Genetics and cardiovascular risk in a primary care population : studies from the HIPPOCRATES project

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**Genetics and cardiovascular risk
in a primary care population
Studies from the HIPPOCRATES project**

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**Genetics and cardiovascular risk
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PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof. mr. G.P.M.F. Mols
volgens besluit van het College van Decanen in het openbaar te verdedigen
op vrijdag 29 mei 2009 om 14.00 uur

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de morgen breekt aan
de stormen van vannacht zijn
in sneeuw begraven
(Shiro)

*Voor Ma
Ter herinnering aan Pa
Aan Leen*

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List of abbreviations

ACE	angiotensin-converting enzyme
AGT	angiotensinogen
ADD1	alpha adducin
AGTR1	angiotensin II type 1 receptor
AngI	angiotensin I
AngII	angiotensin II
ANOVA	analysis of variance
AUC	area under the curve
BMI	body mass index
CBS	statistics Netherlands
CIMT	carotid intima media thickness
CVD	cardiovascular disease
DBP	diastolic blood pressure
DM	diabetes mellitus
DNA	desoxy-ribonucleic acid
GNB3	G-protein $\beta 3$ subunit
GP	general practitioner
FHQ	family history questionnaire
HDL	high-density lipoprotein
HGP	human genome project
HR	heart rate
ICPC	international classification of primary care
I/D	insertion/deletion
IHD	ischemic heart disease
LDL	low-density lipoprotein
mRNA	messenger ribonucleic acid
RNH	registration network general practices
NOS3	endothelial nitric oxide synthase
OR	odds ratio
PCR	polymerase chain reaction
PP	pulse pressure
PHARMO RLS	PHARmacoMOrbidity record linkage system
PWV	pulse wave velocity
RAS	renin-angiotensin system
ROC	receiver operating characteristic
SBE	multiplex single base extension
SBP	systolic blood pressure
SD	standard deviation
SEM	standard error of the mean
SNP	single nucleotide polymorphism

Chapter 1

INTRODUCTION

Introduction

Cardiovascular genetics

Atherosclerotic cardiovascular disease (CVD) - involving the heart, brain and peripheral arteries of the legs - is a major health problem worldwide.^{1,2} The aetiology of CVD is multifactorial, with both genetic and environmental factors playing important roles. Several monogenetic disorders contribute to CVD, such as familial hypercholesterolemia and hypertrophic cardiomyopathy.^{3,4} Although most of these monogenetic mutations are rare, they are associated with an elevated cardiovascular risk. Moreover, the most common forms of CVD are believed to be multifactorial, resulting from the interaction of several genes, each with a relatively small effect, working alone or in combination with other modifiers, genes and/or environmental factors. Little is currently known about which genes or mutations therein are involved in atherosclerosis. However, there is accumulating evidence that a positive family history for CVD in a first-grade family member is associated with an increased cardiovascular risk in other (affected) family members.⁵⁻⁷ Therefore, there is active research (predominantly epidemiologic studies) aimed at elucidating the combination of genes or genetic factors that play active roles in the aetiology of CVD.

Genetic epidemiology involves the aetiology, distribution and control of disease in groups of relatives and also in populations.^{8,9} Major topics considered in the genetic epidemiology of CVD include cholesterol and lipid levels, blood coagulation, leukocyte adhesion, endothelial cells of blood vessels, the renin-angiotensin system, homocysteine and left ventricular hypertrophy.

Intermediate endpoints

The role of the renin-angiotensin system (RAS) in the development of CVD has been studied for several years at the Department of Internal Medicine, Maastricht University Medical Center. Figure 1.1 presents a schematic of the RAS, which is an important system involved in blood pressure regulation, cardiovascular cellular growth and remodelling, and thus also in the aetiology of atherosclerotic vascular disease. Many association studies have connected specific genes of the RAS with myocardial infarction, hypertrophic cardiomyopathy, hypertension, left ventricular hypertrophy and stroke.¹⁰⁻¹² Other association studies have found correlations between certain polymorphisms of RAS genes and so-called intermediate vascular endpoints, such as carotid intima media thickness (CIMT) and arterial stiffness¹³⁻¹⁵ as assessed by pulse wave velocity (PWV) measurements. CIMT measurements provide a non-invasive assessment of the (preclinical) atherosclerotic burden caused by cardiovascular risk factors such as hypertension, diabetes mellitus and hypercholesterolemia.¹⁶ Non-invasive PWV measurements have revealed increased systolic and pulse pressures and, consequently, increased arterial stiffness.¹⁷ Both

increased CIMT and PWV have been indicated as independent predictors of future CVD.^{16,17}

Figure 1.1 The renin-angiotensin system and several associated polymorphisms.

Polymorphisms

Polymorphisms are frequently occurring variations in the humane genome. By definition, the frequency of the allele with the lowest prevalence is higher than 1%, but the occurrence can vary between populations.⁹ The Department of Internal Medicine of Maastricht University Medical Center has investigated the following polymorphisms related to the RAS in hypertensive and normotensive patients:^{18–21} angiotensin II (AngII) type 1 receptor [AGTR1 (A1166C)], angiotensinogen [AGT (M235T)], angiotensin-converting enzyme [ACE (4656_{pt})], endothelial nitric oxide (NO) synthase [NOS3 (E298D)], G-protein $\beta 3$ subunit [GNB3 (C825T)] and α -adducin [ADD1 (G460W)].²² Figure 1.1 depicts the location of these polymorphisms, which function as follows: Patients homozygous for the C allele of the AGTR1 polymorphism exhibit increased sensitivity to AngII, which can - in combination with the DD genotype of the ACE (I/D) polymorphism - lead to an increased risk of cardiovascular complications.^{23,24}

The T allele of the AGT polymorphism is associated with high plasma levels of AGT and increased responsiveness to AngII.¹¹ The ACE (I/D) polymorphism was determined by detecting ACE_{4656+CT}. The ACE_{4656+CT} polymorphism in the 3'-untranslated region of the ACE gene consists of a repetition of two or three CT dinucleotides and is in complete linkage disequilibrium with the ACE (I/D) polymorphism.^{25,26} Increased ACE levels have been found in the presence of a 2 allele which is comparable to the D allele of the I/D polymorphism, and they have been associated with an increased risk of cardiovascular complications.²⁷ The NOS3 (E298D) polymorphism is associated with reduced NO production in endothelial cells of the arterial wall, inducing elevation of the arterial pressure.^{19,28} The T allele of the GNB3 (C825T) polymorphism is associated with increased intracellular signal transduction that stimulates the Na⁺/H⁺ exchanger²⁹ to increase Na⁺ transport across renal tubules and cause chronic volume expansion that might lead to proliferation of vascular smooth muscle cells, vascular remodelling and increased peripheral arterial resistance.³⁰ Finally, the ADD1 (G460W) polymorphism is of interest because the rare allele is also associated with increased Na⁺ transport, though in this case the Na⁺/K⁺ exchanger is stimulated, which also causes chronic volume expansion with related cardiovascular complications.³¹

Patient care

Collins *et al.* (1999) stated that genomics research will dramatically accelerate the development of new strategies for the diagnosis, prevention and treatment of disease, not just for single-gene disorders but for most of the more common complex diseases, such as CVD.³² It was thought that the completion of the Human Genome Project would lead naturally to the clinical application of medical genetics in both hospital and general practice. The clinical meaning of both diagnostics and therapeutics is strongly focused on the function of genes in both pathophysiological (e.g. CVD) and healthy circumstances. As a consequence, the expectation is that genetic knowledge will eventually have a major impact on daily clinical decision-making by every doctor. Therefore, doctors need to know the genetic background of a disease, not only for the treatment and prevention in an individual patient, but also as a consequence in all first-degree family members.

Hypothesis

At the start of our studies, we reasoned that the above-mentioned polymorphisms might be relevant individually or in combination as additional risk factors for CVD. We hypothesized that these polymorphisms would lead to better cardiovascular risk stratification, not only for an individual but probably also for all first-degree family members. Furthermore, it might offer opportunities for genetically tailored treatments. To explore this hypothesis, we studied the relationships between this set of polymorphisms and biological (risk factors) and clinical (intermediate endpoints)

cardiovascular outcome parameters in a non-selected patient population. This led to the initiation of the HIPPOCRATES project.

HIPPOCRATES

The **H**ypertension: **I**nteraction and **P**revalence of **P**OLymorphisms related to **C**ardiovascular **R**isk and the **A**ssociation to **T**reatment **E**fficacy **S**tudy (HIPPOCRATES) was designed to compare genetic information (genotype) with clinical data on cardiovascular risk factors and morbidity (clinical phenotype) in primary care. We selected the six aforementioned polymorphisms - AGTR1 (A1166C), AGT (M235T), ACE (4656_{rpt}), NOS3 (E298D), GNB3 (C825T) and ADD1 (G460W) - in this non-selected population for several reasons: (1) when HIPPOCRATES was designed, there was strong evidence in the literature for the role of these polymorphisms in the development of CVD, (2) these polymorphisms appeared very relevant to this study population, and (3) a complete infrastructure was available to determine these polymorphisms accurately and quickly.

Though our study had a cross-sectional design, we developed the project so as to facilitate the investigation of longitudinal relationships. A strong aspect of the HIPPOCRATES project was that the study subjects were primary care patients, and hence the entire range of low-to-high-risk patients was available. The subjects were derived from a rather small geographical area (and hence had similar social living conditions) that previously had been described in unpublished reports for the Heerlen/Kerkrade region as a population with a cardiovascular risk that was higher than that in the general Dutch population. All participants were recruited from one general practice in Kerkrade. This practice is located in a region with high burdens of CVD and cardiovascular risk factors, so the number of patients with CVD was higher than in other general practices. Consequently, it has well-implemented cardiovascular consulting hours and well-trained general practitioners and practice assistants with many years of experience in cardiovascular risk management.

We reasoned that this setting would provide an ideal environment for studying genetic associations of the aforementioned polymorphisms. The acronym HIPPOCRATES suggests that we focused on hypertension, but of course we did not restrict ourselves to this condition. To assess the effects of both genotype and cardiovascular risk factors on the arterial wall, we chose two intermediate endpoints: CIMT and carotid-femoral PWV (CF-PWV). Figure 1.2 summarizes the specific relationships that we intended to study in HIPPOCRATES.

Figure 1.2 Specific relationships studied in the HIPPOCRATES project. Arrows indicate mutual associations.

This thesis describes several studies that aimed to elucidate the contribution of six cardiovascular polymorphisms - AGTR1 (A1166C), AGT (M235T), ACE (4656_{rpt}), NOS3 (E298D), GNB3 (C825T) and ADD1 (G460W) - to cardiovascular risk assessment in primary care, by investigating the relationship between genotype, biological and clinical cardiovascular outcomes (phenotype).

Chapter 2 compares the burden of CVD in the Heerlen/Kerkrade region in the south of the Netherlands with those in a nearby region (Maastricht) and in the average Dutch population. We performed this comparison since there were indications that the prevalence of CVD was elevated in the Heerlen/Kerkrade region. Knowledge of interregional differences in cardiovascular risk factor profiles was necessary to assess the possible role of genetic factors in our study. **Chapter 3** describes the results of our baseline cross-sectional data analyses in the HIPPOCRATES population. We first show the allele frequency distribution of our set of six polymorphisms and compare these data to that in a large Dutch control sample from the PHARMO RLS database. We then assess the contribution of these six polymorphisms to the occurrence of CVD. **Chapter 4** reports on the contribution of the six cardiovascular polymorphisms to the occurrence of a first event of ischemic heart disease, which constitutes a specific phenotype of CVD, with the data presented according to gender and age. The study described in **Chapter 5** investigated the relationship between the six polymorphisms and CIMT as an intermediate endpoint for preclinical atherosclerosis. **Chapter 6** reports on the relationships between arterial stiffness as measured by CF-PWV measurements, the six polymorphisms and medication use. Since family history is closely related to the genetic background, we studied the practice and outcomes of family history-taking. **Chapter 7** reviews the literature on family history-taking in primary care as a genetic tool for identifying individuals and families at an increased risk of multifactorial disorders, especially CVD. Finally, **Chapter 8** summarizes the main findings, discusses possible clinical implications and provides suggestions for further research.

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Chapter 2

REGIONAL DIFFERENCES IN CARDIOVASCULAR RISK FACTOR PROFILE CANNOT FULLY EXPLAIN DIFFERENCES IN CARDIOVASCULAR MORBIDITY IN THE NETHERLANDS: A COMPARISON OF TWO URBAN AREAS

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PW de Leeuw, K-J Hajema, HEJH Stoffers*

The Netherlands Journal of Medicine 2005;63:309-315

Abstract

Background

Our objective was to investigate whether a region in the south of the Netherlands (Heerlen/Kerkrade) had a high burden of cardiovascular disease in comparison with a nearby region (Maastricht) and the average Dutch population, respectively. We also wanted to determine if there are interregional differences in cardiovascular risk factor profile.

Design

Cross-sectional study.

Method

Data from a nationwide registry (CBS) were used to analyse cardiovascular mortality in the two regions and the average in the Netherlands. Data from a primary care morbidity registration network (RNH) were used to compare cardiovascular morbidity and cardiovascular risk factors in both regions. A standardisation procedure was carried out for age and sex. Data were analysed using logistic regression analyses.

Results

The overall cardiovascular mortality rate was higher in the Heerlen/Kerkrade region (7.8‰) compared with Maastricht (6.1‰), (OR=1.3 [1.2-1.5]) and the average in the Netherlands (5.7‰). Similarly, most cardiovascular morbidity rates for Heerlen/Kerkrade were more elevated compared with the RNH overall and with Maastricht. Prevalence rates of risk factors such as diabetes mellitus (7.2%), (OR=1.5 [1.3-1.7]) and overweight (10.8%), (OR=2.0 [1.8-2.2]) were significantly higher in the Heerlen/Kerkrade region compared with Maastricht. There were no differences with regard to hypertension (15.2%), (OR=1.0 [0.9-1.1]).

Conclusion

Heerlen/Kerkrade is indeed a region with a high burden of cardiovascular disease. Differences in morbidity between Heerlen/Kerkrade and Maastricht cannot be fully explained by differences in cardiovascular risk factor profile.

Introduction

Recently our group started the HIPPOCRATES project (**H**ypertension: **I**nteraction and **P**revalence of **P**OLymorphisms related to **C**ardiovascular **R**isk and the **A**ssociation to **T**reatment **E**fficacy **S**tudy).¹ The main objective of this study is implementation of genetic polymorphisms in the assessment of cardiovascular risk in primary care. This study utilises the population of a general practice centre located in the southeast of Limburg, that is, an urbanised area around the cities of Heerlen and Kerkrade. From a population genetic point of view, this region is interesting in two aspects: (1) in unpublished Dutch reports a relatively high cardiovascular mortality has been described; (2) in and out migration figures are relatively low. Therefore, we were interested in the cardiovascular mortality of this region compared with the average in the Netherlands as well as with a region geographically nearby. Comparisons as these are usually limited to mortality data due to scarcity of national morbidity data.

However, morbidity data give a better estimation of the prevalence of cardiovascular disease (CVD). Fortunately, sources for regional morbidity data are available from general practice registration networks.² Since in the Netherlands almost everyone is on the list of a general practitioner, morbidity registrations in these practices reflect the health status of a general population in a specific area. Our department coordinates such a primary care morbidity registration network.² Consequently, we had the opportunity to compare the morbidity figures of various regions.

Moreover, we could explore the prevalence rates of some important cardiovascular risk factors also registered by this network (hypertension, diabetes mellitus, overweight and lipid disorders). Variation in risk factor profile might explain possible differences in cardiovascular mortality and morbidity profile between regions.^{3,4} Comparing data on cardiovascular mortality and morbidity as well as risk factors could give insight into the specific cardiovascular profile of the study region. In this study the main question was whether the Heerlen/Kerkrade region does indeed have a high burden of CVD compared with a nearby region (Maastricht) and the average of the Netherlands. A second question was whether there were interregional differences in cardiovascular risk factor profile.

Methods

Cardiovascular mortality

From the official death certification data managed by Statistics Netherlands (CBS), the latest available mortality data (2000) were used on CVD and risk factors for Heerlen/Kerkrade and Maastricht. Diagnoses were coded according to the International Classification of Diseases (ICD-10).⁵ The validity of the death registry is

generally considered sufficiently good for epidemiological use.^{6,7} The disease categories studied are presented in Table 2.1.

Table 2.1 Mortality and morbidity of categories of CVD and risk factors studied and their ICD-10 and ICPC codes.

