then specified a Cox proportional hazards model including all covariates for the probability of death from D_C. The cumulative hazard function was calculated for each subject *i* as:

$$H_i^C(t) = H_0^C(t)e^{\beta_{age}\,Age + \beta_{gender}\,Gender + \ldots} = H_0^C(t)e^{\sum X_{ij}\beta_j^C} \quad (1)$$

where X_{ij} is the specific determinant *j* of D_C for subject *i* and β_j is the coefficient of the determinant *j*.

For each subject who did not suffer death from disease D_C during follow up, we used the results of model (1) to estimate the probability of being free from fatal disease D_C through the end of their observation period.

$$S_i^C(t) = \text{probability of survival beyond time } t = \Pr(T > t) = e^{-H_i^C(t)} \quad (2)$$

This probability was used to compute a weight for each subject *i*, that is uncensored by fatal disease D_C. This weight was the inverse of the survival probability:

$$w_i^C(t) = \frac{1}{S_i^C(t)} \quad (3)$$

Finally, a weighted Cox proportional hazards model was fitted on disease D_I:

$$H_i^I(t) = H_0^I(t)e^{\sum Z_{ij}\beta_j^I} \quad (4)$$

where Z_{ij} is the specific determinant *j* of disease D_I for subject *i*.

An intuitive explanation for these weights is given here. For a person with a cumulative probability of 25 percent of being free from death from disease D_C through the end of follow up, on average, there would have been three other persons who were censored by death from disease D_C before

the end of follow up, but who would have had a similar set of covariates and risk of disease D_I had censoring been prevented. We therefore assigned this person a weight of four in the final regression analysis on disease D_I. These weights are computed in S-PLUS, which allows sampling weights to be used. The standard errors of the β coefficients of the Cox proportional hazards model are estimated by statistical methods available in S-PLUS that are robust against introducing weights.[25]

The weighed analysis was performed in each of the five imputed data sets. The final result was the pooled estimate of the five analyses according to standard multiple imputation methodology.[26] Suppose that β_i is the coefficient of interest (e.g. of the II or the ID genotypes) obtained from data set i (i = 1,2,…,M). The pooled estimate is the average of the individual estimates:

$$\beta_{pooled} = \frac{1}{M}\sum_{i=1}^{M}\beta_i$$

For the pooled variance, one must first calculate the within-imputation variance:

$$\overline{Var(\beta_i)} = \frac{1}{M}\sum_{i=1}^{M}Var(\beta_i)$$

and the between-imputation variance:

$$Var(\beta_1,\ldots,\beta_M) = \frac{1}{1-M}\sum_{i=1}^{M}\left(\beta_i - \beta_{pooled}\right)^2$$

The total variance will be:

$$Var(\beta_i)_{pooled} = \overline{Var(\beta_i)} + \frac{M+1}{M}Var(\beta_1,\ldots,\beta_M)$$

which, in our study with five imputations, is computed as:

$$Var(\beta_i)_{pooled} = \frac{1}{5}\sum_{i=1}^{5}Var(\beta_i) + \frac{5+1}{5}Var(\beta_1,\ldots,\beta_5)$$

Results

Demographic characteristics of the participants are presented in Table 1 for men and women separately. Women were older than men with a mean difference less than 0.5 year. Men had a higher risk profile than women

Table 1. Demographic characteristics of the study population

	Men (n = 2431)	Women (n = 3281)
Age (years)	66.44 ± 6.52	66.83 ± 6.83*
Body mass index (kg/m^2)	25.78 ± 2.92	26.71 ± 4.11*
Alcohol consumption (%)	87.67	74.52*
Total cholesterol (mmol/L)	6.37 ± 1.12	6.90 ± 1.18*
HDL cholesterol (mmol/L)	1.21 ± 0.31	1.45 ± 0.37*
Systolic BP (mmHg)	138.31 ± 21.60	137.90 ± 21.86
Diastolic BP (mmHg)	74.90 ± 11.41	73.40 ± 10.99*
Diabetes mellitus (%)	9.67	8.57
Prevalence MI (%)†	17.46	7.12*
Family history of MI (%)	16.91	18.84
Ankle-arm index	1.10 ± 0.21	1.07 ± 0.19*
Common carotid IMT (mm×10^{-1})	8.19 ± 1.56	7.56 ± 1.41*
Number of carotid plaques ≥ 3 (%)	26.90	15.60*
Aortic calcification (%)	58.20	54.86*
Current smoking (%)	29.23	20.42*
ACE D allele (%)	52.41	53.15

Continuous values are mean ± standard deviation.
ACE: angiotensin converting enzyme gene; BP: blood pressure; HDL: high density lipoprotein; IMT: intima media thickness; MI: myocardial infarction.
* $p < 0.05$ compared with men.
† Myocardial infarction, verified by cardiologist, general practitioner or electrocardiogram.

considering most of the CVD risk factors (Table 1). There was no difference in the D allele frequency between the two genders and the distribution of the genotype and allele frequencies were in Hardy-Weinberg equilibrium in both genders ($p = 0.59$ in men and $p = 0.62$ in women).

During follow up, 212 fatal cases of CVD (CHD and stroke) and 51 possible and probable cases of Alzheimer's disease were recorded in men. Among women 168 fatal CVD and 89 AD cases were observed. All subjects who died from CVD, but were already screened positive for AD were considered as Alzheimer's disease patients (n = 16 in men and 9 in women). Those who died from CVD without previously being screened positive for AD were considered as CVD cases (n = 196 and 159 in men and women, respectively).

Table 2 shows the results from the Cox proportional hazards regression for fatal CVD in univariable and multivariable models in men. In the

Table 2. Cox proportional hazards model for fatal cardiovascular diseases in men

Variable	Univariable beta* (95% CI)	Multivariable beta (95% CI)
Age (years)	0.124 (0.100, 0.148)	0.088 (0.050, 0.125)
Body mass index (kg/m^2)	–0.030 (–0.077, 0.017)	–0.056 (–0.133, 0.021)
Alcohol consumption	–0.329 (–0.725, 0.067)	–0.673 (–1.189, –0.158)
Total cholesterol (mmol/L)	0.005 (–0.117, 0.127)	–0.087 (–0.276, 0.103)
HDL cholesterol (mmol/L)	–0.781 (–1.281, –0.281)	–0.406 (–1.159, 0.347)
Systolic BP (mmHg)	0.011 (0.005, 0.017)	0.010 (–0.003, 0.023)
Diastolic BP (mmHg)	0.008 (–0.004, 0.020)	0.010 (–0.014, 0.033)
Diabetes mellitus	0.768 (0.429, 1.107)	0.841 (0.342, 1.341)
Prevalence MI	1.266 (0.982, 1.550)	1.240 (0.813, 1.666)
Family history of MI	0.186 (–0.175, 0.547)	–0.128 (–0.729, 0.474)
Ankle-arm index	–1.318 (–1.886, –0.750)	–0.069 (–0.992, 0.853)
Common carotid IMT (mm×10^{-1})	1.651 (0.853, 2.449)	0.734 (–0.653, 2.120)
Number of carotid plaques	0.206 (0.124, 0.288)	0.057 (–0.075, 0.189)
Aortic calcification	0.411 (0.078, 0.744)	0.305 (–0.160, 0.770)
Current smoking	0.121 (–0.183, 0.425)	–0.485 (–1.443, 0.473)
ACE I/D polymorphism†	0.028 (–0.164, 0.220)	–0.035 (–0.370, 0.301)
ACE I/D polymorphism × smoking	0.330 (–0.119, 0.779)	0.436 (–0.247, 1.119)

ACE: angiotensin converting enzyme gene; Beta: regression coefficient; BP: blood pressure; CI: confidence interval; HDL: high density lipoprotein; I/D: insertion/deletion; IMT: intima media thickness; MI: myocardial infarction.