Disease category	Mortality (CBS ^a database, ICD-10 codes)	Morbidity (RNH ^b database, ICPC codes)
Ischemic heart disease	I20 – I25	K74 – K76
Stroke	I60 – I69	K89 – K90
Other cardiovascular disease		
- Other heart disease	I00 – I09	K70 – K73
	I30 – I52	K77 – K84
- Other vascular disease	I26 – I28	K93 – K99
	I80 – I89	
	I98 – I99	
- Peripheral arterial occlusive disease	I70	K91 – K92
	I73 – I74	
Risk factor category		
Hypertension	I10 – I13	K85 – K87
	I15	
Diabetes mellitus (I & II)	E10 – E14	T90
Overweight	E66	T82 – T83
Lipid disorders	E78	T93

^a CBS: Statistics Netherlands; ^b RNH: Registration Network Family Practices (RegistratieNet Huisarts-praktijken)

Cardiovascular morbidity

Cardiovascular morbidity rates were retrieved from the Registration Network Family Practices (RNH). This is a continuous and computerised database in which 63 general practitioners (GPs) working in 22 different practices in the south of the Netherlands participate. All relevant health problems are registered. A health problem is defined as ‘anything that has required, does or may require healthcare management and has affected or could significantly affect a person’s physical or emotional well-being’.⁸ Health problems are only coded by the GPs if they are permanent (no recovery expected), chronic (duration longer than six months), recurrent (more than three recurrences within six months), or when they have lasting consequences for the functional status or prognosis of the patient. Problems are coded according to the International Classification of Primary Care (ICPC) using the criteria of the International Classification of Health Problems in Primary Care for diagnoses.^{9,10} The registered data are continuously updated and historically cumulated for each patient. Population membership only ends by migration or death. The quality of the data is ensured by instruction and training sessions, regional consensus groups, quality control experiments and by an automated thesaurus and automated checking for erroneous or missing entries.¹¹

For this study, data from five general practices (n=10,587) in Heerlen/Kerkrade (index population) and from three general practices (n=8,742) in Maastricht (control population) were used. For both regions and the RNH overall (n=56,976) prevalence rates were calculated. We compared the prevalence rates of Heerlen/Kerkrade with Maastricht. Age and sex distribution of the total RNH dataset was reported to be similar to that of the Netherlands.⁸ Comparison of the age and sex distribution of the total RNH dataset (2001) with the Netherlands (2001) still appeared to be the same. Age and sex specific data were drawn from the latest RNH dataset available (1 July 2001). The disease categories studied are presented in Table 2.1.

Cardiovascular risk factors

Prevalence rates of cardiovascular risk factors were also retrieved from the eight general practices in Heerlen/Kerkrade and Maastricht. Age and sex specific data were drawn from the latest RNH dataset available (1 July 2001). The risk factors studied are also presented in Table 2.1.

Statistical analysis

To compare the prevalence of cardiovascular mortality and morbidity between both regions (Heerlen/Kerkrade vs Maastricht), a standardisation procedure was carried out for age and sex, in which the standard population was the population of all RNH practices. To determine whether the observed differences between both regions were statistically significant, logistic regression analyses were performed, using the statistical software programme SPSS 9.0 for Windows. In the analyses regarding mortality, the dependent variable was presence or absence of a specific cause or causes of death; in those regarding morbidity and risk factors, the dependent variable was presence or absence of the disease category or risk factor. The independent variable was 'region' (Heerlen/Kerkrade vs. Maastricht) with potential confounders sex and age distribution. First, all variables were entered in the model, followed by all possible interaction terms (method: enter). The model was then further fitted, based on the statistical significance of the various interaction terms (region times age, region times sex, sex times age) according to the likelihood ratio test. In all analyses, age was entered as a categorical variable since this improved the Hosmer-Lemeshow goodness-of-fit tests considerably.¹²

Results

General characteristics

The general characteristics of the study populations are summarised in Table 2.2. With regard to mortality data (CBS), there were hardly any differences in age distribution

between both regions. With regard to morbidity data (RNH), the population of Heerlen/Kerkrade was slightly older than that of Maastricht. Both CBS and RNH populations contained slightly more females than males. Overall, both study populations were very comparable regarding to age and sex.

Table 2.2 Sex and age distribution of the study populations for mortality and morbidity.

Characteristic	Mortality analyses ^a %			Morbidity analyses ^a %		
	Netherlands (n=8,544,381)	Maastricht (n=67,975)	Heerlen/ Kerkrade (n=87,444)	RNH (n=56,976)	Maastricht (n=8,742)	Heerlen/ Kerkrade (n=10,587)
Sex						
Male	48.2	47.3	47.7	48.1	46.9	48.3
Female	51.8	52.7	52.3	51.9	53.1	51.7
Age (year)						
35-44	29.6	27.2	27.8	27.5	28.8	26.5
45-54	26.7	25.4	25.1	26.4	23.7	25.9
55-64	18.5	18.9	18.9	19.6	18.7	19.5
65-74	14.0	15.8	16.0	15.4	16.4	15.9
75-84	8.6	9.8	9.8	9.0	9.6	10.0
≥85	2.6	2.8	2.4	2.1	2.8	2.1

^a Based on CBS database 2000; ^b Based on RNH database 2001

Cardiovascular mortality

Mortality figures of some CVDs or risk factors were so small that no meaningful analyses could be performed. This was the case for 'other vascular disease' and 'peripheral arterial occlusive disease' and for all the risk factors. Table 2.3 shows standardised mortality rates of CVDs for both regions and the average in the Netherlands. The overall trend for all categories was that mortality rates were higher in Heerlen/Kerkrade compared with Maastricht. The most distinct difference was found for 'ischemic heart disease'. Figure 2.1 shows the age specific mortality rates for the two regions. For every age group, the mortality rate was higher in Heerlen/Kerkrade compared with Maastricht. However, the pattern of the mortality rates was comparable in the two regions.

Logistic regression analyses confirmed the statistical significance of the differences between the two regions. The variable 'region' had an independent association with mortality in the categories 'ischemic heart disease' (OR=1.4 [1.2-1.7]), 'other cardiovascular disease' (OR=1.2 [1.1-1.5]) and 'other heart disease' (OR=1.3 [1.1-1.7]). No independent association was observed for the mortality category 'stroke'.

Table 2.3 Standardized mortality rates of CVDs for two urban regions (n=155,419) and the Netherlands (n=8,544,381).

	Netherlands (%)	Maastricht (%)	Heerlen/Kerkrade (%)	Significance of the variable 'region'
Mortality category				
Ischemic heart disease	2.0	2.2	3.1	OR=1.4 (1.2-1.7) ^{b,c}
Stroke	1.4	1.5	1.8	OR=1.2 (0.9-1.5) ^{b,c}
Other CV disease	- ^a	2.4	2.9	OR=1.2 (1.1-1.5) ^{b,c}
Other heart disease	- ^a	1.6	2.2	OR=1.3 (1.1-1.7) ^{b,c}
Overall CV diseases	5.7	6.1	7.8	OR=1.3 (1.2-1.5) ^{b,c}

^a No comparable data available; ^b Heerlen/Kerkrade versus Maastricht; ^c 95% confidence interval. CV=cardiovascular.

Figure 2.1 Age specific mortality rates for ischemic heart disease (CBS mortality data).

Cardiovascular morbidity

In Table 2.4 standardised prevalence rates of cardiovascular morbidity are presented for both regions. Overall, the Heerlen/Kerkrade region consistently showed a higher prevalence of cardiovascular morbidity in comparison with Maastricht. The most distinct differences between the regions were found with respect to 'ischemic heart disease' and 'other heart and vascular diseases', in which the main contribution came from 'other vascular disease'. Figure 2.2 shows the trend of the prevalence rates of ischemic heart disease in both regions. The prevalence rate of Heerlen/Kerkrade for every age group was consistently above Maastricht, except for the youngest and the oldest age category. For all other cardiovascular morbidity categories, the prevalence rate of Heerlen/Kerkrade lay consistently above Maastricht. Comparatively, the relative risk was the highest for peripheral arterial occlusive disease. Logistic regression analyses confirmed the statistical significance of the differences between both regions in most cases. The variable 'region' had an independent association with

morbidity in the categories ‘other heart and vascular disease’ (OR=1.2 [1.1-1.3]), ‘other vascular disease’ (OR=1.2 [1.1-1.3]) and ‘peripheral arterial occlusive disease’ (OR=1.4 [1.2-1.7]). It was significant in interaction with age for ‘ischemic heart disease’, indicating that in all age categories the prevalence of ischemic heart disease was higher in Heerlen/Kerkrade than in Maastricht, except for the age category 35 to 44 and ≥85. Differences in prevalence rates for stroke and ‘other heart disease’ were small, which was confirmed by the results of the logistic regression analysis (Table 2.4).

Figure 2.2 Age specific prevalence rates for ischemic heart disease (morbidity data from the RNH database).

Cardiovascular risk factors

In Table 2.4 also standardised prevalence rates of available cardiovascular risk factors are presented for both regions. The prevalence of the cardiovascular risk factors studied was higher in Heerlen/Kerkrade compared with Maastricht. This holds especially for ‘diabetes mellitus’, ‘overweight’ and ‘lipid disorders’. Regarding ‘hypertension’, the difference was small.

Logistic regression analyses confirmed the statistical significance of the differences between both regions in most cases. With respect to the cardiovascular risk factors studied, the variable ‘region’ had an independent association with ‘diabetes mellitus’ (OR=1.5 [1.3-1.7]) and with ‘overweight’ (OR=2.0 [1.8-2.2]). For ‘lipid disorders’, it was significant in interaction with age and sex, indicating that if age rises, more males develop lipid disorders in the region Heerlen/Kerkrade than in Maastricht. The difference in prevalence rates of hypertension was small, which was confirmed by the results of the logistic regression analysis.

Table 2.4 Standardized prevalence rates of CVD and risk factors for two urban regions (n=19 329) and the RNH[#] overall (n=56 976).

	RNH (%)	Maastricht (%)	Heerlen/Kerkrade (%)	Significance of the variable 'region'
Cardiovascular Disease category				
Ischemic heart disease	8.5	7.8	9.6	In interaction with age
Stroke	3.7	3.9	4.0	OR=1.0 (0.9-1.2) ^{b,c}
Other heart and vascular disease	19.6	20.1	22.3	OR=1.2 (1.1-1.3) ^{b,c}
- Other heart disease	7.4	7.6	7.5	OR=1.0 (0.9-1.1) ^{b,c}
- Other vascular disease	11.6	12.4	14.1	OR=1.2 (1.1-1.3) ^{b,c}
- Peripheral arterial occlusive disease	3.2	2.8	3.9	OR=1.4 (1.2-1.7) ^{b,c}
Overall diseases of the heart and circulation system	25.8	25.9	28.9	OR=1.2 (1.1-1.3) ^{b,c}
Cardiovascular Risk factor category				
Hypertension	14.6	15.3	15.2	OR=1.0 (0.9-1.1) ^{b,c}
Diabetes Mellitus (I & II)	6.3	5.0	7.2	OR=1.5 (1.3-1.7) ^{b,c}
Overweight ^a	7.5	5.8	10.8	OR=2.0 (1.8-2.2) ^{b,c}
Lipid disorders	6.4	5.5	8.0	In interaction with age and sex

^a BMI≥25; ^b 95% confidence interval; ^c Heerlen/Kerkrade versus Maastricht; [#]RNH: Registration Network Family Practices

Discussion

The present study showed that cardiovascular mortality rates were higher in the region Heerlen/Kerkrade compared with both the average in the Netherlands and in Maastricht in particular. Similarly, most cardiovascular morbidity rates for Heerlen/Kerkrade were more elevated compared with the RNH overall and more specifically with Maastricht. In accordance with these results prevalence rates of cardiovascular risk factors – in particular diabetes mellitus, overweight and lipid disorders, but not hypertension – were significantly higher in the Heerlen/ Kerkrade region.

Before discussing possible explanations and the relevance and implications of these findings, it is important to discuss in short the validity of the registration systems used. In the case of mortality data (CBS), validity depends on the accuracy of the registration of the primary cause of death. Several studies have assessed the validity of the Dutch mortality registration and confirmed the completeness of this registration.^{6,7,13} However, discrepancies have been found between the judgement of physicians and subsequent findings at autopsy and between physicians coding identical cases for research purposes.^{6,14} Using broad categories, as was done in this study, is known to lead to fewer discrepancies than analysing single disorders.^{14,15} We have no reason to assume there are regional differences with regard to registration of death certificates by doctors. Regarding data on morbidity and risk factors (RNH), validity depends on the accuracy of the registration of the diagnostic problems by the general practitioners involved. The quality is ascertained by instruction and training

sessions, regional consensus groups, quality control experiments and by an automated thesaurus and automated checking for erroneous or missing entries.⁸ There were no differences between the selected general practices in both regions with regard to participation in cardiovascular research projects over the last 13 years. Our results demonstrate that Heerlen/Kerkrade is indeed a region with a high burden of CVD, in comparison with Maastricht and the average in the Netherlands. Given the fact that five general practices in Heerlen/Kerkrade are included in the RNH overall, the estimates of the prevalence rates for cardiovascular morbidity in the RNH overall are probably higher than the average in the Netherlands. Consequently, average cardiovascular morbidity figures for the Netherlands will probably be lower than presented here.

Data from the Framingham study show that important cardiovascular risk factors such as hypertension, diabetes mellitus, overweight and lipid disorders have a mutual amplifying effect.¹⁶ Our results show a relatively high prevalence of diabetes mellitus, overweight and lipid disorders in Heerlen/Kerkrade and this will contribute to a higher prevalence rate of cardiovascular mortality and morbidity. However, the prevalence rates for hypertension did not differ in the two regions. A Dutch study using CBS data for the period 1950 to 1984 suggested that excess cardiovascular mortality appearing in the south of the Netherlands could be explained by Roman Catholic lifestyle and relatively lower income.¹⁷ Unpublished reports for the Heerlen/Kerkrade region and Maastricht on these cardiovascular risk factors as well as for alcohol use, showed inconsistent differences. No data were available on risk factors such as elevated homocysteine levels and unfavourable nutrition patterns.¹⁸⁻²¹

Our results are consistent with the complex relationship between cardiovascular morbidity and multiple cardiovascular risk factors.²² However, the excess risk observed in the Heerlen/Kerkrade region as compared with the Maastricht region can not be fully explained on the basis of a higher prevalence of risk factors. For instance, in subjects from Heerlen/Kerkrade with hypertension, the risk of coronary complications was substantially greater than that predicted from placebo-treated patient populations in major clinical trials.²³ In the pathogenesis of CVD many factors, including genetic and environmental factors, play a role.^{24,25} Genetic factors that modulate the individual susceptibility to CVD are common, functionally different types of genes (polymorphisms).²⁶⁻²⁹ These polymorphisms generally have a modest effect at an individual level, but because of their high frequency in the population can be associated with a high attributable risk.³⁰ Environmental factors can reveal or facilitate the phenotypic expression of such susceptibility genes. There is now accumulating evidence that most of the susceptibility genes for common diseases do not have a primary aetiological role in predisposition to disease, but rather act as response modifiers to exogenous factors such as stress, environment, disease, and drug intake.³⁰ A better characterisation of the interactions between environmental and

genetic factors constitutes a key issue in the understanding of the pathogenesis of CVD.³¹⁻³³ Therefore it is important to study all these factors on an individual level.

Conclusion

The Heerlen/Kerkrade region is indeed a region with a high burden of CVD. However, the differences in morbidity between Heerlen/Kerkrade and Maastricht cannot be fully explained by differences in cardiovascular risk factor profile. Therefore a better characterisation of the interactions between environmental and genetic factors is important in cardiovascular research in the Heerlen/ Kerkrade region.

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Chapter 3

THE CONTRIBUTION OF SIX POLYMORPHISMS TO CARDIOVASCULAR RISK IN A DUTCH HIGH RISK PRIMARY CARE POPULATION: THE HIPPOCRATES PROJECT

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Abstract

This study was designed to examine the contribution of six cardiovascular polymorphisms to the occurrence of cardiovascular disease (CVD) in a Dutch primary care population with a high prevalence of cardiovascular risk factors. In this cross sectional case-control study, 232 patients with CVD and 571 event-free controls were studied. Patients were genotyped for the AGTR1 (A1166C), AGT (M235T), ACE (4656_{rpt}), NOS3 (E298D), GNB3 (C825T) and ADD1 (G460W) polymorphisms. Univariate and multivariate odds ratios (ORs) were calculated to assess the relationship between genotypes and CVD. Receiver operating characteristic (ROC) analysis was used to quantify the contribution of the polymorphisms to the prediction of CVD. No differences in either genotype or allele frequencies were found between CVD cases and controls. Multivariate analyses showed significant protective associations for the T allele of AGT (OR=0.55 [0.34-0.84]) and for the T allele of ADD1 (OR=0.52 [0.31-0.82]). ROC analysis showed only a very small improvement of CVD risk prediction by adding the six polymorphisms to a model with traditional risk factors. Our data suggests that a major attribution of the six polymorphisms to the cardiovascular risk prediction in a primary care population such as HIPPOCRATES is unlikely.