* All estimates were adjusted for age.

† Number of the D alleles was used in the model.

univariable model, all the estimates were adjusted for age. All four measurements of atherosclerosis (ankle-arm index, aortic calcification, carotid IMT, and plaques) showed significant association with CVD mortality. Based on our previous findings on association between the *ACE* I/D polymorphism and cardiovascular mortality in the Rotterdam Study,[23] we also included an interaction between the D allele and smoking status in the model. No significant association was observed between the D allele or its interaction with smoking and mortality from CVD in men.

Results of similar analyses in women are presented in Table 3. In general, cardiovascular risk factors showed stronger association with CVD mortality in women than in men. There was a significant interaction between the D allele and smoking in both the univariable and multivariable models. The multivariable models were used to compute weights using equations (2)

Table 3. Cox proportional hazards model for fatal cardiovascular diseases in women

Variable	Univariable beta* (95% CI)	Multivariable beta (95% CI)
Age (years)	0.140 (0.113, 0.167)	0.098 (0.052, 0.144)
Body mass index (kg/m^2)	0.004 (–0.033, 0.041)	0.003 (–0.055, 0.061)
Alcohol consumption	–0.444 (–0.803, –0.085)	–0.442 (–0.926, 0.042)
Total cholesterol (mmol/L)	0.026 (–0.101, 0.153)	–0.066 (–0.260, 0.128)
HDL cholesterol (mmol/L)	–0.345 (–0.782, 0.092)	–0.167 (–0.849, 0.516)
Systolic BP (mmHg)	0.017 (0.009, 0.025)	0.002 (–0.012, 0.017)
Diastolic BP (mmHg)	0.026 (0.012, 0.040)	0.028 (0.003, 0.054)
Diabetes mellitus	0.863 (0.493, 1.233)	0.482 (–0.163, 1.128)
Prevalence MI	1.052 (0.660, 1.444)	0.732 (0.103, 1.362)
Family history of MI	0.112 (–0.274, 0.498)	–0.330 (–0.934, 0.275)
Ankle-arm index	–2.394 (–2.960, –1.828)	–1.712 (–2.696, –0.729)
Common carotid IMT (mm×10^{-1})	3.217 (2.351, 4.083)	2.602 (1.404, 3.800)
Number of carotid plaques	0.309 (0.209, 0.409)	0.195 (0.039, 0.350)
Aortic calcification	0.569 (0.142, 0.996)	–0.157 (–0.758, 0.445)
Current smoking	0.816 (0.461, 1.171)	–0.921 (–2.115, 0.272)
ACE I/D polymorphism†	–0.092 (–0.312, 0.128)	–0.439 (–0.823, –0.056)
ACE I/D polymorphism × smoking	0.660 (0.152, 1.168)	1.457 (0.618, 2.295)

ACE: angiotensin converting enzyme gene; Beta: regression coefficient; BP: blood pressure; CI: confidence interval; HDL: high density lipoprotein; I/D: insertion/deletion; IMT: intima media thickness; MI: myocardial infarction.

* All estimates were adjusted for age.

† Number of the D alleles was used in the model.

and (3) for those who did not experience a fatal CVD event during follow up, in men and women separately.

The un-weighted survival analyses showed a prominent increased risk of AD among the II carriers compared with the DD carriers in women (hazard ratio = 1.71; 95% confidence interval (CI): 0.97, 3.01) (Table 4). Although separate analyses in men also showed an increased risk of AD related to the I allele, the estimates were far from statistical significance (hazard ratio for II carriers = 1.13; 95% CI: 0.49, 2.60). In the weighted analyses, hazard ratios did not materially change among women, and declined somewhat in men compared with the un-weighted analyses (Table 4).

Table 4. Un-weighted and weighted hazard ratios of Alzheimer's disease for the *ACE* I/D genotypes in men and women

	Men			Women		
	ID	II	*p* for trend	ID	II	*p* for trend
Un-weighted	1.39 (0.71, 2.71)	1.13 (0.49, 2.60)	0.69	1.00 (0.59, 1.68)	1.71 (0.97, 3.01)	0.09
Weighted	1.16 (0.57, 2.35)	0.98 (0.41, 2.35)	0.97	0.98 (0.58, 1.66)	1.71 (0.97, 3.03)	0.09

Data are adjusted for age.
The DD genotype is used as the reference group.
ACE: angiotensin converting enzyme gene; I/D: insertion/deletion.

Discussion

In this study, we examined the effect of co-morbidity by CVD on the association between the angiotensin converting enzyme gene polymorphism and Alzheimer's disease. We used a methodology, previously used in a clinical trial, for the first time in a large-scale population based cohort study. The results did not show major changes between the standard (un-weighted) and corrected (weighted) models, indicating that the increased risk of AD associated with the I allele is not due to selective cardiovascular mortality related to the D allele.

When dealing with competing risks in which one of the diseases is highly fatal, the effects of determinants on the other disease could be biased due to the co-morbidity of these diseases. This, in turn, means that those who died from one disease may have been at a higher risk of developing the other disease than those who survived. Robins[12,27] showed that, having available data on all time-dependent prognostic factors that predict censoring, one can correct for the dependent censoring by using the inverse probability of censoring weighted methodology. In the present study, we used this method to adjust for dependent censoring in an observational study on the risk of Alzheimer's disease. The weights were derived from the inverse of the probability of death from CVD. We assume that we have data on a sufficient number of covariates, such that censoring due to death on CVD is independent of the risk of developing Alzheimer's disease.

We used multiple imputation techniques to generate five imputed data sets in order to deal with missing data on the determinants of cardiovascular mortality. Multiple imputation has proven to be one of the most efficient methods to deal with missing data. Rubin[26] showed that the efficiency of an estimate based on multiple imputation is dependent on the rate of missing information and the number of imputations performed. One can easily show that, in practice, between 3 to 10 imputed data sets is sufficient and in most situations there is simply little advantage in producing and analysing more than a few imputed data sets.[26]

Our study failed to show any clear differences between the un-weighted and weighted models. Particularly in women, with the highest AD risk associated with the II genotype, the risk estimates were the same in the un-weighted and weighted analyses. We found a slight decline in the hazard ratio estimates in men. Although these changes are in the expected direction of a co-morbidity effect, it is important to note that the differences between the weighted and un-weighted analyses could occur due to random variation introduced by the weighting factor (if the weighting model is imperfect).

There could be several explanations for the fact that the co-morbidity effect was not observed in our study. Firstly, it is important to use a large enough number of covariates as predictors of CVD. In our study, we used the conventional cardiovascular risk factors and the majority of them were significantly related to the CVD outcome in the Cox regression model. At the same time, it is necessary that the same model predicts the incidence of AD. The assumption of the inverse probability weighting method is that, conditional on covariates, censoring due to CVD is independent of the risk of AD. It is likely that our model was not strongly predicting disease in either direction and thereby failed to demonstrate the co-morbidity effect.

Secondly, the effect of co-morbidity might be larger in a younger population than the Rotterdam Study. In other words, if dependent censoring is present at a young age, it might very well have happened before the start of our study, which only includes a cohort aged 55 years or older. Finally, the possibility remains that there is no co-morbidity effect in the association between the *ACE* I/D polymorphism, CVD mortality, and incidence of AD.

In summary, we performed the inverse probability of censoring weighted analyses method for the first time in a population based cohort study. Our results did not provide an evidence for a co-morbidity effect introduced by cardiovascular mortality in association with the *ACE* I/D polymorphism and Alzheimer's disease. The increased risk of AD related to the II genotype appears not to be due to excess of cardiovascular mortality among the DD carriers.

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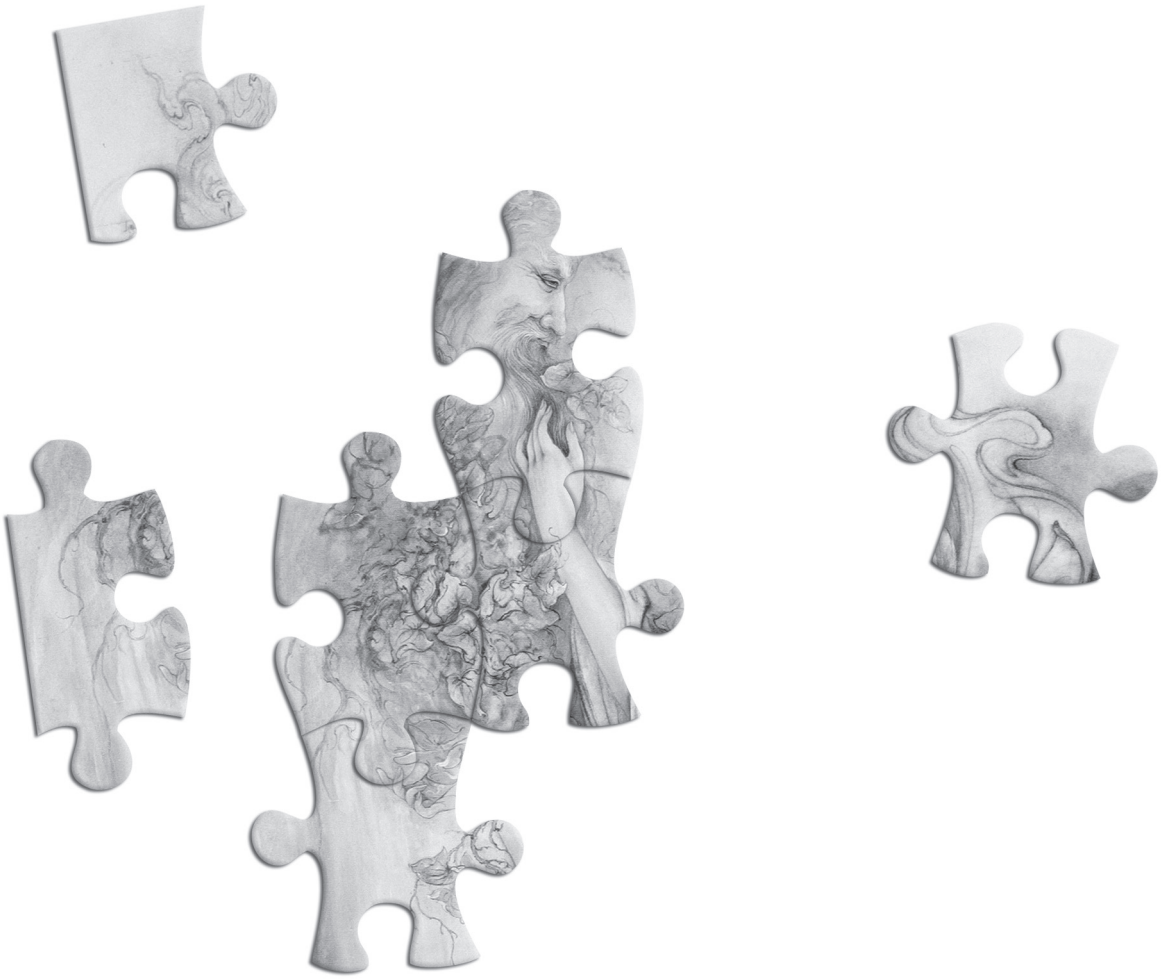

Chapter 5

General discussion

Introduction

Although many developing countries are still fighting against infectious diseases and malnutrition among children, most western countries are moving toward older populations. In the 21st century, diseases of the elderly are more frequently showing their faces, and, among them, atherosclerosis is a key feature. It is the main cause of myocardial infarction, stroke, and several other organ damaging conditions, such as kidney failure. About half of the mortality in the western world and many Asian countries is caused by atherosclerotic diseases.[1]

In the late 15th century, Leonardo da Vinci (1452-1519) described the atherosclerosis condition for the first time. In his book, The Anatomy of the Old Man, he stated, "vessels in the elderly, through the thickening of the tunics, restrict the transit of the blood". It was not, however, until the mid 20th century that epidemiologists tried to reveal its complexity by determining the risk factors involved in this condition. By now, numerous factors have been related to atherosclerosis;[2] some of them are purely environmental, such as smoking, lack of exercise, and high-fat diet. Other risk factors for atherosclerosis are (partly) of genetic origin, which means they are passed from parents to their children. Hypertension, hyperlipidaemia, and diabetes are among these factors.[2] As in other complex disorders, interactions between environmental and genetic risk factors also play an important role in the formation and progression of atherosclerosis.

Known risk factors of atherosclerosis only explain between 20% to 40% of the total variance for this trait.[3,4] This means that a large portion of atherosclerosis risk factors is still unknown. It is expected that these unknown factors consist of environmental and genetic components. Our knowledge of the key genes involved in the development of atherosclerosis is still limited despite the large number of studies conducted to date.[2] The contribution of genetic factors (heritability) can be estimated by measuring atherosclerosis in people who are related to each other. Based on the work performed in this thesis (chapter 2) and the work of other researchers,[5] we conclude that around 30% to 40% of the unknown portion of atherosclerosis risk factors is genetically determined. One important fact, however, is that this proportion could vary between different populations. Considering the complex interaction between genetic and environmental factors, it is expected that a certain genetic factor may not become involved in the process of atherosclerosis unless another gene or environmental factor is present. For instance, genes involved in salt sensitivity will not express in a population with low salt intake. This implies that the contribution of genes to a trait

may differ between populations.

Another characteristic of the arteries, namely arterial stiffness, is also an important factor related to cardiovascular diseases.[6,7] Atherosclerosis and arterial stiffness have been found to be correlated,[8] although the interrelation of the two traits at different stages of atherosclerosis is not completely known. Studies on the genetic factors of arterial stiffness are limited in number. A few candidate genes have been identified so far, most of them focused on genetic polymorphisms in the renin angiotensin system.[9-13] To our knowledge, there has been no published report on the heritability of this trait. In chapter 2, we studied the heritability of arterial stiffness, measured by carotid-femoral pulse wave velocity (PWV), in a genetically isolated population. We found a heritability estimate of about 35% that is comparable to the heritability of carotid intima media thickness (IMT) in the same population.