Introduction

Cardiovascular diseases are the leading cause of mortality and morbidity in the world. Smoking, obesity, gender, metabolic disorders and hypertension are traditional risk factors, which have been intensively investigated in population surveys.¹ The appearance of cardiovascular genetics as a new discipline in medicine has led to the publication of numerous studies, investigating whether specific genetic polymorphisms may either cause cardiovascular disease (CVD) in a monogenetic or a polygenetic fashion or predispose subjects to various cardiovascular disorders.² Among these, many studies have aimed to investigate the influence of genetic variability of the renin-angiotensin system (RAS) on the development of CVD.³ Among the many potential genes, those related to activation of the RAS or to an increased tendency to retain sodium rank high and so do genes which may cause reduced variability of nitric oxide (NO). To this date, the results from human studies on their role in the development of CVD are not beyond dispute.³ An important reason is that most of these observations have been obtained in selected populations.⁴

Earlier, we have suggested that the excess risk observed in the south-east region of the province of Limburg, the Netherlands, could not be fully explained on the basis of a higher prevalence of cardiovascular risk factors.⁵ Furthermore, a pilot study suggested a role for certain genetic variants of the RAS in healthy normotensive patients in this population.⁶ This prompted us to perform a genetic association study on CVD in a primary care population in this region. On the basis of the available literature at the start of the study, we restricted ourselves to the analysis of six polymorphisms. These were the angiotensinogen [AGT (M235T)], angiotensin converting enzyme [ACE (4656_{rs17997})], and the angiotensin II type 1 receptor [AGTR1 (A1166C)] polymorphism, that are involved in the generation and action of angiotensin II (AngII). In addition, α -adducin [ADD1 (G460W)], G-protein β 3 subunit [GNB3 (C825T)], and the endothelial nitric oxide synthase [NOS3 (E298D)] polymorphisms, as markers of sodium retaining tendency and altered NO availability, were selected, respectively. We hypothesized that the presence of one or more rare alleles of the aforementioned six polymorphisms would affect the occurrence of CVD in a high risk primary care population. Therefore, in this study we assessed the contribution of these six polymorphisms to the occurrence of CVD in patients with known CVD in comparison to control subjects without CVD.

Methods

Study population

The HIPPOCRATES (**H**ypertension: **I**nteraction and **P**revalence of **P**olymorphisms related to **C**ardiovascular **R**isk and the **A**ssociation to **T**reatment **E**fficacy **S**tudy) is an

ongoing study, in which the implementation of genetic polymorphisms in the assessment of cardiovascular risk in primary care is investigated.⁵⁻⁷ This study utilizes the population of one large general practice in Kerkrade, which is located in the south-east of the Netherlands, and is characterised with a high burden of CVD compared with the average of the Netherlands.⁵ This academic general practice takes part in the Registration Network Family Practices (RNH), which is a continuous and computerized morbidity registration network in which 63 general practitioners (GPs), working in 22 different practices, in the south of the Netherlands participate. All relevant health problems, including a full medical history of all cardiovascular events have been registered since 1990.^{8,9} Medical problems are coded according to the International Classification of Primary Care (ICPC) using the criteria of the International Classification of Health Problems in Primary Care for diagnoses.^{10,11} The general practice in Kerkrade has a list of approximately 4400 patients, among whom are more than 2300 patients of 40 years and older. The practice has well implemented cardiovascular consulting hours. For HIPPOCRATES, all known patients aged ≥ 40 years with CVD or hypertension were invited. From the group with neither CVD nor hypertension but with other cardiovascular risk factors, that is, smoking, alcohol, diabetes mellitus, dyslipidemia, overweight including obesity, a random 1:3 sample was taken. Of all patients without known CVD, hypertension or other cardiovascular risk factors, also a random 1:3 sample was taken. The initial classification was validated by a thorough assessment of the patients' records combined with additional measurements of risk factors for all subjects. The total HIPPOCRATES population consisted of 803 patients (382 males, 421 females), who were recruited between May 2003 and July 2005.

For the present study, 232 patients with known CVD were compared with 571 event-free controls. The presence of CVD was defined as a diagnosis of ischemic heart disease, stroke, peripheral arterial occlusive disease or heart failure. Control subjects had no documented CVD. The study was approved by the Medical Ethics Committee of the Maastricht University Medical Center. Written informed consent was obtained from each of the participants.

Clinical data collection

All clinical measurements were performed in the practice by one of the authors (AWP; first measurement) and a nurse practitioner (second, third and fourth measurement with in-between periods of three months). The nurse practitioner measured weight and blood pressure. All persons were questioned on their smoking behaviour and alcohol consumption. Weight and height were measured. Body-mass index (BMI) was calculated as body weight divided by squared height. BMI values ≥ 27 were considered to be too high. The size of the waist was measured. Blood pressure and heart rate were measured three times per visit after ten minutes rest in sitting position using a

sphygmomanometer. Fasting blood samples were drawn to determine serum creatinine, total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, serum triglycerides and glucose levels and analyzed in one regional laboratory. Microalbuminuria was determined semiquantitatively in a first-morning urine sample.

Definitions

Hypertension was defined as a systolic blood pressure (SBP) ≥ 140 mmHg and/or diastolic blood pressure (DBP) ≥ 90 mmHg according to the Guidelines for Hypertension specified by the European Society of Hypertension¹² or the use of antihypertensive drugs. The presence of dyslipidemia was defined as total cholesterol to HDL cholesterol ratio exceeding 4.0 or use of lipid lowering drugs. Diabetes mellitus was considered to be present in case of fasting plasma glucose levels >6.9 mmol/l or the use of oral antidiabetic drugs or insuline. Creatinine values higher than 115 $\mu\text{mol/l}$ (males) or 107 $\mu\text{mol/l}$ (females) were considered to be a risk factor for CVD. Microalbuminuria was defined as albumin excretion above 20 mg/l.

Genetic analyses

The various polymorphisms were determined at the Cardiovascular Genotyping (CAGT) Laboratory of the Department of Internal Medicine of the Maastricht University Medical Center. DNA was extracted from whole blood with the use of the QIAmp Blood Kit (Qiagen Inc., Valencia, California, USA). Genotyping of the angiotensin II type 1 receptor [AGTR1 (A1166C)/rs5186], angiotensinogen [AGT (M235T)/rs699], angiotensin converting enzyme [ACE (4656_{rpt})/rs1799752], endothelial nitric oxide synthase [NOS3 (E298D)/rs1799983], G-protein $\beta 3$ subunit [GNB3 (C825T)/rs5443] and α -adducin [ADD1 (G460W)/rs4961] polymorphisms was performed with a multiplex single base extension (SBE) assay (Multiplex SNaPshot, Applied Biosystems, Foster City, USA) similar to previously published protocols.¹³ Briefly, in the first stage a multiplex PCR is carried out to amplify the gene fragments that contain the polymorphisms. In the second stage, the polymorphic positions are interrogated by a SBE reaction using fluorescently labelled ddNTPs. The oligos used for the single base extension have specific lengths, varying from 22-50 nt. After the SBE assay, the fragments are size-resolved on an ABI3100 Genetic Analyser: the size of the peaks identifies the locus (e.g. ADD1) and the color of the peak(s) identifies the genotype (e.g. Gly/Trp). Raw data were analyzed with Genescan Analysis software (v 3.7), then genotypes were scored independently by two researchers and entered in the database. In 27 patients, a second analysis was necessary to obtain all the genotypes. The ACE (I/D) polymorphism was determined by detecting ACE_{4656+CT} observed on the SNaPshot as ACE (4656_{rpt}). The use of this SNaPshot method does not allow the direct determination of the insertion/deletion polymorphism. The ACE_{4656+CT} polymorphism in the 3'-untranslated region of the ACE gene consists of a repetition of

two or three CT dinucleotides and is in complete linkage disequilibrium with ACE (I/D).^{14,15} The multiplex assay was validated on a set of 100 DNA samples that had previously been genotyped with an alternative technique; this technique concerned a multilocus genotyping assay for candidate markers of CVD risk (Roche Molecular systems Inc., Pleasanton, USA) and has been described in detail previously.¹⁶ Only four samples gave a discordant result for the NOS3 polymorphism. All other genotypes were concordant. The discordant results have been adjusted.

Genetic validation of control group

To evaluate the validity of our control group, we compared the genotype- and allele frequencies with subjects who use antihypertensive drugs but without CVD in another general population sample in the Netherlands, that is, the PHARMAcoMorbidity Record Linkage System (PHARMO RLS), which has allele frequencies comparable with those found in large European healthy control groups.^{17,18} In short, PHARMO RLS links drug dispensing histories from a representative sample of Dutch community pharmacies to the national registrations of hospital discharges (LMR) from 1985 onwards. Currently, the base population of PHARMO covers more than 2.5 million community-dwelling inhabitants of several population-defined areas in the Netherlands.^{19,20}

Statistical analysis

Statistical analysis was performed by using SPSS statistical software, version 13.0 (SPSS, Inc., Chicago, IL, USA). A *P*-value of 0.05 or less was considered statistically significant. Bonferroni correction was applied for multiple testing. Statistical significance for differences in quantitative variables between CVD patients and controls was tested by unpaired Student's *t*-tests. In case of categorical variables the chi-square test was used. Genotype and allele frequencies were calculated to test the Hardy-Weinberg equilibrium. Allele frequencies were analyzed using chi-square tests.

Logistic regression analyses

Both univariate and multivariate odds ratios (ORs) were calculated with a binary-logistic regression model (method: enter) to evaluate the relationship between polymorphisms and prevalent CVD. For that purpose, dummy variables were created using the homozygous wild-type genotype as reference category. Age and gender, both demographic variables, were incorporated in both the univariate as well as in the multivariate linear regression analyses. As indicated, we selected control subjects from different groups. Therefore, in the logistic regression analyses we had to adjust for this selection and dummy variables had to be created using the normotensive group without cardiovascular risk factors as the reference category. Adjustment for potential confounders was performed by incorporating smoking, alcohol, diabetes

mellitus, waist circumference, serum creatinine, mean systolic and diastolic blood pressure, microalbuminuria and dyslipidemia into these models. To avoid collinearity, waist circumference was used instead of waist-to-hip ratio or BMI and condensed measures were used like diabetes and dyslipidemia as defined earlier. Recent research has shown that waist circumference is a better marker of abdominal fat accumulation than waist-to-hip ratio or BMI²¹, which is strongly associated with increased cardiovascular risk.²² The simultaneous contribution of the six polymorphisms to the prediction of CVD was tested, using a likelihood ratio test. If two or more polymorphisms reached statistical significance, interactions (effect modification) between two polymorphisms with respect to CVD were assessed. Again binary-logistic regression analysis (method: enter) was used, in which the alleles at risk, the interaction term between the alleles and the other covariates were incorporated. If an interaction term became statistically significant, the model was explored further.

ROC analysis

The quantitative impact of the six polymorphisms on the prediction of CVD was evaluated by comparison of the receiver operating characteristic (ROC) curve incorporating the traditional risk factors (that is, age, gender, smoking, alcohol, diabetes mellitus, waist circumference, serum creatinine, mean systolic and diastolic blood pressure, microalbuminuria, and dyslipidemia) and the polymorphisms versus the ROC curve without the polymorphisms, that is, based only on the traditional risk factors. The area under the curve (AUC) is a quantitative summary for the ROC curve. Values between 0.70 and 0.80 are considered acceptable, and those between 0.80 and 0.90 are considered excellent.²³

Results

General characteristics

Characteristics of the study population are summarized in Table 3.1.

Both groups differed with respect to established cardiovascular risk factors. Subjects with CVD were more frequently older males and showed greater proportions of dyslipidemia, diabetes and microalbuminuria. They also had a higher waist-to-hip ratio and serum creatinine as compared with the control group. However, probably because of treatment in the CVD group, (that is antihypertensive and lipid-lowering drugs), diastolic blood pressure, heart rate, total and LDL cholesterol were lower in subjects with CVD. There were no significant differences with respect to smoking and alcohol use between both groups.

Table 3.1 Characteristics of the study population (n= 803).

	CVD patients (n=232)	Controls (n=571)	P
Males, n (%)	142 (61.2)	240 (42.0)	<0.001 ^a
Age (years)	69.5 ± 9.6	59.9 ± 11.3	<0.001
Morbidity			
History of ischemic heart disease, n (%)	162 (69.8)		
History of stroke, n (%)	77 (33.2)		
Peripheral artery occlusive disease, n (%)	36 (15.5)		
Heart failure, n (%)	20 (8.6)		
Risk factors			
Body-mass index (kg/m ²)	29.0 ± 4.8	28.1 ± 4.8	0.011
Waist (cm)	102.1 ± 11.0	95.1 ± 14.2	<0.001
Systolic blood pressure (mmHg)	145.7 ± 21.3	140.1 ± 21.0	0.001
Diastolic blood pressure (mmHg)	78.8 ± 10.2	80.7 ± 10.2	0.019
Heart rate (beats per minute)	73.2 ± 12.4	75.4 ± 11.4	0.019
Current smoking, n (%)	52 (22.4)	107 (18.7)	0.232 ^a
Alcohol, n (%)	5 (2.2)	10 (1.8)	0.658 ^a
Hypertension, n (%)	182 (78.4)	339 (59.4)	<0.001 ^a
Diabetes, n (%)	68 (29.3)	69 (12.1)	<0.001 ^a
Dyslipidemia, n (%)	164 (70.7)	285 (49.9)	<0.001 ^a
Microalbuminuria, n (%)	50 (21.6)	51 (8.9)	<0.001 ^a
Biochemical			
Creatinine (μmol/l)	98.9 ± 36.7	91.9 ± 14.8	<0.001
Total cholesterol (mmol/l)	5.2 ± 1.2	5.7 ± 1.0	<0.001
LDL-cholesterol (mmol/l)	3.1 ± 1.1	3.5 ± 0.9	<0.001
HDL-cholesterol (mmol/l)	1.3 ± 0.4	1.6 ± 0.4	<0.001
Triglycerides (mmol/l), fasting	1.7 ± 0.9	1.5 ± 0.8	<0.001
Glucose (mmol/l), fasting	6.3 ± 2.2	5.7 ± 1.5	<0.001
Medication			
Antihypertensive, n (%)	191 (82.3)	221 (38.7)	<0.001 ^a
Antidiabetic, n (%)	47 (20.3)	33 (5.8)	<0.001 ^a
Antiplatelet, n (%)	166 (71.6)	40 (7.0)	<0.001 ^a
Hypolipidemic, n (%)	121 (52.2)	47 (8.2)	<0.001 ^a

Values are given as mean ± SD or as number of subjects (with corresponding percentages between brackets); CVD, cardiovascular disease; P, P values are the results of Student's *t*-test (continuous variables) or ^a chi square test (categorical variables); HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Genetic Analyses

Genotype and allele frequencies of all polymorphisms were in Hardy-Weinberg equilibrium. No differences in either genotype or allele frequencies were found between CVD cases and controls in the HIPPOCRATES population (Table 3.2). Comparison between the control subjects of the PHARMO and the HIPPOCRATES database showed that there were no differences in genotype- and allele frequencies between both populations.

Table 3.2 Allele frequencies of polymorphisms in the HIPPOCRATES and the PHARMO population.

Polymorphism		HIPPOCRATES		PHARMO
		CVD patients (n=232)	Controls (n=571)	Controls (n=1,240)
AGTR1 (A1166C)	A	0.724	0.703	0.679
	C	0.276	0.297	0.321
AGT (M235T)	M	0.634	0.606	0.590
	T	0.366	0.394	0.410
ACE (4656 _{rpt})	C	0.524	0.512	0.535
	G	0.476	0.488	0.465
NOS3 (E298D)	G	0.636	0.637	0.654
	T	0.364	0.363	0.346
GNB3 (C825T)	C	0.679	0.718	0.695
	T	0.321	0.282	0.305
ADD1 (G460W)	G	0.793	0.778	0.800
	T	0.207	0.222	0.200

Logistic regression analyses

Both univariate and multivariate ORs were calculated to evaluate the relationship between polymorphisms and CVD (Table 3.3). In the multivariate analysis, the simultaneous contribution of the six polymorphisms to the prediction of CVD was statistically significant (likelihood ratio test; $P<0.001$), showing a protective association for carriership of the T allele of the AGT polymorphism (OR=0.55 [0.34–0.84]) and for carriership of the T allele of the ADD1 polymorphism (OR=0.52 [0.31–0.82]). Both associations were still statistically significant after Bonferroni correction, that is, AGT ($P=0.02$) and ADD1 ($P=0.01$). In addition, we found a trend towards a protective effect for the rare-allele of the AGTR1 (OR=0.69 [0.47–1.01]). In all other polymorphisms, carriership of the rare allele of the investigated polymorphisms was not associated with an increased risk of CVD. Evaluation of possible interactions between AGT and ADD1 showed no statistically significant interaction terms.

Table 3.3 Odds ratios for prevalent cardiovascular disease according to the gene polymorphisms.

Polymorphism	Rare allele*	OR (95% CI)	
		Univariate	Multivariate
AGTR1 (A1166C)	C*	0.76 (0.53 – 1.09)	0.69 (0.47 – 1.01)
AGT (M235T)	T*	0.67 (0.46 – 0.96) [#]	0.55 (0.34 – 0.84) [#]
ACE (4656 _{rpt})	G*	1.00 (0.67 – 1.50)	0.97 (0.64 – 1.47)
NOS3 (E298D)	T*	1.10 (0.76 – 1.59)	1.20 (0.82 – 1.76)
GNB3 (C825T)	T*	1.09 (0.76 – 1.56)	1.13 (0.78 – 1.64)
ADD1 (G460W)	T*	0.69 (0.48 – 1.01)	0.52 (0.31 – 0.82) [#]

* The wild-type alleles were used as reference category; multivariate adjustment was performed for age, gender, waist circumference, smoking, alcohol, diabetes mellitus, mean systolic and diastolic blood pressure, serum creatinine, microalbuminuria and dyslipidemia; [#] $P\leq 0.05$; CI denotes 95% confidence interval.