This thesis also includes some work on one of the genetic risk factors contributing to the genetic complexity of atherosclerosis. We focused on the angiotensin converting enzyme (ACE), which is a key component in the renin angiotensin system and some other physiological systems, by using one of the variations in its gene, the insertion/deletion polymorphism (I/D).

The renin angiotensin system

Renin angiotensin system is a hormonal system that helps to regulate long-term blood pressure and blood volume in the body. Renin is released by the juxtaglomerular cells in the kidneys under conditions of salt or volume loss or sympathetic activation. It cleaves the inactive peptide angiotensinogen (synthesised in the liver), producing angiotensin I, which in turn is converted to angiotensin II by the act of angiotensin converting enzyme.

Angiotensin II has a variety of effects on the body. In the kidneys, it constricts glomerular arterioles, which decreases the glomerular filtration rate and, in turn, raises systemic arterial blood pressure. It also acts on the adrenal cortex, causing the release of aldosterone, which acts on the tubules in the kidneys, allowing them to reabsorb more sodium and water from the urine.[14] These effects directly act to increase the amount of fluid in the blood, making up for a loss in volume, and to increase blood pressure.

In addition, angiotensin II is a potent vasoconstrictor. It also mediates cell growth and the proliferation of vascular smooth muscle cells, cardiomyocytes and coronary endothelial cells by stimulating various

cytokines and growth factors, such as insulin-like growth factor-I and platelet derived growth factor.[15] Furthermore, angiotensin II may induce endothelial dysfunction by reducing nitric oxide biovailability.[16] These findings emphasise the importance of angiotensin II in cardiovascular pathophysiology and stimulate exploration of the role of the genetic polymorphisms present in the renin angiotensin system in the search for genetic factors involved in atherosclerosis and other cardiovascular outcomes.

Angiotensin converting enzyme insertion/deletion polymorphism

ACE is a zinc metallopeptidase widely distributed on the surface of endothelial and epithelial cells. It converts angiotensin I to angiotensin II, the main active product of the renin angiotensin system.[17] At the same time, ACE also plays an important role in the kinin kallikrein system by inactivating bradykinin, which is a strong vasodilator. In the central nervous system (CNS), ACE is also expressed, with the primary function of degrading some of the neurotransmitters involved in the transmission of pain, the regulation of emotions, and the alteration of inflammatory and immune responses.[18,19]

The human *ACE* gene is located on chromosome 17q23 and includes 26 exons. In 1990, Rigat and co-workers published an important report that provided the impetus to further study polymorphisms on this gene.[20] They found a polymorphism involving the presence (insertion, I) or absence (deletion, D) of a 287 bp sequence of DNA in intron 16 of the gene. Interestingly, the mean ACE activity level in DD genotype individuals was about twice that found in II genotype individuals.[20] Subjects with ID genotype had intermediate levels indicating co-dominancy; that is, the effects of both alleles are detectable in heterozygotes. It was hypothesised that the higher ACE levels could result in increased angiotensin I to angiotensin II conversion. Considering the importance of angiotensin II in cardiovascular pathophysiology, it is not surprising that many studies have tried to find an association between the *ACE* I/D polymorphism and different cardiovascular diseases.

In this thesis, we investigated the relation between this polymorphism and atherosclerosis. As the first step, we made an effort to put together the findings from previously published studies in a meta-analysis (chapter 3.1) and we found a positive association between the D allele and carotid IMT in populations who are at high risk of atherosclerosis. This suggests the

presence of some interaction between the D allele and other atherosclerosis risk factors. Based on this finding, we looked for other determinants of ACE plasma level using data from the Rotterdam Study, and we found that smoking increases plasma ACE levels (chapter 3.2). Therefore, we hypothesised that the D allele and smoking might interact to cause atherosclerosis. We did, indeed, find this interaction using IMT as a measurement of atherosclerosis (chapter 3.2). Further support for this interaction was provided when we studied the I/D polymorphism in relation to cardiovascular mortality and we found a higher risk of mortality related to the D allele only among smokers. This association was mainly present in younger subjects in our population (chapter 4.1). We also studied the relation between this polymorphism and arterial stiffness using two different measurements, carotid-femoral PWV and carotid distensibility. No relation between the polymorphism and PWV was found, while the D allele was related to a higher stiffness in the carotid artery. In stratified analyses, the association was stronger in subjects younger than 70 years in this population of individuals who were 55 years or older (chapter 3.3).

Other polymorphisms in the *ACE* gene

The I/D is not the only polymorphism in the *ACE* gene. The National Centre of Biological Information (NCBI) lists more than 160 polymorphisms in this gene, most of which are single nucleotide polymorphisms (SNPs). The I/D polymorphism is located in a non-coding region of the *ACE* gene, meaning that presence or absence of the *alu* repeat is not likely to modify the structure, and thus, the function and activity of the synthesised protein. Many studies investigated the association between the other variants in this gene and plasma ACE levels, and some researchers could narrow down the region where the functional polymorphism is likely to be located.[21,22] However, there is no final agreement as to which variant is responsible for the changes in ACE levels. Although one would like to know the functional polymorphism in this gene, the I/D polymorphism is still useful in association studies. This issue will be explained in more detail in the section on methodological aspects in this chapter.

I/D polymorphism and other complex diseases

Besides relationship between the *ACE* I/D and atherosclerosis related outcomes, we also examined its relationship with total mortality (chapter 4.2). We observed that there is a higher risk of mortality related to the DD genotype, especially at younger ages among smokers. Although this effect modification was similar to our finding on the *ACE* I/D and cardiovascular mortality in chapter 4.1, unpublished data from the Rotterdam Study suggest that this finding cannot singularly be ascribed to the relationship between the polymorphism and cardiovascular mortality. In fact, this observation might be explained by other causes of death, which is supported by a large body of literature on wide range of ACE actions in multiple physiological systems.

In the renin angiotensin system, angiotensin II production is mediated by ACE. Experimental studies demonstrate that angiotensin II promotes angiogenesis, an important determinant in the growth and spread of many human cancers. Angiotensin II induces vascular endothelial growth factor, which plays a pivotal role in tumour angiogenesis and correlates with aggressive behaviour and a poor prognosis.[23,24] In addition, angiotensin II stimulates the production of growth factors such as transforming growth factor-I, platelet-derived growth factor, and basic fibroblast growth factor.[25] Given those observations, many researchers investigated the relationship between the *ACE* I/D polymorphism and several types of cancer, such as prostate, endometrial, and breast cancer.[26-28]

Besides its role in the renin angiotensin system, ACE also plays an important role in the kinin kallikrein system by degrading bradykinin, a powerful vasodilator. In skeletal muscle tissue, a local kinin kallikrein system exists that liberates kinins and express bradykinin B-2 receptors.[29] Bradykinin infused at physiological doses produces an endothelial dependent increase in muscle blood flow and glucose extraction rates.[30] Recent studies have suggested that the I allele of the *ACE* I/D polymorphism might be associated with some aspects of endurance performance, being found with increased frequency in elite distance runners,[31] rowers[32] and mountaineers.[33] A recent case-control study also suggested that DD carriers are eight times more likely to develop chronic fatigue syndrome than those with the II genotype,[34] suggesting a lower metabolic efficiency in DD carriers.

There are still other functions associated with ACE. In the CNS, ACE is responsible for degeneration of a family of neurotransmitters known as neurokinins, which appear to play a key role in the regulation of

emotions.[18,19] Although the role of ACE in degenerating these neurotransmitters is not always replicated,[35] it has triggered studies on the *ACE* I/D polymorphism and neurological diseases such as depression,[36-38] other affective disorders,[39] and Parkinson disease.[40] The role of ACE in the CNS, however, is not limited to these neurotransmitters. It has been demonstrated that ACE degrades amyloid beta peptide *in vitro*,[41] one of the primary adverse biological agents implicated in Alzheimer's disease (AD) pathogenesis.[42,43] Thus, if present in sufficient levels *in vivo*, ACE may preclude or lessen the formation of senile plaques, which are neuropathological hallmarks of AD. A recent large-scale study involving multiple markers spanning *ACE*, in conjunction with a meta-analysis of previously published data on the I/D polymorphism, supports the hypothesis that the I allele contributes to AD.[44]

The *ACE* I/D has also studied with respect to many other diseases, with various theories on the possible pathways. To mention them briefly, the role of the *ACE* polymorphism has been examined in relation to conditions such as insulin resistance,[45-47] asthma, [48,49] pneumonia,[50] sarcoidosis,[51] and systemic lupus erythematosus.[52,53]

Methodological aspects

Causality and linkage disequilibrium

Association studies, by their nature, may show a statistical correlation between a given polymorphism and a certain phenotype (a change in the level of an enzyme, risk of developing a disease, etc.). From a pathogenetic point of view, however, this does not prove a causal relationship. If a non-functional allele under study and the allele that cause the phenotype are not physically close to each other, they are in "linkage equilibrium" and inherited independently according to Mendelian laws. If, however, the distance between these DNA segments is close enough, it results in "linkage disequilibrium", a situation in which the allele under study does appear together with the phenotype more often than would be expected according to the allelic distribution in the population. In other words, the non-functional polymorphism may act as a marker for the mutation responsible for the phenotype.

In Caucasian populations, most of the polymorphisms on the *ACE* gene are in close linkage disequilibrium with the I/D polymorphism. The fact that the I/D may not be the functional polymorphism could only dilute the effect and increase the chance of false negative results in association studies.

Despite this, the positive association between the *ACE* I/D polymorphism and atherosclerosis (or other outcomes) could still be an important finding even if this polymorphism is only a marker flagging the functional mutation.

Cause versus consequence

An important aspect of studying the relation between candidate genes and (patho)physiological conditions is that the temporal relationship is mostly unharmed. Environmental or physiological factors may have changed due to a disease condition and, in most epidemiological designs, such as cross-sectional or case-control studies, it is extremely difficult to distinguish between the causes and consequences of the disease. A cohort or a nested case-control study design, of course, gives us the opportunity to measure the risk factors before the outcome has occurred. With regard to complex diseases that develop in an elderly population, however, the subclinical processes of the disease generally start long before the disease becomes manifest. In this situation, defining a temporal relationship between the risk factor and the disease becomes problematic. The genetic make-up of a person, however, is unique throughout his life. In other words, genetic risk factors are not altered by any disease status, which is one of the advantages of genetic association studies. The only point of concern is that selection may have occurred based on genotypes in a study on prevalent patients. This problem can be overcome by using only the incident cases in a study.

Co-morbidity effect

A possible source of bias, which also occurs in studies using incident cases, is the presence of a co-morbidity effect. In an association study on a given risk factor and a certain disease, the presence of selective censoring could be a cause of bias. The association between ACE and multiple diseases on the one hand, and the common pathophysiological pathways of these diseases on the other hand, provided us with the opportunity to study the *ACE* I/D polymorphism in relation to Alzheimer's disease as an example of such a situation. The evidence of an association between atherosclerosis and AD[44] and the possible relationship between the D allele and cardiovascular diseases,[54] lead to the hypothesis that the observed association between the *ACE* I allele and AD could be due to selective censoring of carriers of the D allele due to cardiovascular mortality. In chapter 4.3, we used the method of weighted analyses using the inverse probability of censoring to evaluate this problem. We compared the standard (un-weighted) and corrected (weighted) risk estimates using the inverse probability of censoring analyses

and we observed no differences in estimated risks, indicating that the relation between the I allele and AD is not likely to be due to a selective censoring of the cases with cardiovascular mortality among the DD carriers.

Genetic research in isolated populations

Due to the complexity of the genetic basis of atherosclerosis, any attempt to eliminate part of that complexity may eventually lead to more feasible approaches toward understanding this disorder. One way to accomplish this is to use genetically isolated populations, an idea that has emerged over the last few years. The general assumption is that an isolated population is descended from a limited number of founders and, therefore, genetic heterogeneity in a sample from such a population is reduced compared with a sample from the general population. In this thesis, we presented the first report on atherosclerosis in the Erasmus Rucphen Family (ERF) study, an ongoing study in an isolated population in the southwest of The Netherlands. Until now, we only studied the heritability estimates of atherosclerosis and arterial stiffness. The purpose of the ERF study is to search for new genetic factors in these traits by the use of extensive genealogy data, which allow us to conduct family based studies. Candidate gene studies as well as a genome wide screen will be performed in this design. In addition, when a new genetic risk factor is found in this isolated population, we can determine the contribution of the gene to the trait in the general population by using the design of the Rotterdam Study.

Multiple testing

Recently, automation of laboratory techniques has made it possible to genotype large number of SNPs from all over the genome very quickly and accurately. Using the information from these polymorphisms in the whole genome and testing the association between each of them and a selected outcome is a very tempting idea. By performing large number of statistical tests with no other a priori hypothesis than that a polymorphism is involved, the possibility increases that the positive findings are simply due to chance.[55] One method for dealing with this scenario is to correct for multiple testing by known statistical methods, such as the Bonferroni correction. A point of concern is the chance of false negative findings. Using a Bonferroni correction, a larger sample size is needed in order to execute a study with sufficient statistical power. An alternative way might be to test the significance of associations using conventional statistical significance levels and, in a second step, make an attempt to replicate the positive findings in

a second population. In this way, we decrease the chance of false negative findings at the cost of a higher chance of false positives that will be judged in the replication stage.

Future studies

In this thesis, we observed associations between the D allele and IMT, arterial stiffness, cardiovascular mortality, and total mortality. Although a lot of work has been done,[56,57] it is clear that we are in great need of more basic physiological studies that investigate the relation between the *ACE* gene and atherosclerosis, especially with regard to possible interactions. The pathophysiological mechanism underlying the interaction between the D allele and smoking that has been found in the studies comprising this thesis has yet to be evaluated. Experimental studies utilizing animal models may show if this interaction is due to an increased level of ACE and an alteration in the renin angiotensin system, or whether it works through other pathophysiological pathways. Furthermore, the findings of this thesis on the relationship between the *ACE* I/D and atherosclerosis, cardiovascular endpoints, or other clinical outcomes need to be replicated. Especially our findings on the interaction between the D allele and smoking, which has not been reported before, needs to be confirmed in other studies. The only way to show if these are real effects or false positive findings is to replicate them with a different study sample. In the next step, the role of other genetic factors that contribute to the renin angiotensin system or the kinin kallikrein system should be studied. Proteins that are involved in a single system (or inter-connected systems) are more likely to modify the effects of each other. Studying the interactions between the genetic variations responsible for changes in the function or level of these proteins is a plausible research plan.