Cardiovascular risk prediction

To quantify the contribution of the polymorphisms ROC curves were constructed for prediction of CVD by traditional risk factors only versus prediction of CVD by risk factors supplemented with the six polymorphisms, respectively (Figure 3.1). The area under the curve (AUC) for the first prediction was 0.83 [0.80–0.86] and for the second 0.84 [0.81–0.87], indicating that the six polymorphisms have hardly any additional influence on the assessment of CVD in this population. Adding only the AGT and ADD1 polymorphism, which showed a significant association in the multivariate analyses, did not change the AUC (0.84 [0.81–0.87]).

Figure 3.1 Receiver operating characteristic (ROC) analysis of the six cardiovascular polymorphisms for the prediction of cardiovascular disease in a primary care population; traditional risk factors were age, gender, waist circumference, smoking, alcohol, diabetes mellitus, mean systolic and diastolic blood pressure, serum creatinine, microalbuminuria and dyslipidemia.

Discussion

In this primary care study (HIPPOCRATES) no statistically significant differences in allele frequencies were found between CVD cases and controls with regard to the six polymorphisms. The allele frequencies were comparable to another Dutch database, PHARMO, consisting of subjects from other regions of the Netherlands. Multivariate analyses showed a statistically significant contribution of six polymorphisms ($P < 0.001$) to the presence of CVD, predominantly consisting of protective associations with carriership of the rare T alleles of AGT (OR=0.55 [0.34–0.84]) and of ADD1 (OR=0.52 [0.31–0.82]), and a borderline statistically significant association with CVD for the

AGTR1 polymorphism (OR=0.69 [0.47–1.01]). Addition of all six or only the AGT and ADD1 polymorphisms to the traditional cardiovascular risk factors showed, however, only a 1% improvement of the prediction of CVD, as determined by ROC curve analysis.

Allele frequencies

To the best of our knowledge, no data on allele frequencies of RAS polymorphisms are available for a primary care population. Therefore, comparison of our allele frequencies with other studies is difficult. Moreover, allele frequencies differ from population to population. Therefore, the best comparison should be done on a similar population. However, a control group from the same population may not differ with respect to the genetic background compared to subjects with CVD. Therefore, we compared our allele frequencies with frequencies from the PHARMO cohort, which has comparable allele frequencies with those found in large European healthy control groups.^{17,18} Consequently, we found an equal allele frequency for all six polymorphisms in HIPPOCRATES and PHARMO control subjects. Besides, if we compared the observed allele frequencies in HIPPOCRATES of cases and controls with those found in larger European studies (with at least 500 cases and 500 controls), comparable allele frequencies were found (data not presented). This comparability of the allele frequencies indicates that the observed high cardiovascular risk in the HIPPOCRATES population is probably not related to marked differences in genetic make up of the investigated polymorphisms.^{17,18,24-26}

Polymorphisms

Angiotensinogen (M235T) polymorphism

The observed decreased risk for CVD for carriers of the rare T allele of the AGT polymorphism needs further explanation. From literature we know that angiotensinogen (AGT) is significantly elevated in patients with the AGT (M235T) polymorphism and is particularly associated with hypertension, coronary heart disease and myocardial infarction.^{27,28} Recently, Xu *et al.* performed a meta-analysis on the AGT (M235T) polymorphism studying 43 association studies on coronary artery disease including a total of 13,478 cases and 17,024 controls.²⁹ This meta-analysis showed that overall the effect of this polymorphism is weak, showing both protective and harmful associations.²⁹ A possible explanation for the observed decreased risk for CVD in our study might be a low-renin state. The protective association for the T allele of AGT under these conditions may be related to lower angiotensin levels. This was confirmed by a study performed by Fernández-Arcás *et al.*, who indeed found a suppressed RAS in carriers of the T allele of AGT.³⁰ In addition, misclassification of the AGT (M235T) genotype should also be considered, as has been indicated recently.³¹ However, this is less likely an explanation in our study, since the extensive validation

process of the multiplex assay did not result in discordant cases for the AGT (M235T) polymorphism.

Alpha-adducin (G460W) polymorphism

The ADD1 (G460W) polymorphism is associated with salt sensitivity, or high sodium intake, and an increased risk of cardiovascular events and mortality.³² The results of our study, that is, decreased risk for CVD for carriers of the T allele of ADD1, are not in line with these findings. A possible explanation may be related to the treatment status of the subjects. Earlier, it has been reported that there is an interaction between carriers of the rare T allele of ADD1 and diuretic therapy.³³ Hypertensive subjects carrying the rare T allele, who were treated with diuretics, were at lower risk of myocardial infarction and stroke compared with subjects treated with other antihypertensive therapies. We checked our database for the use of diuretics and found no association with diuretics. So, although attractive, the concurrent use of diuretics does not explain our findings. Our finding of a protective association for the rare allele of ADD1 is however not an isolated unique observation. Tobin *et al.*, also found such a protective association for ADD1 in a multilocus case-control study in which patients with myocardial infarction were compared with healthy controls.¹⁸ They suggested that this was attributable to the premature death of T allele carriers or to a protective effect of this allele through a cellular mechanism different from its effect leading to hypertension.¹⁸ Since the design of both our study and the one of Tobin *et al.*, was cross sectional, we think this need to be evaluated in future prospective studies.

Angiotensin II type 1 receptor (A1166C) polymorphism

Over the last decade, the angiotensin II type I receptor (A1166C) polymorphism has been intensively investigated for its possible role in hypertension and CVD.³⁴ An explanation for the borderline significant protective association with the rare C allele of the AGTR1 polymorphism may be found in the fact that these subjects have a poor cardiovascular prognosis and as a consequence were not included in our study. Although the data collection for this study already started in 1990, this possibility can not be excluded completely. Importantly, the data of this AGTR1 polymorphism were of borderline significance, and the relevance should not be overstretched therefore.

Strengths and limitations

A unique aspect of a primary care population is that the whole range of low-to-high-risk patients is available. Moreover, our population was selected using strict inclusion criteria and was derived from a rather small area, including subjects with similar social living conditions. Although our study has an exploratory character, we performed this study with a clear a priori hypothesis, therefore reducing multiple hypothesis testing. Albeit even after Bonferroni correction for multiple testing the statistical significant

associations maintained. We realize that our population was not designed to obtain realistic ROC estimates; the ROC results should be interpreted as indicative of the contribution of genetic information, not as quantitatively accurate. The last decades, investigators developed multivariable risk prediction algorithms to predict the risk for occurrence of CVD.^{35,36} For instance, the Framingham risk score uses age, gender, total and HDL cholesterol levels, blood pressure, smoking and diabetes mellitus as variables to predict risk.³⁷ When ROC analyses were performed, containing these traditional risk factors, in various epidemiological cohorts, the area under the curve (AUC) ranged from 0.66 to 0.88.³⁵ In our analyses we found an AUC of 0.83 for the prediction of CVD by traditional cardiovascular risk factors. This indicates that our multivariate model was already an excellent standard even before the six polymorphisms were added and compared.²³ This may be why the ROC analysis with the addition of six polymorphisms only improved the predictive value of traditional cardiovascular risk factors by approximately 1 per cent.

One may argue the presence of other limitations of our study. Firstly, we only studied the effect of one polymorphism per gene, which does not exclude the role of other polymorphic alleles, which are not in linkage disequilibrium. Another limitation, as is for many genetic association studies, is sample size. It has been emphasized that sample sizes of thousands are required to have adequate power to detect genes conferring ORs as low as 1.2, with minor allele frequencies in the range of 5-10%.³⁸ In our study the actual allele frequencies of the rare alleles ranged between 0.21 and 0.48, and the ORs for prevalent CVD were much larger than 1.2, indicating that the sample size of our study, at least for the AGT and the ADD1 polymorphism, was acceptable.

In conclusion, although pilot data suggested a role for genetics in explaining the cardiovascular risk in the HIPPOCRATES population, and although we observed practically no differences in allele frequencies of six RAS related polymorphisms between patients with CVD and the control group, and although some alleles showed a protective effect, these findings were of little clinical significance for the group as a whole. This does not exclude relevance of our findings for an individual patient with regard to other (intermediate) endpoints. Anyhow, our data suggest that a major contribution of the genetic background, that is, the studied six polymorphisms, to the cardiovascular risk prediction in this south Netherlands primary care population is unlikely.

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Chapter 4

THE INFLUENCE OF SIX CARDIOVASCULAR
POLYMORPHISMS ON A FIRST EVENT OF
ISCHEMIC HEART DISEASE IS MODIFIED BY
GENDER AND AGE

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Submitted

Abstract

This case-control study aimed to examine the contribution of six cardiovascular polymorphisms to the occurrence of a first event of ischemic heart disease (IHD) in a primary care population with a high burden of cardiovascular disease (CVD) including hypertension. Furthermore, we specified the data for gender and age. Patients with a first event of IHD (n=157) were compared with event free controls (n=571). Both groups were genotyped for the AGTR1 (A1166C), AGT (M235T), ACE (4656rpt), NOS3 (E298D), GNB3 (C825T) and ADD1 (G460W) polymorphisms. To assess the association between a first ischemic event and these polymorphisms, univariate and multivariate odds ratios (ORs) were calculated. Sliding mean analyses were performed to show age specific associations. Multivariate ORs indicated a protective association for the carrier status of the T allele of AGT, overall (OR=0.69 [0.34–0.90]) and for males (OR=0.58 [0.27–0.89]). Sliding mean analyses showed for males a continuous protective association with IHD of the T allele of AGT with increasing age, whereas in females an increased risk for IHD was observed with a maximum OR of 1.6 at the age of 56 years. In conclusion, in this population the T allele of the AGT polymorphism is protective for a first event of IHD in males.

Introduction

The renin-angiotensin system (RAS) plays an important role in vascular homeostasis. The biochemical cascade concerns the sequential cleavage of angiotensinogen (AGT) by renin to angiotensin I, and subsequently by angiotensin converting enzyme (ACE) to angiotensin II (Ang II). Besides regulating blood pressure, the vasoactive peptide Ang II has a number of other actions, eventually leading to formation of atherosclerotic lesions.¹⁻³ Therefore, it is commonly accepted that components of the RAS play a role in atherosclerosis and thromboembolic events, e.g. ischemic heart disease (IHD) and stroke.⁴⁻⁶ Many studies have attempted to relate genetic variants of components of RAS to cardiovascular disease (CVD).⁷ Among the many potential genes, those related to activation of the RAS or to an increased tendency to retain sodium rank high and so do genes which may cause reduced availability of nitric oxide (NO). To this date, however, findings from association studies involving the RAS genes remain inconsistent and controversial.^{6,8} Important reasons for this inconsistency is that most of these observations have been obtained in selected populations and the variability of cardiovascular endpoints.⁸

This prompted us to perform a genetic association study on IHD in a primary care population. On the basis of the available literature at the start of the study, we restricted ourselves to the analysis of six polymorphisms. These were the angiotensinogen [AGT (M235T)], angiotensin converting enzyme [ACE (4656_{rpt})], and the angiotensin II type 1 receptor [AGTR1 (A1166C)] polymorphism, that are involved in the generation and action of AngII. In addition, α -adducin [ADD1 (G460W)], G-protein $\beta 3$ subunit [GNB3 (C825T)], and the endothelial nitric oxide synthase [NOS3 (E298D)] polymorphisms, as markers of sodium retaining tendency and altered NO availability, were selected, respectively. The selection criterium for IHD was narrowed down to a first event of IHD. To further reduce the variability of the association with the first event of IHD, we specified the data for gender and age.⁸⁻¹⁰ We hypothesized that the presence of one or more rare alleles of aforementioned six polymorphisms would affect the occurrence of a first event, and that these associations are modified by gender and age. To do so, in the present study, we assessed the contribution of these parameters to patients with a first event of IHD in comparison to control subjects without IHD.

Methods

Participants

For the present study we used data from the HIPPOCRATES project. The HIPPOCRATES (Hypertension: Interaction and Prevalence of **P**OLymorphisms related to Cardiovascular Risk and the **A**ssociation to Treatment Efficacy Study) is an ongoing

study, in which the implementation of genetic polymorphisms in the assessment of cardiovascular risk in primary care is investigated.¹¹⁻¹³ This study utilizes the population of one large general practice in Kerkrade, which is located in the south-east of the Netherlands, and is characterised with a high burden of CVD compared with the average of the Netherlands.¹³ This academic general practice takes part in the Registration Network Family Practices (RNH), which is a continuous and computerized database in which 63 general practitioners (GPs), working in 22 different practices, in the south of the Netherlands participate. All relevant health problems, including a full medical history of all cardiovascular events have been registered since 1990.^{14,15} Medical problems are coded according to the International Classification of Primary Care (ICPC) using the criteria of the International Classification of Health Problems in Primary Care for diagnoses.^{16,17} The general practice in Kerkrade has a list of approximately 4400 patients, among whom are more than 2300 patients of 40 years and older. The practice has well implemented cardiovascular consulting hours. For HIPPOCRATES, all known patients aged ≥ 40 years with CVD or hypertension were invited. From the group with other cardiovascular risk factors, that is, smoking, alcohol, diabetes mellitus, hypercholesterolemia, overweight including obesity, a random 1:3 sample was taken. Of all patients without known CVD, hypertension or other cardiovascular risk factors, also a random 1:3 sample was taken. The initial classification was validated by a thorough assessment of the patients' records combined with additional measurements of risk factors for all subjects. The total HIPPOCRATES population consisted of 803 patients (382 males, 421 females), who were recruited between May 2003 and July 2005.

The study population for the present cross sectional case-control study consisted of 728 Caucasian subjects: 157 patients with a known first event of IHD (cases) and 571 event-free subjects without documented CVD (controls). IHD was defined as having a diagnosis of angina pectoris, myocardial infarction, or heart failure. A first event was defined as the first diagnosis of IHD which was present in the medical registration. An event-free control subject had no IHD, stroke, peripheral artery occlusive disease, or heart failure.

Clinical data collection

Data on risk factors were collected from the RNH from the period before the first event till one year after the event. The latter was done, since data on risk factors are not immediately available due to delay of data entry of medical problems in the registration. Overweight was defined as having a body-mass index (BMI) ≥ 27 (ICPC: T82, T83). Data on smoking behaviour (P17) and alcohol consumption (P15) were collected. Hypertension was defined as a systolic blood pressure (SBP) ≥ 140 mmHg and/or diastolic blood pressure (DBP) ≥ 90 mmHg (diagnosis K86 or K87, respectively) according to the Guidelines for Hypertension specified by the European Society of Hypertension¹⁸ or use of antihypertensive drugs. Hypercholesterolemia was defined

as having a diagnosis of T93 (dyslipidemia) or use of lipid-lowering drugs. Diabetes mellitus was considered to be present in case of fasting plasma glucose levels >6.9 mmol/l (diagnosis T90) or the use of oral antidiabetic drugs or insulin. The computerized medication lists were analysed for antihypertensive, antidiabetic, antiplatelet, and hypolipidemic medication. All relevant data on medication were collected in the period before the first event. All patients gave written informed consent to participate in the study. The study was approved by the Medical Ethics Committee of the Maastricht University Medical Center.

Genetic analyses

The various polymorphisms were determined at the Cardiovascular Genotyping (CAGT) laboratory of the Department of Internal Medicine of the Maastricht University Medical Center. DNA was extracted from whole blood with the use of the QIAmp Blood Kit (Qiagen Inc. Valencia, USA). Genotyping of the angiotensin II type 1 receptor [AGTR1 (A1166C)/rs5186], angiotensinogen [AGT (M235T)/rs699], angiotensin converting enzyme [ACE (4656_{rp1})/rs1799752], endothelial nitric oxide synthase [NOS3 (E298D)/rs1799983], G-protein β 3 subunit [GNB3 (C825T)/rs5443] and α -adducin [ADD1 (G460W)/rs4961] polymorphisms was performed with a multiplex single base extension (SBE) assay (Multiplex SNaPshot, Applied Biosystems, Foster City, USA) similar to previously published protocols.¹⁹ Briefly, in the first stage a multiplex PCR is carried out to amplify the gene fragments that contain the polymorphisms. In the second stage, the polymorphic positions are interrogated by a SBE reaction using fluorescently labeled ddNTPs. The oligos used for the single base extension have specific lengths, varying from 22-50 nt. After the SBE assay, the fragments are size-resolved on an ABI3100 Genetic Analyser: the size of the peaks identifies the locus (e.g. ADD1) and the color of the peak(s) identifies the genotype (e.g. Gly/Trp). Raw data were analysed with Genescan Analysis software (v 3.7), then genotypes were scored independently by two researchers and entered in the database. In 27 patients, a second analysis was necessary to obtain all the genotypes. The ACE insertion/deletion (I/D) polymorphism was determined by detecting ACE_{4656+CT} observed on the SNaPshot as ACE (4656_{rp1}). The use of this SNaPshot method does not allow the direct determination of the I/D polymorphism. The ACE_{4656+CT} polymorphism in the 3'-untranslated region of the ACE gene consists of a repetition of two or three CT dinucleotides and is in complete linkage disequilibrium with ACE (I/D) polymorphism.^{20,21} The multiplex assay was validated on a set of 100 DNA samples that had previously been genotyped with an alternative technique; this technique concerned a multilocus genotyping assay for candidate markers of CVD risk (Roche Molecular systems Inc., Pleasanton, USA) and has been described in detail previously.²² Only four samples gave a discordant result for the eNOS polymorphism. All other genotypes were concordant. The discordant results have been adjusted.

Statistical analyses

Statistical analysis was performed by using SPSS statistical software, version 13.0 (SPSS, Inc., Chicago, IL, USA). A *P*-value of 0.05 or less was considered statistically significant. Bonferroni correction was applied for multiple testing. Statistical significance for differences in quantitative variables between males and females with IHD and event free male and female controls was tested in case of continuous variables by one-way analysis of variance (ANOVA) and the chi-square test was used in case of categorical variables. Genotype and allele frequencies were calculated to test the Hardy-Weinberg equilibrium. Allele frequencies were analyzed using chi-square tests. Only differences in allele frequencies larger than 0.04 between and within males and females were tested for significance.

Logistic regression analyses

Both univariate and multivariate odds ratios (ORs) were calculated with a binary-logistic regression model (method: enter) to evaluate the relationship between polymorphisms and a first event of IHD. For that purpose, dummy variables were created using the homozygous wild-type genotype as reference category. For females only univariate ORs could be calculated, due to the low number of patients that were available to perform meaningful multivariate regression analyses. Age and gender were incorporated in both the univariate as well as in the multivariate logistic regression analyses. As indicated, control subjects were selected from different groups. Therefore, in the logistic regression analyses we had to adjust for this selection and dummy variables had to be created using the normotensive group without cardiovascular risk factors as the reference category. Adjustment for potential confounders was performed by incorporating smoking, alcohol, overweight, diabetes mellitus and hypercholesterolemia into these models. The simultaneous contribution of the six polymorphisms to the prediction of a first event of IHD was tested, using a likelihood ratio test. If two or more polymorphisms reached statistical significance, interactions (effect modification) between two polymorphisms with respect to a first event of IHD were assessed. Again binary-logistic regression analysis (method: enter) was used, in which the alleles at risk, the interaction term between the alleles and the other covariates were incorporated. If an interaction term became statistically significant, the model was explored further.

Sliding means analyses

To explore the influence of increasing age on the risk of a first event of IHD within males or females the sliding means technique for ORs was applied.²³ According to this approach, subgroups of 328 subjects were formed based on increasing age. Each subgroup differed from the preceding or succeeding subgroup by ten individuals. For each subgroup a binary-logistic regression analysis (method: enter) was carried out, to explore the relation between carriership of the rare allele and a first event of IHD.

Covariates were not incorporated because the number of patients available per data point did not allow meaningful regression analyses. For both males and females a curve was fitted using STATA Graph 8.2 (Lowess smoothing technique).²³ Differences between males and females were tested by comparing the area under the curve (AUC), which represents the mean OR calculated for the relevant polymorphism in the univariate analyses.

Results

General characteristics

Baseline characteristics of the study population are summarized in Table 4.1. Overall, the first event group differed from the control group with respect to established cardiovascular risk factors, except for the occurrence of hypertension and diabetes. For both male and female cases, the mean age at which the first cardiovascular event occurred was below 60 years. On average, male cases smoked more cigarettes and used more alcohol. Cases with a first IHD event used more often antihypertensive, antidiabetic, antiplatelet, and hypolipidemic medication as compared to controls. There was no statistically significant difference between male and female cases in terms of the presence of overweight, hypertension, diabetes and hypercholesterolemia. The control group contained more females than males; their mean age was comparable with the first event group.

Table 4.1 Characteristics of the HIPPOCRATES study population (n=728).

	First event of IHD		Controls		P
	Male (n=105)	Female (n=52)	Male (n=240)	Female (n=331)	
Age (years)	57.6 ± 10.4	58.5 ± 11.3	59.3 ± 11.3	60.4 ± 11.4	0.135
Risk factors					
Overweight, n (%)	28 (26.7)	18 (34.6)	32 (13.3)	46 (13.9)	<0.001 ^a
Smoking, n (%)	66 (62.9)	22 (42.3)	41 (17.1)	50 (15.1)	<0.001 ^a
Alcohol, n (%)	8 (7.6)	0 (0.0)	5 (2.1)	1 (0.3)	<0.001 ^a
Hypertension, n (%)	47 (44.8)	27 (51.9)	88 (36.7)	135 (40.8)	0.169 ^a
Diabetes, n (%)	14 (13.3)	7 (13.5)	20 (8.3)	26 (7.9)	0.240 ^a
Hypercholesterolemia, n (%)	31 (29.5)	12 (23.1)	22 (9.2)	22 (6.6)	<0.001 ^a
Medication					
Antihypertensive, n (%)	93 (88.6)	45 (86.5)	86 (35.8)	135 (40.8)	<0.001 ^a
Antidiabetic, n (%)	19 (18.1)	7 (13.5)	13 (5.4)	20 (6.0)	<0.001 ^a
Antiplatelet, n (%)	67 (63.8)	26 (50.0)	15 (6.3)	25 (7.6)	<0.001 ^a
Hypolipidemic, n (%)	53 (50.5)	19 (36.5)	16 (6.7)	31 (9.4)	<0.001 ^a

Values are given as mean ± SD or as number of subjects (with corresponding percentages between brackets); IHD, ischemic heart disease; P, P-values are the results of one-way analysis of variance (continuous variables) and ^achi square test (categorical variables) for overall differences between males and females with a first event of IHD event free male and female controls.

Genetic analyses

Genotype and allele frequencies of all polymorphisms were in Hardy-Weinberg equilibrium. No statistically significant differences in allele frequencies were found between cases and controls, except between male cases with IHD and male controls for GNB3 ($P=0.03$) and between male and female controls for ecNOS ($P=0.04$) (Table 4.2). Additionally, for AGT a borderline statistically significant difference in allele frequency was observed ($P=0.08$) between males with IHD and male controls. Other differences in allele frequency, that is, larger than 0.04 between and within males and females, were not statistically significant.

Table 4.2 Allele frequencies of cardiovascular polymorphisms in a primary care population.

Gene polymorphism		First event of IHD			Controls		
		Overall (n=157)	Male (n=105)	Female (n=52)	Overall (n=571)	Male (n=240)	Female (n=331)
AGTR1 (A1166C)	A	0.729	0.719	0.750	0.703	0.704	0.702
	C	0.271	0.281	0.250	0.297	0.296	0.298
AGT (M235T)	M	0.653	0.652	0.654	0.606	0.583	0.622
	T	0.347	0.348	0.346	0.394	0.417	0.378
ACE (4656 _{rrt})	C	0.545	0.562	0.510	0.512	0.510	0.514
	G	0.455	0.438	0.490	0.488	0.490	0.486
ecNOS (E298D)	G	0.650	0.676	0.596	0.637	0.671	0.612
	T	0.350	0.324	0.404	0.363	0.329*	0.388*
GNB3 (C825T)	C	0.672	0.657	0.702	0.718	0.738	0.704
	T	0.328	0.343 [†]	0.298	0.282	0.262 [†]	0.296
ADD1 (G460W)	G	0.780	0.786	0.769	0.778	0.790	0.770
	T	0.220	0.214	0.231	0.222	0.210	0.230

IHD, ischemic heart disease; *Male versus female controls: $P=0.04$; [†]Males with IHD versus male controls: $P=0.03$.

Logistic regression analyses

Both univariate and multivariate ORs for a first IHD event were calculated (Table 4.3). In the multivariate analysis, the simultaneous contribution of the six polymorphisms to the prediction of a first event was statistically significant for all subjects and males, respectively (likelihood ratio test; $P<0.001$), showing a protective association for carriership of the T allele of the AGT polymorphism (OR=0.58 [0.27-0.89]). After Bonferroni correction, this association was still statistically significant ($P=0.04$). In addition, we found a trend towards a protective effect for AGTR1 (OR=0.65 [0.42-1.01]), which was also observed for females in the univariate analysis (OR=0.56 [0.29-1.09]). In all other polymorphisms, carriership of the rare allele of the investigated polymorphisms was not associated with an increased risk of IHD. Evaluation of possible interactions between AGT and AGTR1 showed no statistically significant interaction terms for both males and females together or for males alone.

Table 4.3 Univariate and multivariate odds ratios for a first event of ischemic heart disease according to the gene polymorphisms.

Polymorphism	Rare-allele*	Univariate odds ratio (95% CI)			Multivariate odds ratio (95% CI)		
		Overall	Male	Female	Overall	Male	Female
AGTR1 (A1166C)	C*	0.73 (0.48–1.11)	0.87 (0.50–1.52)	0.56 (0.29–1.09)	0.65 (0.42–1.01)	0.71 (0.39–1.29)	n.d.
AGT (M235T)	T*	0.60 (0.39–0.91) [#]	0.54 (0.31–0.95) [#]	0.66 (0.34–1.27)	0.69 (0.34–0.90) [#]	0.58 (0.27–0.89) [#]	n.d.
ACE (4656 _{ins})	G*	0.97 (0.60–1.58)	0.91 (0.51–1.90)	0.93 (0.48–1.80)	0.98 (0.58–1.66)	0.93 (0.48–1.80)	n.d.
NOS3 (E298D)	T*	1.00 (0.66–1.51)	0.90 (0.53–1.53)	1.11 (0.56–2.18)	1.05 (0.67–1.65)	1.08 (0.58–1.98)	n.d.
GNB3 (C825T)	T*	1.19 (0.79–1.79)	1.26 (0.74–2.14)	1.08 (0.57–2.07)	1.11 (0.71–1.73)	1.19 (0.65–2.18)	n.d.
ADD1 (G460W)	T*	0.85 (0.56–1.30)	0.75 (0.42–1.32)	1.00 (0.52–1.94)	0.84 (0.53–1.34)	0.70 (0.37–1.33)	n.d.

*The wild-type alleles were used as reference category; n.d., indicates: no meaningful multivariate regression analyses could be performed due to few numbers available; CI denotes 95% confidence interval; [#] $p \leq 0.05$; multivariate adjustment was performed for age, sex, smoking, alcohol, overweight, diabetes mellitus and hypercholesterolemia.

Sliding means analyses

For AGT the effect of increasing age was explored by the sliding means technique for ORs (Figure 4.1). For males a protective trend was observed, whereas in females the T allele is harmful till the age of approximately 65. This observed difference between males and females was statistically significant ($P<0.001$). The maximum OR for the Lowess-curve in female cases was estimated 1.6 at the age of 56 years old.

Figure 4.1

Sliding OR analysis of the interaction between carriership of the T allele of AGT and age for males and females with respect to the risk of a first event of ischemic heart disease. Each endpoint represents the unadjusted OR (T allele versus MM genotype) of 328 individuals according to age and gender and differs from the preceding or succeeding point by 10 individuals. The observed difference between males and females is statistically significant ($P<0.001$).

Discussion

In the present study, no statistically significant differences in allele frequencies were found between cases and controls, except for a higher frequency of the T allele of GNB3 ($P=0.03$) in male cases compared to male controls and a higher frequency of the T allele of ecNOS ($P=0.04$) in female controls compared to male controls. However, multivariate analyses showed a protective association for carriership of the T allele of AGT, overall (OR=0.69 [0.34–0.90]) and for males (OR=0.58 [0.27–0.89]). Sliding mean analyses indicated a statistically significant, but opposite age-associated trend for this polymorphism between males and females.

Gender and age

Gender-specific associations of cardiovascular phenotypes with a variety of candidate genes have been reported previously.²⁴⁻²⁸ In most cases, they appear as rather

unexpected findings and are difficult to explain in terms of straightforward pathophysiological mechanisms. These kind of results are often marked as type I errors, due to sample stratification with consequent multiple comparisons and in some cases a small number of individuals in a subgroup.²⁹ Our study is certainly not protected from such a risk. However, a few considerations may support the plausibility of a gender-specific association. First of all, gender was a statistically significant co-variate in the overall multivariate analyses, irrespective of correction for multiple testing. Secondly, it is known from literature, that hypertension and IHD are age-dependent^{30,31}, and that females develop both conditions at an older age.³⁰⁻³³ However, in our study population the age at onset for IHD is approximately the same for both males and females (Table 4.1). Since, age-related phenotypes (e.g. overweight, hypertension, diabetes and hypercholesterolemia) are present to the same extent for both male and female cases, a gender-specific association is plausible in our study.

The influence of age on the association of cardiovascular polymorphisms with cardiovascular phenotypes has not yet been completely elucidated. Several authors have underlined the importance of taking into account pathophysiological changes related to ageing when analysing the phenotypic effect of genetic variants.^{34,35} For instance, Province *et al.*, found in a study in which they investigated the association of higher blood pressure values with ADD1 (G460W), that only in older individuals this association was observed. They discussed that this is probably due to a reduced efficiency of compensatory mechanisms at an older age.³⁴

Angiotensinogen (M235T) polymorphism

The opposing associations of the T allele of AGT with IHD for males and females need further explanation. Recently, a meta-analysis by Sethi *et al.*, including 127 studies (n=45,267) stratified by gender, concluded that the M235T polymorphism did not predict risk of IHD or myocardial infarction, neither for males nor females.³⁶ However, when looking in detail in their data these authors also showed a harmful association for women homozygous for the T allele and a protective association for males. These data were however not statistically significant. Another recent study performed by Sekuri *et al.*, found that patients with the TT genotype had a decreased risk of premature coronary heart disease.³⁷ Such an association was reported earlier for myocardial infarction.³⁸ However, in both studies no subgroup analysis was performed on gender.

A speculative explanation for our observations might be a low-renin state, which can be enhanced by the GNB3 (C825T) polymorphism and older age.³⁹⁻⁴¹ A protective association for the T allele of AGT may under these conditions be related to lower angiotensin levels. This was confirmed by a study performed by Fernández-Arcás *et al.*, who indeed found a suppressed RAS in carriers of the T allele of AGT.³⁸ In combination with the higher frequency of the T allele of GNB3 in males, this could

translate to the observed ORs. This is also suggested by Schunkert *et al.*, who hypothesized that the difference between males and females is rather a genetic alternation than an effect induced by for instance blood pressure.³⁹ Another explanation might be the recently indicated misclassification of the AGT (M235T) genotype.⁴² However, in our study the validation process of our multiplex assay did not reveal discordant results for this polymorphism.

Strengths and limitations

The present study has several limitations. Firstly, we only studied the effect of one polymorphism per gene, which does not exclude the role of other polymorphic alleles, which are not in linkage disequilibrium. Another limitation for the present study as is for many genetic association studies is sample size. It has been emphasized that sample sizes of thousands are required to have adequate power to detect genes conferring ORs as low as 1.2, with minor allele frequencies in the range of 5-10%.⁴³ In our study the actual allele frequencies of the rare alleles ranged between 0.22 and 0.55, and the ORs for a first event were much larger than 1.2, indicating that the sample size of our study, at least for the AGT polymorphism, was acceptable. Furthermore, one could object that the definition of IHD included heart failure. However, in primary care the diagnosis heart failure is mostly caused by an event of angina pectoris or myocardial infarction. Nevertheless, the association maintained after exclusion of this type of event.

Our study has also some strong aspects, including a primary care population in which the whole range of low-to-high-risk patients is available. Although our study has an exploratory character, we performed this study with a clear *a priori* hypothesis, therefore reducing multiple hypothesis testing. Additionally, our population was selected using strict inclusion criteria and was derived from a rather small area, including subjects with similar social living conditions.

In conclusion, this study provides evidence that in this primary care population the T allele of the AGT polymorphism is protective for males with a first event of IHD. Our data confirm that age and gender are not simply confounders in genetic epidemiology but represent a key to a better understanding of the underlying mechanisms of pathological phenotypes.

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Chapter 5

GENDER-SPECIFIC EFFECT OF THE α -ADDUCIN (G460W) AND AGTR_I (A1166C) POLYMORPHISM ON CAROTID INTIMA MEDIA THICKNESS

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Submitted

Abstract

Objective

In a primary care population covering a broad spectrum of cardiovascular risk (HIPPOCRATES project) the relationship between carotid intima media thickness (CIMT) and six cardiovascular polymorphisms was analysed in a cross sectional study.

Methods

CIMT was assessed in 618 patients, who were genotyped for the AGTR1 (A1166C), AGT (M235T), ACE (4656_{pt}), NOS3 (E298D), GNB3 (C825T) and ADD1 (G460W) polymorphisms. Linear regression analyses were performed to assess the relationship between CIMT and the polymorphisms.

Results

The study population included 289 males (46.8%) and 329 (53.2%) females of whom 52.3% were treated with cardiovascular medication. CIMT increased for both males and females with age ($P<0.001$) after correction for the arterial diameter. CIMT was significantly associated with age, female gender, use of cardiovascular medication, waist circumference and dyslipidemia. After correction for these covariates, multivariate linear regression analyses showed that only in females the C allele of AGTR1 was associated with a thicker CIMT ($P=0.03$). The T allele of ADD1 was associated with a smaller CIMT in both males and females, but this only reached statistical significance in females ($P=0.03$).

Conclusion

Although the effect of both genetic variants on CIMT was small, this study showed a statistically significant effect of AGTR1 and ADD1 in females. However, our findings should be viewed as hypothesis generating and require further confirmation in prospective epidemiological primary care studies.