Genetic association studies can be done in a cohort study design in a general population (like the Rotterdam Study) as well as in genetically isolated populations (like the ERF study). A case-control design might be a good alternative if incident cases are used to avoid a possible selection bias. Nested case-controls studies, therefore, are preferable. Considering the relatively small effect of each polymorphism and the multiple subgroups that should be examined for possible interactions, studies with large sample sizes are needed in order to have enough power to detect any interaction. In addition, it will be very useful to perform a systematic literature review (for example by using a meta-analysis), before the start of a new association

study. It may provide useful information to build up an accurate a priori hypothesis.

Another important issue when conducting a candidate gene association study is to choose the appropriate polymorphisms in the candidate genes. Since the completion of the Human Genome Project, a huge amount of information on SNPs throughout the genome has become available. Although some of those SNPs may show functional properties, it might be a haplotype (combination of several SNPs) that indicates the changes of the protein in a more precise way. Using only one SNP or an inappropriate combination of SNPs will lead to a misclassification of the risk factor and increase the chance of false negative findings.

Besides studies on candidate genes, it is, of course, interesting to know more about unknown genetic factors involved in cardiovascular diseases. As mentioned earlier, arterial stiffness is a trait that has been related to different cardiovascular outcomes but few studies have been performed on genetic risk factors for this trait. We made the first step toward a better understanding of the genetic basis of this trait by reporting, for the first time, the heritability of carotid-femoral PWV in an isolated population. This heritability estimate was close to the heritability of IMT. This finding supports the search for genetic determinants of arterial stiffness as well as for atherosclerosis.

In recent years, there has also been increasing interest in pharmacogenomics.[58] Studies aiming to identify genes involved in medication response conducted to date have typically followed a population based approach.[59,60] The lower genetic complexity of an isolated population and the availability of extensive genealogical data will provide us with unique opportunities to conduct research with a family based approach to localise and identify new genes, such as genes involved in patients' response to medication.[61] If there are genes involved in a patients' response to medications, these genes will segregate in families. As a consequence, non-response to treatment will cluster in families and we should be able to detect the genes involved using classical family based approaches such as linkage analysis. A proof of principle of this design will be to find genes that are involved in response to antihypertensive treatment in the ERF study.

Vast amounts of work will be necessary to grasp the whole picture of a complex disease such as atherosclerosis. Modern laboratory techniques, super computers and new statistical packages are important tools for the future that should be used carefully. In lay terms, using these tools is like driving a fast car on a highway: a careless driver will harm both himself and the others on the road.

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Summary

Summary

Atherosclerosis, a progressive disorder of the large arteries, is the major factor in the pathophysiology of cardiovascular diseases. Around half of all the deaths in western society is due to these diseases. Knowing the risk factors involved in the formation and progress of atherosclerosis will eventually lead to a better understanding of this pathophysiological condition and, thereby, better ways to prevent or treat it. In the last 50 years, many epidemiological studies attempted to discover the risk factors for atherosclerosis. Many such factors have been discovered; some are purely environmental and others have important genetic components. In this thesis we worked on the genetic basis of atherosclerosis, mainly by focusing on one of the famous genetic polymorphisms, the angiotensin converting enzyme (*ACE*) insertion/deletion (I/D) polymorphism.

Chapter 1 is a general introduction on known aspects of atherosclerosis and the different kinds of measurements that have been used in this thesis. The concept of genetics in atherosclerosis is discussed and some basic information about the *ACE* I/D polymorphism is given.

In chapter 2, we present heritability estimates, showing the extent to which genetic factors contribute to different measures of arterial wall structure and function. We used carotid-femoral pulse wave velocity (PWV) as an indicator of arterial stiffness, and carotid intima media thickness (IMT) and plaque score as indicators of atherosclerosis. The analyses were performed on a sample from a genetically isolated population in the southwest of The Netherlands, participants in the Erasmus Rucphen Family (ERF) study. ERF is an ongoing study conducted among 2500 participants from the isolate who belong to a single large pedigree. This pedigree was not selected on the basis of any disease status. The results presented in chapter 2 are the first report on atherosclerosis from the ERF study, using data from 930 participants available at the time of the analyses. Heritability estimates of PWV, IMT, and carotid plaques are presented. Age and gender adjusted heritability estimates were 0.36 for PWV, 0.41 for carotid IMT, and 0.28 for plaque score. After adjustment for appropriate risk factors, the heritabilities were 0.26, 0.35, and 0.21 for PWV, IMT and plaque score, respectively. All heritability estimates were statistically significant. To our knowledge, this is the only report on the heritability of PWV, which is quite similar to the heritability estimates of IMT and plaque score in our study. These findings suggest that the search for genes responsible for arterial stiffness might be as valuable as the search for genes involved in atherosclerosis.

Chapter 3 consists of studies on the *ACE* I/D polymorphism and atherosclerosis measurements. Previously published studies on the relationship between the *ACE* I/D and carotid IMT measurements have been inconsistent. In chapter 3.1, we present a meta-analysis that shows the results of the previous studies combined. Based on those results, there is a stronger association among the purportedly "high-risk" populations. After making that observation, we performed an association study using data from the Rotterdam Study, which is presented in chapter 3.2. In a combined functional and population based approach, we showed evidence for an interaction between smoking and the D allele on IMT. Further, in chapter 3.3, we present the association between the *ACE* I/D and measurements of arterial stiffness in the same population. This study shows that there is a positive relation between the D allele and carotid stiffness.

In chapter 4, associations between the *ACE* I/D and clinical outcomes are detailed. We investigated the relationship between the I/D polymorphism and morbidity and mortality due to cardiovascular diseases in chapter 4.1. Although there is no significant association between the polymorphism and cardiovascular morbidity, carriers of the DD genotype show an increased risk of mortality as a result of cardiovascular diseases among current smokers. This association is mainly present in younger age groups. In addition, we used total mortality as the outcome of interest and observed the same finding among smokers at younger ages. We present these results in chapter 4.2. A methodological study is presented in chapter 4.3 using the inverse probability of censoring weighted analyses to investigate a possible co-morbidity effect. The plausibility of selective censoring by cardiovascular mortality is tested in regard to the association between the *ACE* I/D and Alzheimer's disease. The standard and corrected analyses show no differences, suggesting that a co-morbidity effect is not playing a role in that association.

Finally, chapter 5 provides a general discussion on the *ACE* gene, the linkage between the I/D and other polymorphisms in this gene, the relationship between the I/D and other complex diseases, some methodological aspects, and ideas for the future studies.