Introduction

Carotid intima media thickness (CIMT) is often increased in patients with cardiovascular risk factors, such as hypertension, diabetes mellitus and hypercholesterolemia.^{1,2} It has also been suggested that an increased CIMT is an independent predictor of future myocardial infarction, stroke and peripheral artery disease.¹⁻³ Consequently, CIMT is regarded as a surrogate marker for pre-clinical atherosclerosis.^{1,4} Current research has shown that CIMT is not only associated with traditional cardiovascular risk factors, but also with certain genetic variants. Among the many potential genes, those related to activation of the renin-angiotensin system (RAS) or to an increased tendency to retain sodium rank high, and so do genes which may cause reduced availability of nitric oxide (NO). Indeed, some studies suggest that certain genetic polymorphisms of the RAS may be important modifiers of CIMT.^{5,6} Most of these observations, however, have been obtained in selected populations and this prompted us to address such associations in a primary care population. On the basis of the available literature at the start of the study, we restricted ourselves to the analysis of only six polymorphisms. These were the angiotensinogen [AGT (M235T)], angiotensin converting enzyme [ACE (4656_{rpt})] and the angiotensin II type 1 receptor [AGTR1 (A1166C)] polymorphism, because these genes are involved in the generation and action of angiotensin II (Ang II).⁷⁻⁹ In addition, we assessed the role of polymorphisms which may be related to an enhanced reabsorption of sodium, that is, the G-protein $\beta 3$ subunit [GNB3 (C825T)] and α -adducin [ADD1 (G460W)] polymorphism.^{10,11} Finally, we looked at the endothelial nitric oxide synthase [NOS3 (E298D)] polymorphism because this may be associated with altered NO availability.¹² We hypothesized that the presence of one or more rare alleles of the aforementioned polymorphisms would affect CIMT values. Therefore, in the present study, we aimed to assess the association of these six polymorphisms with CIMT.

Methods

Study population

For this study we used data from the HIPPOCRATES project. The HIPPOCRATES project (**H**ypertension: **I**nteraction and **P**revalence of **P**OLymorphisms related to **C**ardiovascular **R**isk and the **A**ssociation to **T**reatment **E**fficacy **S**tudy) is an ongoing study, in which the implementation of genetic polymorphisms in the assessment of cardiovascular risk in primary care is investigated.¹³⁻¹⁵ All participants for this project were recruited from one general practice in Kerkrade, The Netherlands, between May 2003 and July 2005. This academic general practice takes part in the Registration Network Family Practices (RNH), which is a continuous and computerized database. All relevant health problems, including a full medical history of all cardiovascular events have been registered since 1990.¹⁶ Medical problems are coded according to

the International Classification of Primary Care (ICPC) using the criteria of the International Classification of Health Problems in Primary Care for diagnoses.^{17,18} The general practice in Kerkrade has a list of approximately 4400 patients, among whom are more than 2300 patients of 40 years and older. The practice is located in a region with a high burden of cardiovascular disease (CVD) and cardiovascular risk factors and, therefore, it has well implemented cardiovascular consulting hours. For the HIPPOCRATES project, all known patients aged at least 40 years with CVD and hypertension were invited. From the group with cardiovascular risk factors other than hypertension, that is, smoking, alcohol, diabetes mellitus, hypercholesterolemia, overweight including obesity, a random 1:3 sample was taken. Of all patients without known CVD, hypertension or other cardiovascular risk factors, also a random 1:3 sample was taken. Classification was validated by thorough analysis of the patients' records combined with additional measurements of risk factors for all patients. The total HIPPOCRATES population consisted of 803 patients (382 males, 421 females). During their first visit, participants were asked permission to measure their CIMT. Included patients did not differ in baseline characteristics from those who did not consent (185 patients). CIMT was measured successfully in all 618 patients: 289 males and 329 females. These were included in the present study. The study was approved by the Medical Ethics Committee of the Maastricht University Medical Center. All patients gave written informed consent to participate in the study.

Clinical data collection

All clinical measurements were performed in the practice by one of the authors (AWP; first measurement) and a nurse practitioner (second, third and fourth measurement with in between periods of three months). The nurse practitioner measured weight, blood pressure and heart rate. All persons were questioned on their smoking behaviour and alcohol consumption. Weight and height of all patients were measured. Body-mass index (BMI) was calculated as body weight divided by squared height. The size of the waist circumference was measured. Blood pressure and heart rate were measured after ten minutes rest in sitting position, using a sphygmomanometer. Fasting blood samples were drawn to determine serum creatinine, total cholesterol, low-density lipoprotein-cholesterol (LDL-c), high-density lipoprotein-cholesterol (HDL-c), serum triglycerides and glucose levels and were analyzed in one regional laboratory. Microalbuminuria was determined semi quantitatively in a first morning urine sample.

Treatment status

The computerized medication lists were analyzed for antihypertensive, hypolipidemic, antiplatelet and antidiabetic medication. Furthermore, all persons were questioned about their medication use at the start of the study. Cardiovascular medication was based on the anatomical therapeutic classification (ATC) and defined as having at least

one prescription of antihypertensive (ATC level 2: C02, C03, C04, C07, C08, C09), hypolipidemic (ATC level 2: C10), or antiplatelet (ATC level 5: B01AC) medication in the selected study period. This definition was chosen since these drugs are most frequently prescribed for the prevention of CVD in primary care. Combination therapy was defined as having at least two different prescriptions of antihypertensive, hypolipidemic or antiplatelet medication used concomitantly during the study period. Antidiabetic medication was defined as having at least one prescription of A10 (ATC level 2) in the study period.

Definitions

Hypertension was defined as a systolic blood pressure (SBP) at least 140 mmHg, diastolic blood pressure (DBP) at least 90 mmHg or both according to the Guidelines for Hypertension specified by the European Society of Hypertension.¹⁹ Mean arterial pressure (MAP) was calculated as $MAP = (2 \times DBP + SBP) / 3$. The presence of dyslipidemia was defined as the total cholesterol to HDL-c ratio exceeding 4.0. Diabetes mellitus was considered to be present in case of fasting plasma glucose levels above 6.9 mmol/l. Creatinine clearance was calculated by the Cockcroft-Gault formula. Microalbuminuria was defined as albumin excretion above 20 mg/l.

Carotid IMT

CIMT measurements were performed by one of the authors (AWP) at the Circulation Laboratory of the Department of Internal Medicine of the Maastricht University Medical Center. To measure CIMT, high-resolution B-mode ultrasonography was performed with ESAOTE PICUS 3 equipped with a 7.5-MHz. linear array transducer. The left and right common carotid arteries were examined in anterolateral, posterolateral and medialateral directions. Measurements were performed 1 cm proximal to the beginning of the dilatation of the carotid bulb. The mean CIMT of the far wall of the common carotid artery was measured automatically over 1 cm, during seven heartbeats and was accepted as correct as the mean SD <0.05, according to the Mannheim Carotid Intima Media Thickness Consensus.¹ In order to correct for asymmetry of the arterial vessel and to enhance reproducibility, CIMT was measured at four different angles and mean values were calculated for both the left and the right carotid artery. The internal diameter of the carotid artery was defined as the distance between the media-adventitia interfaces of the near and far arterial wall. Carotid atheromatous plaques were defined as focal structures encroaching into the arterial lumen and where its thickness was at least 50% greater than that of the adjacent sites.¹ For this article, plaques were not analyzed.

Genetic analyses

The various polymorphisms were determined at the Cardiovascular Genotyping (CAGT) Laboratory of the Department of Internal Medicine of the Maastricht

University Medical Center. DNA was extracted from whole blood with the use of the QIAmp Blood Kit (Qiagen Inc., Venlo, the Netherlands). Genotyping of the angiotensin II type 1 receptor [AGTR1 (A1166C)/rs5186], angiotensinogen [AGT (M235T)/rs699], angiotensin converting enzyme [ACE (4656_{rpt})/rs1799752], endothelial nitric oxide synthase [NOS3 (E298D)/rs1799983], G-protein β 3 subunit [GNB3 (C825T)/rs5443] and α -adducin [ADD1 (G460W)/rs4961] polymorphisms was performed with a multiplex single base extension (SBE) assay (Multiplex SNaPshot, Applied Biosystems, Foster City, California, USA) similar to previously published protocols.²⁰ Briefly, in the first stage a multiplex PCR is carried out to amplify the gene fragments that contain the polymorphisms. In the second stage, the polymorphic positions are interrogated by a SBE reaction using fluorescently labeled ddNTPs. The oligos used for the single base extension have specific lengths, varying from 22-50 nt. After the SBE assay, the fragments are size-resolved on an ABI3100 Genetic Analyser (Applied Biosystems): the size of the peaks identifies the locus (e.g. ADD1) and the color of the peak(s) identifies the genotype (e.g. Gly/Trp). Raw data were analyzed with Genescan Analysis software (v 3.7), then genotypes were scored independently by two researchers and entered in the database. In 27 patients, a second analysis was necessary to obtain all the genotypes. The ACE (I/D) polymorphism was determined by detecting ACE_{4656+CT} observed on the SNaPshot as ACE (4656_{rpt}). The use of this SNaPshot method does not allow the direct determination of the insertion/deletion polymorphism. The ACE_{4656+CT} polymorphism in the 3'-untranslated region of the ACE gene consists of a repetition of two or three CT dinucleotides and is in complete linkage disequilibrium with ACE (I/D).^{21,22} The multiplex assay was validated on a set of 100 DNA samples that had previously been genotyped with an alternative technique; this technique concerned a multilocus genotyping assay for candidate markers of CVD risk (Roche Molecular systems Inc., Alameda, California, USA) and has been described in detail previously.²³ Only four samples gave a discordant result for the NOS3 polymorphism. All other genotypes were concordant. The discordant results have been adjusted.

Statistical analysis

Statistical analysis was performed using SPSS statistical software, version 13.0 (SPSS, Inc., Chicago, IL, USA). A *P*-value of 0.05 or less was considered statistically significant. Statistical significance for differences in quantitative variables was tested by Student's *t*-test or Mann-Whitney *U* test when appropriate. In case of categorical variables, the chi-square test was used. Overall mean CIMT values were calculated by taking the average of the left and right mean CIMT values. Correction for gender differences in arterial diameter was provided by calculation of the CIMT/internal carotid diameter ratio. Since we expected potential differences between males and females, we analyzed data with respect to gender separately.

A power calculation indicated that the HIPPOCRATES population was large enough to calculate differences between groups. Assuming allele frequencies ranging between 0.51 and 0.79, a total group number of 590–635 would be sufficient to detect a clinical

significant difference in CIMT of 0.035 mm with a power of 80% ($\alpha=0.05$). Genotype and actual allele frequencies were calculated to test the Hardy-Weinberg equilibrium. Furthermore, allele frequencies were analyzed using chi-square tests. Differences in frequency between groups larger than 0.04 were tested for significance. Significant co-variables for the relationship between CIMT and polymorphisms were traced by multivariate linear regression analysis using backward model selection. To avoid collinearity, waist circumference was used instead of waist-to-hip ratio or BMI and condensed measures were used like diabetes and dyslipidemia as defined earlier. Recent research has shown that waist circumference is a better marker of abdominal fat accumulation than waist-to-hip ratio or BMI²⁴, which is strongly associated with increased cardiovascular risk.²⁵ MAP was used because it is constant along the arterial tree and hence reflects accurately the impact of pressure on the carotid segments. *P*-values for co-variables to stay in the model were set at 0.10. Because of the skewed distribution of CIMT, log transformation was applied to achieve normality for further analysis. The contribution of all co-variables to CIMT was calculated as the estimated effect on population mean CIMT. Univariate and multivariate linear regression analyses were performed to evaluate the relationship between polymorphisms and CIMT. For that purpose, the homozygous wild-type genotype was used as reference category. Age and gender, both demographic variables, were incorporated in both the univariate as well as the multivariate linear regression analyses. As indicated, we selected patients from different groups. Therefore, in the regression analyses, we had to adjust for this selection by creating dummy variables using the normotensive group without cardiovascular risk factors as the reference category. Adjustment for potential confounders was performed by incorporating age, gender, waist circumference, dyslipidemia and use of cardiovascular medication into the model (enter procedure). The simultaneous contribution of the six polymorphisms to the prediction of increased CIMT was determined, using the overall *F*-test. If two or more polymorphisms reached statistical significance, interactions (effect modification) between these polymorphisms with respect to CIMT were assessed. Again linear regression analysis was used, in which the alleles at risk, the interaction term between the alleles and the other covariates were incorporated. If an interaction became statistically significant, the model was explored further.

Results

Characteristics of the participants

Baseline characteristics of the study population are summarized in Table 5.1. There were slightly more females (53.2%) than males (46.8%). Overall, 52.3% of these patients were treated with cardiovascular medication. Males more often had a history of ischemic heart disease, used more antiplatelet and hypolipidemic medication as compared to females. Additionally, they used more often alcohol, they had a larger

waist circumference and a lower HDL-c. Their creatinine clearance was higher as compared with females and they had more often microalbuminuria. The observed difference in CIMT between males and females (Table 5.1) was not statistically significant, except for the age category 50 – 59 ($P=0.02$). However, females have a higher CIMT / diameter ratio than males, because the internal diameter of their carotid artery is smaller (Table 5.1). For both males and females this ratio increased with age (Figure 5.1; $P<0.001$).

Table 5.1 Characteristics of the HIPPOCRATES study population (n= 618).

	Overall (618)	Males (289)	Females (329)	P
Age (years)	60.5 ± 10.6	60.8 ± 10.4	60.3 ± 10.8	0.592
Morbidity				
History of ischemic heart disease, n (%)	117 (18.9)	78 (27.0)	39 (11.9)	<0.001 ^a
History of stroke, n (%)	39 (6.3)	24 (8.3)	15 (4.6)	0.056 ^a
Peripheral artery occlusive disease, n (%)	23 (3.7)	12 (4.2)	11 (3.3)	0.596 ^a
Heart failure, n (%)	12 (1.9)	7 (2.4)	5 (1.5)	0.417 ^a
Risk factors				
Body-mass index (kg/m ²)	27.8 ± 4.5	27.5 ± 3.9	28.0 ± 5.0	0.171
Waist (cm)	95.1 ± 13.0	100.0 ± 10.9	93.4 ± 14.9	<0.001
MAP (mmHg)	103.3 ± 12.1	102.9 ± 11.3	103.7 ± 12.8	0.393
Heart rate (beats per minute)	74.3 ± 11.2	72.3 ± 12.0	76.1 ± 10.1	<0.001
Current smoking, n (%)	117 (18.9)	59 (20.4)	58 (17.6)	0.378 ^a
Alcohol, n (%)	14 (2.3)	11 (3.8)	3 (0.9)	0.016 ^a
Diabetes, n (%)	85 (13.8)	40 (13.8)	45 (13.7)	0.953 ^a
Dyslipidemia, n (%)	342 (55.3)	166 (57.4)	176 (53.5)	0.275 ^a
Microalbuminuria, n (%)	66 (10.7)	41 (14.2)	25 (7.6)	0.008 ^a
Biochemical				
Creatinine clearance ^c (ml/min)	77.9 ± 22.7	83.4 ± 23.4	73.0 ± 20.9	<0.001 ^b
Total cholesterol (mmol/l)	5.6 ± 1.1	5.4 ± 1.0	5.7 ± 1.1	0.001
LDL-cholesterol (mmol/l)	3.4 ± 1.0	3.3 ± 0.9	3.4 ± 1.0	0.721
HDL-cholesterol (mmol/l)	1.5 ± 0.4	1.3 ± 0.4	1.7 ± 0.5	<0.001
Triglycerides (mmol/l)	1.6 ± 0.9	1.6 ± 0.9	1.5 ± 0.8	0.077
Glucose (mmol/l)	5.7 ± 1.5	5.8 ± 1.4	5.6 ± 1.6	0.003 ^b
Medication				
Antihypertensive, n (%)	293 (47.4)	146 (50.5)	147 (44.7)	0.147 ^a
Hypolipidemic, n (%)	124 (20.1)	69 (23.9)	55 (16.7)	0.027 ^a
Antiplatelet, n (%)	144 (23.3)	85 (29.4)	59 (17.9)	0.001 ^a
Antidiabetic, n (%)	48 (7.8)	21 (7.3)	27 (8.2)	0.663 ^a
Arterial measurements				
Carotid atheromatous plaques, n (%)	92 (14.9)	39 (13.5)	53 (16.1)	0.362 ^a
Carotid intima media thickness (μm)	684 ± 137	680 ± 142	688 ± 132	0.342 ^b
Internal diameter of carotid artery (mm) ^d	6.2 ± 0.9	6.5 ± 0.8	5.9 ± 0.8	<0.001
Carotid IMT / diameter ratio	0.091 ± 0.02	0.086 ± 0.02	0.095 ± 0.01	<0.001 ^b

Values are given as mean ± SD or as number of patients (with corresponding percentages in brackets); P, P values are the results of ^a chi square test (categorical variables) or Student's *t*-test or ^b Mann-Whitney *U* test (continuous variables) for differences between males and females; ^c creatinine clearance by the Cockcroft-Gault formula; ^d The distance between the media-adventitia interfaces of the near and far arterial wall; MAP, mean arterial pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Allele frequencies

Genotype and allele frequencies of all polymorphisms were in Hardy-Weinberg equilibrium. No statistically significant differences in allele frequencies were found between males and females, except for NOS3 ($P=0.01$). For this polymorphism, females had a higher frequency of the T allele (Table 5.2).