Samenvatting (summary in Dutch)

Atherosclerose, een progressieve aandoening van de grote arteriën, is de belangrijkste factor in de pathofysiologie van cardiovasculaire ziekten. Ongeveer de helft van alle sterfte in de Westerse wereld is het gevolg van deze ziekten. Het identificeren van de risico factoren betrokken bij het ontstaan en de progressie van atherosclerose, zou uiteindelijk kunnen leiden tot een beter inzicht in de pathofysiologie van deze aandoening, waardoor betere behandeling en preventie mogelijk worden. In de laatste 50 jaar hebben veel epidemiologische studies een poging gedaan de risico factoren voor atherosclerose op te sporen. Vele factoren zijn ontdekt, waaronder pure omgevingsfactoren, maar ook factoren met een belangrijke genetische component. In dit proefschrift hebben we de genetische basis van atherosclerose onderzocht, voornamelijk door ons te richten op één zeer bekend genetische polymorphisme, het angiotensin converting enzyme insertie/deletie (*ACE* I/D) polymorphisme.

Hoofdstuk 1 is een algemene introductie over de bekende aspecten van atherosclerose en de verschillende soorten metingen die in dit proefschrift gebruikt zijn. Het concept van de genetica in atherosclerose wordt besproken en algemene informatie over het *ACE* I/D polymorphisme wordt gegeven.

In hoofdstuk 2 hebben we de erfelijkheid berekend van verschillende maten van vaatwandstructuur en -functie, waarmee we laten zien in hoeverre genetische factoren hierin een rol spelen. Wij gebruikten de carotid-femoral pulse wave velocity (PWV) als een indicator voor vaatstijfheid en intima media thickness (IMT) van de carotiden en plaque score als indicatoren voor atherosclerose. De analyses werden uitgevoerd in deelnemers aan de Erasmus Rucphen Familieonderzoek (ERF). Dit is een studie uitgevoerd onder 2500 deelnemers, woonachtig in een genetisch geïsoleerd gebied in het Zuidwesten van Nederland, die allen deel uitmaken van één grote stamboom. Deze stamboom is niet geselecteerd op basis van een ziekte. De uitkomsten in hoofdstuk 2 zijn de eerste resultaten van de ERF studie, waarbij data van 930 deelnemers gebruikt is, welke beschikbaar waren op het moment van de analyses. De erfelijkheid van PWV, IMT en plaque score wordt gepresenteerd. De voor leeftijd en geslacht gecorrigeerde schatting van de erfelijkheid was 0.36 voor PWV, 0.41 voor carotid IMT en 0.28 voor plaque score. Na correctie voor andere risico factoren waren deze schattingen 0.26, 0.35 en 0.21 voor respectievelijk PWV, IMT en plaque score. Alle schattingen waren statistisch significant. Voor zover ons bekend, is dit het enige verslag van de erfelijkheid van PWV, welke in grote mate gelijk is aan de schattingen van de erfelijkheid

van IMT en plaque score in onze studie. Deze bevinding suggereert dat het zoeken naar genen verantwoordelijk voor vaatstijfheid van even grote waarde kan zijn als het zoeken naar genen voor atherosclerose.

Hoofdstuk 3 beschrijft studies over het *ACE* I/D polymorphisme en atherosclerose metingen. Eerdere gepubliceerde studies over de relatie tussen *ACE* I/D en IMT van de carotiden lieten inconsistente resultaten zien. In hoofdstuk 3.1 presenteren we een meta-analyse die al deze resultaten combineert. Gebaseerd op dit resultaat blijkt er een sterkere associatie te bestaan in de zogenaamde "hoge-risico" populaties. Uitgaande van deze bevinding hebben we een associatie studie gedaan met data van de Rotterdam Studie, welke beschreven staat in hoofdstuk 3.2. In een gecombineerd functioneel en populatie onderzoek, vonden we aanwijzingen voor interactie tussen roken en het D allel op IMT. Verder presenteren we in hoofdstuk 3.3 de associatie tussen het *ACE* I/D polymorphisme en metingen van vaatstijfheid in dezelfde populatie. Deze studie laat zien dat er een positieve relatie is tussen het D allel en vaatstijfheid.

In hoofdstuk 4 beschrijven we de associaties tussen het *ACE* I/D polymorphisme en klinische gevolgen. We onderzochten in hoofdstuk 4.1 de relatie tussen *ACE* I/D, morbiditeit en mortaliteit als gevolg van cardiovasculaire ziekten . Er was geen significante associatie tussen het polymorphisme en cardiovasculaire morbiditeit, echter, onder rokers vonden wij dat personen met het DD genotype een verhoogd risico hebben op sterfte als gevolg van cardiovasculaire ziekten. Deze associatie was voornamelijk aanwezig in de jongere leeftijdsgroep. Verder vonden we dezelfde associatie wanneer we onder jonge rokers de totale sterfte als eindpunt namen. Deze resultaten presenteren wij in hoofdstuk 4.2. In hoofdstuk 4.3 beschrijven we een methodologische studie waarin we gebruik maakten van de "inverse probability of censoring weighted analyses" om het effect van co-morbiditeit te onderzoeken. De mogelijkheid van selectieve censoring bij cardiovasculaire sterfte werd getest met betrekking tot de associatie tussen *ACE* I/D en de ziekte van Alzheimer. De standaard en gecorrigeerde analyses lieten geen verschil zien, wat suggereert dat het effect van co-morbiditeit geen rol speelt in deze associatie.

Tenslotte beschrijft hoofdstuk 5 een algemene discussie over het *ACE* gen, linkage met andere polymorphismen en het I/D polymorphisme in dit gen, de relatie tussen het I/D polymorphisme en andere complexe ziekten en een aantal methodologische aspecten en ideeën voor toekomstige studies.

«پر خطر» رابطه محکم‌تری بین این پلی‌مرفیسم و IMT وجود دارد. با داشتن این یافته و با استفاده از اطلاعات Rotterdam Study، مطالعه‌ای را انجام دادیم که در فصل ۲-۳ نشان داده شده است. این مطالعه مدارکی از تداخل عمل آلل D از ژن *ACE* و مصرف سیگار بر روی کاروتید IMT را نشان می‌دهد. بعد از آن، در فصل ۳-۳، رابطه بین I/D *ACE* و اندازه‌گیری‌های مختلف از سفتی سرخرگ در همان نمونه جمعیتی ارائه شده است، که نشان می‌دهد یک رابطه مثبت بین آلل D و سفتی رگ کاروتید وجود دارد.

در فصل ۴، رابطه بین I/D *ACE* و یافته‌های بالینی گزارش شده است. رابطه بین پلی‌مرفیسم I/D و ابتلا و میرائی از بیماری‌های قلب و عروق در فصل ۱-۴ بررسی شده است. اگرچه هیچ رابطه معنی‌داری بین پلی‌مرفیسم و ابتلا به سکته قلبی وجود ندارد، در بین افراد سیگاری کسانی که ژنوتیپ DD را دارند افزایشی در میرائی از بیماری‌های قلب و عروق را نشان می‌دهند. این رابطه اساساً در گروه سنی جوان‌تر دیده می‌شود. در مطالعه بعدی، مرگ و میر کلی را به عنوان هدف در نظر گرفتیم و همان نتیجه را در بین افراد سیگاری در گروه افراد جوان‌تر مشاهده کردیم. این نتایج در فصل ۲-۴ ارائه شده است. یک مطالعه متودولوژیک با استفاده از روش inverse probability of censoring weighted analyses برای بررسی اثر ابتلای همزمان به دو بیماری در فصل ۳-۴ آورده شده است. در این فصل، تأثیر حذف شدن انتخابی به دلیل میرائی از بیماری‌های قلب و عروق را در رابطه بین I/D *ACE* و بیماری آلزایمر بررسی کرده‌ایم. در این مطالعه، نتایج حاصل از آنالیزهای استاندارد و تصحیح شده هیچ تفاوتی را نشان ندادند، به این معنا که اثر ابتلای همزمان نقشی در رابطه بین I/D و آلزایمر بازی نمی‌کند.

در پایان، فصل ۵ یک بحث عمومی است که مطالبی درباره ژن *ACE*، اتصال (linkage) بین I/D و پلی‌مرفیسم‌های دیگر در این ژن، رابطه بین I/D و دیگر بیماری‌ها، چند مورد متودولوژیک، و ایده‌هائی درباره مطالعات آینده را شامل می‌شود.

خلاصه (summary in Persian)

سختر گی یا تصلب شرایین (atherosclerosis) یک عارضه پیشرونده در سرخرگ‌های بزرگ بدن است و یکی از عوامل مهم در پاتوفیزیولوژی بیماری‌های قلب و عروق به شمار می‌آید. حدود نیمی از مرگ و میر در جوامع غربی به واسطه این بیماری‌ها اتفاق می‌افتد. دانستن اینکه چه عواملی در ایجاد و پیشرفت سختر گی دخیل هستند میتواند به درک بهتر این عارضه و در نتیجه یافتن راه‌هائی برای پیشگیری یا معالجه آن کمک کند. در طی ۵۰ سال گذشته، بسیاری از مطالعات اپیدمیولوژیک تلاش کرده‌اند تا عوامل مؤثر در سختر گی را شناسائی کنند. بسیاری از این عوامل تاکنون شناخته شده‌اند: بعضی از آنها کاملاً محیطی هستند و برخی دیگر منشأ ژنتیکی دارند. در این پایان‌نامه ما منشأ ژنتیکی سختر گی را بررسی کرده‌ایم؛ با تمرکز بر روی یکی از جهش‌های ژنتیکی معروف، به نام جهش insertion/deletion (I/D) در ژن (*ACE*) angiotensin converting enzyme .

فصل ۱ مقدمه‌ای کلی درباره دانش کنونی از سختر گی است، به همراه توضیحاتی درباره روش‌های مختلفی که برای اندازه‌گیری سختر گی در این پایان‌نامه به کار رفته است. مفهوم اثر ژنتیک در سختر گی توضیح داده شده و اطلاعاتی کلی درباره جهش *ACE* I/D بیان شده است.

در فصل ۲ مقادیر وراثت‌پذیری ارائه شده است، یعنی میزانی که عوامل ژنتیکی در ساختمان و عمل سرخرگ‌ها تأثیر دارند. ما از سرعت حرکت نبض بین سرخرگ‌های کاروتید و فمور (Pulse wave velocity, PWV) به عنوان شاخصی از سفتی سرخرگ (arterial stiffness) و از ضخامت دیواره سرخرگ کاروتید (intima media thickness, IMT) به عنوان شاخصی برای سختر گی استفاده کردیم. این تحقیق بر روی نمونه‌ای از یک جامعه منزوی (isolated population) در جنوب غربی هلند، که در مطالعه Erasmus Rucphen Family (ERF) شرکت کرده‌اند، انجام گرفته است. مطالعه ERF یک تحقیق در حال انجام است که ۲۵۰۰ نفر از این جامعه منزوی را شامل می‌شود. این افراد همگی اعضای یک خانواده بزرگ هستند و هیچکدام بر اساس بیماری خاصی انتخاب نشده‌اند. نتایجی که در فصل ۲ ارائه شده، اولین گزارش از مطالعه ERF درباره سختر گی است، که بر اساس اطلاعات مربوط به ۹۳۰ نفر که در زمان انجام این تحقیق در دسترس بودند انجام گرفته است. این مطالعه شامل مقادیر وراثت‌پذیری مربوط به PWV، IMT، و پلاک‌های کاروتید می‌شود. مقادیر وراثت‌پذیری کنترل شده برای سن و جنس، ۳۶٪ برای PWV، ۴۱٪ برای IMT، و ۲۸٪ برای پلاک‌های کاروتید بودند. بعد از کنترل برای دیگر عوامل خطر، اندازه های وراثت پذیری برای PWV، IMT، و پلاک‌های کاروتید به ترتیب عبارت بودند از ۲۶٪، ۳۵٪، و ۲۱٪. تمامی مقادیر وراثت‌پذیری از نظر آماری معنی‌دار بودند. تا آنجائی که ما اطلاع داریم، این اولین گزارش از وراثت‌پذیری PWV است، که در این مطالعه به مقادیر وراثت‌پذیری IMT و پلاک‌های کاروتید بسیار نزدیک است. این یافته‌ها به این معنا است که جستجو برای ژن‌های مؤثر در سفتی سرخرگ می‌تواند به اندازه جستجو برای ژن‌های مؤثر در سختر گی ارزشمند باشد.

فصل ۳ شامل مطالعاتی در مورد اثر پلی‌مرفیسم *ACE* I/D بر روی اندازه‌گیری‌های مختلف از سختر گی است. مطالعات مربوط به *ACE* I/D و کاروتید IMT که قبلاً منتشر شده‌اند یافته‌های یکسانی ندارند. در فصل ۱-۳ ما یک متاآنالیز انجام داده‌ایم که یافته‌های مطالعات قبلی را با هم تلفیق می‌کند. بر اساس نتایج این مطالعه، در جمعیت‌های به اصطلاح

Appendix

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Back to the Monday meetings, I will skip the GE staff meeting that we have every other week, since I have already mentioned all of its members. The day, however, is not over! There is still the weekly seminar of the Department of Epidemiology & Biostatistics at 16:00. This is a good opportunity to thank all of my colleagues on the 21st floor. Special thanks go to prof.dr. Albért Hofman, whose friendliness and enthusiasm always gives a positive atmosphere to the whole research group. Special thanks also go to prof.dr. Jacobus Lubsen who introduced me to Bert Hofman for the first time; this was the start of my whole new career. Going back in time, my thanks go to dr. Jose Luis Santos, dr. Fernando Rivadeneira, and Carolina Pardo, dear friends of mine from the first year of study, and dr. Pedram Sendi, with whom I spent many unforgettable joyous moments.

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List of abbreviations

ACE:	angiotensin converting enzyme
ACE:	angiotensin converting enzyme gene
AD:	Alzheimer's disease
ANOVA:	analysis of variance
BP:	blood pressure
CHD:	coronary heart disease
CI:	confidence interval
CVD:	cardiovascular diseases
CNS:	central nervous system
D_C:	co-morbidity disease
D_I:	disease of interest
DNA:	deoxyribonucleic acid
ERF:	Erasmus Rucphen Family study
h^2:	heritability
HDL:	high density lipoprotein
HWE:	Hardy-Weinberg equilibrium
I/D:	insertion/deletion polymorphism
ICD:	the international classification of diseases
IMT:	intima media thickness
LDL:	low density lipoprotein
MAP:	mean arterial pressure
MI:	myocardial infarction
MICE:	multivariate imputation by chained equations
NCBI:	the national centre of biological information
NO:	nitric oxide
PCR:	polymerase chain reaction
PWV:	pulse wave velocity
SE:	standard error
SNP:	single nucleotide polymorphism
WMD:	weighted mean difference