Figure 5.1 Carotid intima media thickness corrected for the internal diameter of the carotid artery by age categories (years) for males (n=289) and females (n=329) ($P<0.001$). Values are means $\pm$ standard error.

Table 5.2 Allele frequencies of polymorphisms.

Gene polymorphism		Overall (n=618)	Male (n=289)	Female (n=329)
AGTR1 (A1166C)	A	0.708	0.713	0.704
	C	0.292	0.287	0.296
AGT (M235T)	M	0.607	0.611	0.603
	T	0.393	0.389	0.397
ACE (4656 _{rrt})	C	0.515	0.512	0.517
	G	0.485	0.488	0.483
ecNOS (E298D)	G	0.642	0.678	0.611
	T	0.358	0.322*	0.389*
GNB3 (C825T)	C	0.710	0.711	0.708
	T	0.290	0.289	0.292
ADD1 (G460W)	G	0.786	0.798	0.775
	T	0.214	0.202	0.225

* Males versus females ($P=0.01$)

Intima media thickness and cardiovascular polymorphisms

Stepwise regression analysis identified age, gender, waist circumference, dyslipidemia and use of cardiovascular medication as significant co-variables for the association between the six polymorphisms and CIMT (Table 5.3). In the univariate linear

regression analyses, an association was found between CIMT and carriers of the T allele of ADD1. This association was statistically significant for the overall group (Figure 5.2, $P=0.01$), but when males and females were analyzed separately, only in females statistical significance was reached ($P=0.02$) with or without correction for internal carotid diameter. After correction for all co-variables using multivariate linear regression analyses (Table 5.4), we assessed for the overall group an independent significant relationship between CIMT and ADD1 ($P=0.01$). However, the simultaneous contribution of all six polymorphisms to the change in CIMT (overall F -test) was not statistically significant, except for females ($P=0.04$), due to a statistically significant harmful association for carriers of the C allele of AGTR1 ($P=0.03$) and a protective association for carriers of the T allele of ADD1 ($P=0.03$). For all other polymorphisms, carriers of the rare allele were not statistically significant associated with CIMT. Furthermore, there were no other statistically significant differences for these associations between males and females (Table 5.4). For females, a multivariate post hoc evaluation of possible interactions between presence of the C allele of AGTR1 and homozygosity of the G allele of ADD1 showed overall an increase of 36 μm in CIMT [36.6 (-6.2-76.2)]. This interaction was of borderline statistical significance ($P=0.09$). The CIMT in females with both C*/GG was statistically significant thicker than females with both AA/T* ($P=0.04$, Figure 5.3).

Table 5.3 Relevant co-variables for the association between CIMT and six cardiovascular polymorphisms. Results of multivariate linear regression analysis using backward model selection in 618 participants.

	CIMT (μm)
Coefficient of determination (R^2)	0.5
Intercept	266
Estimated effect on population mean (95% CI) ⁺	
Gender (0,1) ^{**}	23.7 (5.5–43.0) [#]
Age (years)	5.5 (4.1–6.9) [#]
Presence of cardiovascular morbidity (0,1)	-8.2 (-30.8, 15.9)
Use of cardiovascular medication (0,1)	33.6 (9.0, 59.2) [#]
Antidiabetic medication (0,1)	18.0 (-26.8, 65.9)
Current smoking (0,1) [†]	9.0 (-12.8, 31.5)
Alcohol (0,1) [†]	-38.6 (-89.4, 17.3)
Diabetes (0,1) [†]	-20.2 (-53.2, 14.5)
Waist (cm)	27.9 (13.8, 35.1) [#]
Creatinine clearance (ml/min)	0.1 (-0.7, 0.7)
Microalbuminuria (0,1) [†]	-18.9 (-45.6, 9.0)
Dyslipidemia (0,1) [†]	15.9 (1.4, 34.4) [#]
Heart rate (beats per minute)	0.1 (-0.7–1.4)
MAP (10 mmHg)	0.6 (-6.9–6.9)

CIMT, carotid intima media thickness; ⁺ population mean is 684.23 μm ; CI denotes 95% confidence interval; ^{**} Males were coded as 0 and females as 1; [†] Variables were coded 0 or 1 if the condition was absent or present; MAP, mean arterial pressure; [#] $P\leq0.05$.

Figure 5.2 Carotid intima media thickness (μm) for carriers of the normal (GG) as of the rare allele (T*) for the ADD1 (G460W) polymorphism ($P=0.01$). Values are unadjusted means $\pm$ standard error.

Table 5.4 Linear regression model for six polymorphisms and carotid intima media thickness for all patients and for males and females separately.

Polymorphism	Rare allele*	Multivariate analysis (95% CI) ^{†‡}		
		Overall (618)	Male (289)	Female (329)
AGTR1 (A1166C)	C*	10.3 (-6.8, 27.2)	0.1 (-25.5, 26.5)	25.1 (3.4, 47.4) [#]
AGT (M235T)	T*	9.0 (-8.2, 26.5)	14.5 (-12.2, 41.6)	0.7 (-21.5, 24.4)
ACE (4656 _{rpt})	G*	-4.8 (-23.5, 14.5)	-8.8 (-37.2, 20.8)	-5.5 (-30.8, 20.1)
NOS3 (E298D)	T*	6.2 (-10.9, 23.7)	8.3 (-16.9, 34.4)	3.4 (-19.6, 26.5)
GNB3 (C825T)	T*	-4.1 (-20.2, 12.4)	11.7 (-14.2, 38.0)	-19.6 (-41.1, 2.1)
ADD1 (G460W)	T*	-22.9 (-38.6, -6.1) [#]	-19.6 (-44.3, 6.9)	-23.5 (-44.3, -2.7) [#]

* The wild-type alleles were used as reference category; ‡ Data presented as unstandardized β coefficient (95% CI): the β represents the change in carotid intima media thickness (μm) for each unit change in continuous risk factors and for a change (absent or present) in dichotomous risk factors; CI denotes 95% confidence interval; † Multivariate adjustment was performed for age, gender, waist, dyslipidemia and use of cardiovascular medication; # $P \leq 0.05$.

Figure 5.3 Carotid intima media thickness (μm) for carriers of the normal versus the rare alleles of the AGTR1 (A1166C) and ADD1 (G460W) polymorphism. AGTR1 / AA plus ADD1 / T* versus AGTR1 / C* plus ADD1 / GG ($P=0.04$). Values are unadjusted means $\pm$ standard error.

Discussion

To the best of our knowledge, this is the first study on CIMT and cardiovascular polymorphisms in a primary care population reflecting current daily practice. In the present study, we found that with increasing age, females show a greater CIMT compared to males if correction for the diameter of the artery is applied ($P < 0.001$). Other variables that were statistically significant associated with CIMT were use of cardiovascular medication, waist circumference and dyslipidemia, which was in accordance with previous findings.^{2,26} In multivariate linear regression analyses a statistically significant harmful association of the C allele of AGTR1 with CIMT was found for females only ($P = 0.03$). For the T allele of ADD1 a protective association was found in males and females, but statistical significance was only reached in females ($P = 0.03$). Post-hoc analyses revealed a potential synergism between AGTR1 and ADD1 in relation to CIMT (Figure 5.3).

CIMT and gender differences

In most studies male gender is associated with increased CIMT.^{2,27,28} Females have most often the thinnest CIMT.^{2,27,28} For the CIMT/internal carotid diameter ratio, the same kind of results are observed, though this ratio yields the weakest associations.²⁹ Our study results are not in line with these findings, especially if we look at CIMT values corrected for differences in arterial diameter (Table 5.1). A possible explanation might be that males are treated more than females with cardiovascular risk-reducing therapies (Table 5.1).

Angiotensin II type I receptor (A1166C) polymorphism

The observed association between carriers of the rare C allele of AGTR1 and higher CIMT has been reported by other authors.^{30,31} The mechanism for this association remains unclear, because the AGTR1 polymorphisms is located in the 3'-untranslated region (3'-UTR) of the chromosome 3q22³² and, therefore, the amino-acid sequence of the receptor is not altered. However, recently, the 3'-UTR site has been shown to be involved in microRNA gene silencing.³³ MicroRNAs are single-stranded RNA molecules of about 21-23 nucleotides in length, which regulate gene expression. They are processed from primary transcripts and their main function is to down-regulate gene expression (gene silencing). The C allele carriers have impaired microRNA binding and a consequent impairment of translation attenuation, resulting in increased receptor density. Such AGTR1 receptor alterations have a number of possible effects which may influence CIMT, including increased extracellular matrix (ECM) formation, smooth muscle cell (SMC) migration, oxidation of LDL-c and the formation of reactive oxygen species (ROS), eventually initiating the inflammation process in the vascular cells.³⁴ Previously, we have shown that there is also an

increase in angiotensin II sensitivity.³⁵ So, these mechanisms might explain the observed higher CIMT for C allele carriers of the AGTR1 polymorphism. Evidence for gender differences with respect to these mechanisms was not present in the literature.

Alpha-adducin (G460W) polymorphism

Several studies have reported an association between ADD1 (G460W) polymorphism, salt sensitivity, or high sodium intake, and an increased risk of cardiovascular events and mortality.^{36,37} Moreover, the risk of cardiovascular events and mortality was shown to be increased in T allele carriers with high systolic blood pressure.³⁸ Recently, in the Rotterdam study and in another study in young adult males performed by Sarzani *et al.*, an increased CIMT was found for carriers of the T allele.^{39,40} Balkestein *et al.*, found that the T allele of ADD1 was associated with increased femoral artery IMT, but only in the context of the ACE DD genotype.⁴¹ The results of our study are not in line with these findings, that is, female carriers of the T-allele are associated with a lower CIMT, indicating that they are at lower risk for developing CVD in comparison with females with the GG genotype. Additionally, compared with males, female carriers of the T-allele have a significantly lower risk to develop CVD, though we realize that in the overall analysis correction for gender yielded an overall protective association for T-allele carriers. A possible explanation may be related to the treatment status of the patients. Previously, it has been reported that there is an interaction between carriers of the rare T allele of ADD1 and diuretic therapy.⁴² Hypertensive patients carrying the rare T allele, who were treated with diuretics, were at lower risk of myocardial infarction and stroke compared to patients treated with other antihypertensive therapies. We checked our database for the use of diuretics and found no association with diuretics. So, although attractive, the concurrent use of diuretics does not explain our findings. Another explanation might be the use of combination therapy which may modify the behavior of the CIMT. We checked our database, if carriers of the T-allele of the ADD1 polymorphism more often used combination therapies and found no association with combination therapy. Our finding of a protective association with outcome for the rare allele of ADD1 is however not an isolated unique observation. Tobin *et al.*, also found such a protective association for ADD1 in a multilocus case-control study in which patients with myocardial infarction were compared with healthy controls. They suggested that this protective effect is likely to be through a cellular mechanism different from its effect leading to hypertension.⁴³ Other authors also indicated that the duration of treatment with medication reduces the increase in CIMT.^{44,45} This may in our cross-sectional study indeed be an alternative explanation for the observed protective effect of the T allele of ADD1. Carriers of the rare T allele for the ADD1 (G460W) polymorphism are probably treated at earlier age and, consequently, longer with medication leading to reduced CIMT.⁴⁶ We checked our database for treatment duration and found no

statistically significant difference in treatment duration between carriers of the normal (GG) and and rare T allele of the ADD1 (G460W) polymorphism. However, we realize that our design is cross-sectional. This implicates two major problems. First of all, CIMT is influenced by cardiovascular medication, and secondly, channeling plays an important role, that is, the most severe cardiovascular patients are treated more intensively. In a cross-sectional design the contribution of these two factors can not be distinguished and, therefore, we think duration of treatment needs to be evaluated in future prospective studies.

Endothelial nitric oxide synthase (E298D) polymorphism

Although we found a statistically significant higher frequency of the rare T allele for the NOS3 (E298D) polymorphism in females, our multivariate linear regression analyses indicated that NOS3 did not contribute to higher CIMT. Homozygosity of the rare T allele of NOS3 has been shown to be associated with coronary heart disease, carotid atherosclerosis and endothelial function.⁴⁷ Recent studies found statistically significant associations between increased CIMT and NOS3. In additional analyses for NOS3, both univariate and multivariate, for homozygosity of the rare allele, we could not confirm these findings, though the allele frequency of the rare T allele in our population was similar or higher as compared with other studies.^{48,49}

Strengths and limitations

Several limitations of our study need to be discussed. Firstly, we only studied the effect of one polymorphism per gene, which does not exclude the role of other polymorphic alleles that are not in linkage disequilibrium. Secondly, we used a recessive model of inheritance. Although based on results of other studies, we realize this choice is arbitrary. Thirdly, one could object to averaging CIMT values from the left and right side, like we did. There is evidence for a significant difference between left and right CIMT, with higher values on the left side.⁵⁰ When we analyzed our data for left and right CIMT values separately, this did not alter our results. Fourthly, for reasons of comparability, we analyzed mean CIMT values, although the gender differences in CIMT values only were statistically significant when they were corrected for gender differences in internal carotid diameter, except for the age category 50-59. Analyses using CIMT/diameter ratio did not alter our results. Other studies have shown that the cross-sectional area of IMT (CSA-IMT) is also an important correction for measurements of CIMT. However, analyses using the CSA-IMT did again not alter our results. Fifthly, one could argue that we did not correct for multiple testing. We had, however, a clear a priori hypothesis, which limited the number of hypotheses tested. Finally, as is for many genetic association studies, a limitation of the present study is sample size.⁵¹ Therefore, as stated by Ginsburg *et al.*, these findings should be replicated in at least one independent cohort.⁵² However, at the start of our study in 2002 such a confirmation cohort was not included.

Perspectives

Recently it has been shown that CIMT is an important surrogate marker of atherosclerosis in the clinical trial setting.⁵³ Therefore, we think the results of our study are of utmost importance, since our results reflect the use of CIMT in daily practice and not the controlled setting of a clinical trial. Therefore, we were able to assess the influence on CIMT of several factors, including the six polymorphisms. Furthermore, we used a relatively unselected population from primary care, in which the whole spectrum of cardiovascular risk was present and well defined. From the perspective of prevention this is clinically relevant, because the general practitioner is first responsible for the long-term cardiovascular risk management of his patients. Furthermore, results from studies among unselected populations may lead to better cardiovascular risk stratification, not only for the index patient, but possibly for (first degree) family members as well. Eventually, it might offer opportunities for genetically-tailored treatment.

Conclusion

This study in a primary care population showed statistically significant associations between AGTR1 and CIMT in females and between ADD1 and CIMT for both males and females, but only statistically significant in females. The observed protective association of the T allele of ADD1 with CIMT under pharmacological therapy warrants further study on the role of this polymorphism. Our findings should be viewed as hypothesis generating and require further confirmation in prospective epidemiological primary care studies.

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Chapter 6

THE ASSOCIATION BETWEEN ARTERIAL
STIFFNESS AND THE ANGIOTENSIN
II TYPE I RECEPTOR (AII66C)
POLYMORPHISM IS INFLUENCED BY THE
USE OF CARDIOVASCULAR MEDICATION

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Abstract

Objective

To examine in a population with a high prevalence of hypertension, the association between six cardiovascular polymorphisms, arterial stiffness and medication use.

Methods

In this cross-sectional study (HIPPOCRATES project), arterial stiffness was assessed by measuring pulse wave velocity (PWV) in 575 patients in one primary care practice. Patients were genotyped for the angiotensin II type 1 receptor (A1166C), angiotensinogen (M235T), angiotensin-converting enzyme (4656_{rs1}), endothelial nitric oxide synthase (E298D), G-protein $\beta 3$ (C825T), and α -adducin (G460W) polymorphisms. Linear regression analyses were performed to assess the association between polymorphisms and PWV.

Results

Thirty percent of the patients (273 men, 302 women) had a carotid-femoral pulse wave velocity above 12 m/s and more than 60% of the patients had a carotid-femoral/carotid-radial PWV (CF/CR ratio) above 1. The CF/CR ratio was significantly associated with age, gender, dyslipidemia, cardiovascular medication use and pulse pressure. After correction for these covariates, multivariate linear regression analyses showed that the C allele of AGTR1 was associated with a lower CF/CR ratio. This association was significantly influenced by cardiovascular medication use ($P=0.011$), and showed a dose-allele effect, the CF/CR ratio decreasing with the number of C alleles ($P=0.04$).

Conclusion

In a primary care population, this study showed an independent protective dose-allele effect for the presence of C alleles of the AGTR1 polymorphism on PWV. This association, which was influenced by the use of cardiovascular medication, needs further investigations to identify the underlying mechanisms.

Introduction

The renin-angiotensin system (RAS) plays an important role in blood pressure (BP) regulation, cardiovascular cellular growth and remodelling. Many association studies have addressed the possible relationship between variant genes of the RAS with hypertension, left ventricular hypertrophy and myocardial infarction in different populations.¹⁻⁴ In the HIPPOCRATES project (**H**ypertension: **I**nteraction and **P**revalence of **P**OLymorphisms related to **C**ardiovascular **R**isk and the **A**ssociation to **T**reatment **E**fficacy **S**tudy), three well studied polymorphisms of the RAS were determined, that is angiotensinogen [AGT (M235T)], angiotensin converting enzyme [ACE (4656_{rpt})], and the angiotensin II type 1 receptor [AGTR1 (A1166C)] polymorphism. Furthermore, three other polymorphisms closely aligned to the RAS were also determined, that is endothelial nitric oxide synthase [NOS3 (E298D)], G-protein $\beta 3$ subunit [GNB3 (C825T)], and α -adducin [ADD1 (G460W)]. When HIPPOCRATES was designed, evidence in the literature for the role of these six polymorphisms in the development of cardiovascular disease (CVD) was very promising. HIPPOCRATES utilizes the population of a large general practice which is located in a region in the south-east of the Netherlands with a high burden of CVD compared with the average of the Netherlands⁵ and, therefore, these polymorphisms seemed very relevant for this study population. Finally, in Maastricht, the role of the RAS in the development of CVD is being studied for several years. Consequently, a complete infrastructure was available to determine these polymorphisms accurately and quickly. The overall objective of the HIPPOCRATES project is to analyse the contribution of the aforementioned polymorphisms with cardiovascular risk. We started off with a cross-sectional data analysis and adopted arterial stiffness as a major surrogate marker for cardiovascular morbidity.

Increased arterial stiffness is an accepted strong independent predictor of cardiovascular risk and is associated with excess cardiovascular morbidity and mortality.⁶⁻⁸ Both genetic and environmental factors are responsible for structural changes in the arterial wall and progressive arterial stiffening. An index of central arterial stiffness is carotid-femoral pulse wave velocity (CF-PWV). The measurement of CF-PWV has been considered as the 'gold standard' of arterial stiffness in a recent expert consensus document.⁹ It is generally accepted as the simplest non-invasive, robust and reproducible method to determine aortic stiffness, and has been used in many epidemiological studies, demonstrating the predictive value of aortic stiffness for cardiovascular events¹⁰ and all-cause mortality.^{6,9} CF-PWV is mainly enhanced by increasing systolic and pulse pressure (PP) and, therefore, the association between CF-PWV and polymorphisms of the RAS is important to investigate. However, studies on CF-PWV and RAS polymorphisms are rather scarce. The few studies performed thus far, showed that genetic variants of the RAS can influence the CF-PWV.¹¹⁻¹⁴ Therefore, we hypothesized that in the presence of carriership of one or more rare

alleles of the aforementioned polymorphisms, the CF-PWV values will be higher. In the present study, we investigated the association between aforementioned six polymorphisms and CF-PWV. Furthermore, we know from literature that pharmacological treatment is associated with a reduction in CF-PWV.⁶ Therefore, we hypothesized that in the presence of carriership of one or more rare alleles of our six polymorphisms, the CF-PWV will be lower in patients treated with cardiovascular medication. Therefore, we also investigated the influence of cardiovascular medication use on the association between our polymorphisms and CF-PWV.

Methods

Study population

All participants of the HIPPOCRATES project were recruited from one general practice in Kerkrade, The Netherlands between May 2003 and July 2005. This academic general practice takes part in the Registration Network Family Practices (RNH), which is a continuous and computerized database. All relevant health problems, including a full medical history of all cardiovascular events have been registered since 1990.^{15,16} Medical problems are coded according to the International Classification of Primary Care (ICPC) using the criteria of the International Classification of Health Problems in Primary Care for diagnoses.^{17,18} The general practice in Kerkrade has a list of approximately 4400 patients, among whom are more than 2300 patients of 40 years and older. The practice is located in a region with a high burden of CVD and, therefore, it has well implemented cardiovascular consulting hours. For many years the practice nurses conducted special cardiovascular consulting hours, to which patients are invited at least once every three months. For the HIPPOCRATES project, all known patients aged at least 40 years with CVD or hypertension, irrespective of their medication use, were invited. From the group with neither known CVD nor established hypertension, but with other cardiovascular risk factors, that is, smoking, alcohol, diabetes mellitus, hypercholesterolemia, overweight including obesity, a random 1:3 sample was taken. Of all 'healthy' patients (neither CVD, nor hypertension nor other cardiovascular risk factors), also a random 1:3 sample was taken. The initial classification was validated by a thorough assessment of the patients' records combined with additional measurements of risk factors for all patients. The total HIPPOCRATES population consisted of 803 patients (382 males, 421 females). During their first visit, participants were asked permission to measure the pulse wave velocity over the carotid-femoral (CF-PWV) and the carotid-radial (CR-PWV) route. Included patients did not differ in characteristics from those who did not consent (185 patients). PWV data of 22 patients were not included owing to premature withdrawal from the study (5 patients) or to poor quality of the measurements (17 patients), though again, these patients did not differ with respect to their characteristics.

Additionally, 21 patients were excluded owing to the diagnosis of peripheral arterial occlusive disease. For the present study, 575 patients were included: 273 men and 302 women. The study was approved by the Medical Ethics Committee of the Maastricht University Medical Center. All patients gave written informed consent to participate in the study.

Clinical data collection

All clinical measurements in the HIPPOCRATES project were performed in the practice by one of the authors (AWP; first measurement) and a nurse practitioner (second, third and fourth measurement with in between periods of three months). The nurse practitioner measured weight and blood pressure. All persons were questioned on their smoking behaviour. Weight and height were measured. Body-mass index (BMI) was calculated as body weight divided by squared height. BMI values at least 27 were considered to be too high. The size of the waist was measured. BP and heart rate were measured three times per visit, after ten minutes rest in sitting position, using a sphygmomanometer. Fasting blood samples were drawn to determine serum creatinine, total cholesterol, low-density lipoprotein-cholesterol (LDL-c), high-density lipoprotein-cholesterol (HDL-c), serum triglycerides and glucose levels and were analyzed in one regional laboratory. Microalbuminuria was determined semiquantitatively in a first morning urine sample.

Treatment status

The computerized medication lists were analyzed for antihypertensive, hypolipidemic, antiplatelet and antidiabetic medication. Furthermore, all persons were questioned about their medication use at the start of the study. Cardiovascular medication was based on the anatomical therapeutic classification (ATC) and defined as having at least one prescription of antihypertensive (ATC level 2: C02, C03, C04, C07, C08, C09), hypolipidemic (ATC level 2: C10), or antiplatelet (ATC level 5: B01AC) medication in the selected study period. This definition was chosen since these drugs are most frequently prescribed for the prevention of CVD in primary care. Antidiabetics were defined as having at least one prescription of A10 (ATC level 2) in the study period.

Definitions

Hypertension was defined as a systolic blood pressure (SBP) at least 140 mmHg diastolic blood pressure (DBP) at least 90 mmHg or both according to the Guidelines for Hypertension specified by the European Society of Hypertension.¹⁹ PP was calculated as the difference between SBP and DBP. The presence of dyslipidemia was defined as total cholesterol to HDL-c ratio exceeding 4.0. Diabetes mellitus was considered to be present in case of fasting plasma glucose levels above 6.9 mmol/l. Creatinine values higher than 115 $\mu\text{mol/l}$ (men) or 107 $\mu\text{mol/l}$ (women) were

considered to be a risk factor for CVD. Creatinine clearance was calculated by the Cockcroft-Gault formula.²⁰ Microalbuminuria was defined as albumin excretion above 20 mg/l.

Arterial stiffness

The PWV measurements were performed at the circulation laboratory of the Department of Internal Medicine of the Maastricht University Medical Center. PWV was recorded by Complior® (Artech, Pantin, France), a PWV measurement system for arterial stiffness evaluation. This system gives an automated measurement of PWV for two arterial segments simultaneously, with dedicated pulse wave sensors.⁹ This system determines the transit time the wave of dilatation (propagated in the arterial wall as a result of the pressure wave generated by contraction of the left ventricle) takes to arrive at a site over a known distance.⁹ After resting in a supine position for 15 minutes in a quiet, temperature-controlled room, participants were measured by a trained investigator (AWP), who obtained PWV measurements in two arterial segments simultaneously, that is carotid-radial and carotid-femoral. In order to enhance reproducibility, PWV was measured four times and mean values were calculated for both arterial segments.⁹

CF-PWV evaluates a mixed elastic and muscular segment of the arterial tree, whereas the CR-PWV evaluates a predominant muscular segment. The effect of aging has been shown to be more pronounced in the proximal, more elastic, and less muscular arteries.²¹ Expressing PWV as a ratio of the CF/CR PWV improves the relationship with age, as has been demonstrated by Cameron *et al.*²² It is for this reason that we expressed our data as such, using the ratio of CF-PWV to CR-PWV. A CF-PWV >12m/s and a CF/CR ratio above 1 were considered as threshold values.⁷ Consequently, values above these thresholds were considered to be increased. All patients were measured at the same period of the day, that is the afternoon.

Genetic analyses

The various polymorphisms were determined at the Cardiovascular Genotyping (CAGT) laboratory of the Department of Internal Medicine of the Maastricht University Medical Center. DNA was extracted from whole blood with the use of the QIAmp Blood Kit (Qiagen Inc., Venlo, the Netherlands). Genotyping of the angiotensin II type 1 receptor [AGTR1 (A1166C)], angiotensinogen [AGT (M235T)], angiotensin converting enzyme [ACE (4656_{rs1})], endothelial nitric oxide synthase [NOS3 (E298D)], G-protein $\beta 3$ subunit [GNB3 (C825T)], and α -adducin [ADD1 (G460W)] polymorphisms was performed with a multiplex single base extension (SBE) assay (Multiplex SNaPshot, Applied Biosystems, Foster City, California, USA) similar to previously published protocols.²³ Briefly, in the first stage, a multiplex PCR is carried out to amplify the gene fragments that contain the polymorphisms. In the second stage, the polymorphic positions are interrogated by a SBE reaction using fluorescently labeled

ddNTPs. The oligos used for the single base extension have specific lengths, varying from 22–50 nt. After the SBE assay, the fragments are size-resolved on an ABI3100 Genetic Analyser (Applied Biosystems): the size of the peaks identifies the locus (e.g. ADD1) and the color of the peak(s) identifies the genotype (e.g. Gly/Trp). Raw data were analysed with Genescan Analysis software (v 3.7), then genotypes were scored independently by two researchers and entered in the database. In 27 patients, a second analysis was necessary to obtain all the genotypes. The ACE (I/D) polymorphism was determined by detecting ACE_{4656+CT} observed on the SNaPshot as ACE (4656_{rpt}). The use of this SNaPshot method does not allow the direct determination of the insertion/deletion polymorphism. The ACE_{4656+CT} polymorphism in the 3'-untranslated region of the ACE gene consists of a repetition of two or three CT dinucleotides and is in complete linkage disequilibrium with ACE (I/D).^{24,25} The multiplex assay was validated on a set of 100 DNA samples that had previously been genotyped with an alternative technique; this technique concerned a multilocus genotyping assay for candidate markers of CVD risk (Roche Molecular systems Inc., Alameda, California, USA) and has been described in detail previously.²⁶ Only four samples gave a discordant result for the NOS3 polymorphism. All other genotypes were concordant.

Polymorphisms

In short, the presumed function of the included polymorphisms is as follows. Patients homozygous for the C allele of the AGTR1 polymorphism, have increased sensitivity to angiotensin II (AngII) and this may in combination with the DD genotype of the ACE (I/D) polymorphism, lead to an increased risk of cardiovascular complications.^{27,28} The T allele of the AGT polymorphism is associated with high plasma levels of AGT and increased responsiveness to Ang II.⁴ In the presence of a two allele of the ACE_{4656+CT} polymorphism, comparable to the D allele of the I/D polymorphism, increased ACE levels have been found, which have been associated with an increased risk for cardiovascular complications.² The NOS3 (E298D) polymorphism is associated with reduced NO production in endothelial cells of the arterial wall, inducing elevation of the arterial pressure.^{26,29} The T allele of the GNB3 (C825T) polymorphism is associated with increased intracellular signal transduction and this leads to stimulation of the Na⁺/H⁺ exchanger.³⁰ The ADD1 (G460W) polymorphism is of interest because the rare allele is also associated with increased Na⁺ transport, though here the Na⁺/K⁺ exchanger is stimulated.³¹ Both polymorphisms are associated with chronic volume expansion and, consequently, with related cardiovascular complications.^{31,32}

Statistical analyses

Statistical analyses were performed by using SPSS statistical software, version 14.0 (SPSS, Inc., Chicago, Illinois, USA). A *P*-value of 0.05 or less was considered statistically significant. Genotype and allele frequencies were calculated to test the Hardy-

Weinberg equilibrium. Furthermore, allele frequencies were analyzed using chi-square tests.

Multivariate linear regression analyses

Because of the skewed distribution of CF/CR ratio, log transformation was applied to achieve normality for further analyses. Univariate and multivariate linear regression analyses were performed to evaluate the relationship between polymorphisms and CF/CR ratio. For that purpose, dummy variables were created using the homozygous wild-type genotype as reference category. Age and gender, both demographic variables, were incorporated in both the univariate as well as in the multivariate linear regression analyses. As indicated, we selected control individuals from different groups. Therefore, in the regression analyses we had to adjust for this selection and dummy variables had to be created using the normotensive group without cardiovascular risk factors as the reference category. Adjustment for potential confounders was performed by incorporating age, gender, dyslipidemia, cardiovascular medication and PP into these models (enter procedure). The simultaneous contribution of the six polymorphisms to the prediction of increased CF/CR ratio was determined, using the overall *F*-test. If two or more polymorphisms reached statistical significance, interactions (effect modification) between two polymorphisms with respect to CF/CR ratio were assessed. Again linear regression analysis was used, in which the alleles at risk, the interaction term between the alleles and the other covariates were incorporated. If an interaction became statistically significant, the model was explored further.

If one or more polymorphisms reached statistical significance, interaction between cardiovascular medication use and the relevant polymorphism with respect to CF/CR ratio was assessed. Again, linear regression analysis (method: enter) was used, in which the alleles at risk, the interaction between cardiovascular medication use and the alleles and the other covariates were incorporated. If an interaction became statistically significant, the model was explored further.

Results

Characteristics of the participants

Characteristics of the study population are summarized in Table 6.1. Most patients were overweighted hypertensives with dyslipidemia. There were slightly more women than men and their mean age was 60 years. Overall, 30% of the patients had a CF-PWV above 12m/s and more than 60% of the patients had a CF/CR ratio above 1.

Allele frequencies

Genotype and allele frequencies of all polymorphisms were in Hardy-Weinberg equilibrium. For all six polymorphisms, no statistically significant differences in allele frequencies were found.

Table 6.1 Characteristics of the study population (n= 575).

Characteristics	
Men/women, n	273 / 302
Age (years)	60.2 ± 10.6
Risk factors	
Body-mass index (kg/m ²)	27.7 ± 4.4
Waist (cm)	95.0 ± 12.9
Pulse pressure (mmHg)	62.2 ± 18.4
Heart rate (beats per minute)	65.4 ± 10.1
Hypertension, n (%)	360 (62.6)
Current smoking, n (%)	108 (18.8)
Diabetes, n (%)	77 (13.4)
Dyslipidemia, n (%)	311 (54.1)
Microalbuminuria, n (%)	61 (10.6)
Biochemical	
Creatinine clearance (ml/min)	78.3 ± 22.9
Total cholesterol (mmol/l)	5.5 ± 1.0
LDL-cholesterol (mmol/l)	3.3 ± 1.0
HDL-cholesterol (mmol/l)	1.5 ± 0.4
Triglycerides (mmol/l)	1.6 ± 0.9
Glucose (mmol/l)	5.7 ± 1.5
Medication	
Antihypertensive, n (%)	272 (47.3)
Hypolipidemic, n (%)	113 (19.7)
Antiplatelet, n (%)	121 (21.0)
Antidiabetic, n (%)	42 (7.3)
Arterial measurements	
CF-PWV (m/s)	
Overall	11.3 ± 2.3
>12 m/s, n (%)	171 (29.7)
CR-PWV (m/s)	10.2 ± 1.7
CF/CR ratio	
Overall	1.1 ± 0.3
>1, n (%)	349 (60.7)

Values are given as mean ± SD or as number of patients (with corresponding percentages between brackets); creatinine clearance by the Cockcroft-Gault formula; HDL, high-density lipoprotein; LDL, low-density lipoprotein; CF-PWV, carotid-femoral pulse wave velocity; CR-PWV, carotid-radial pulse wave velocity; CF/CR ratio, carotid-femoral to carotid-radial pulse wave velocity ratio.

Arterial stiffness, cardiovascular polymorphisms and treatment status

Stepwise regression analyses identified age, gender, dyslipidemia, cardiovascular medication use and PP as significant co-variables for the relationship between the six polymorphisms and the CF/CR ratio. In the univariate linear regression analyses (Table

6.2), an association was found between carriership (dominant model) of the C allele of AGTR1 and the CF/CR ratio ($P=0.014$). However, in the multivariate linear regression analyses (Table 6.2), the simultaneous contribution of the six polymorphisms to the change in the CF/CR ratio (overall F -test) reached no statistically significance ($P=0.107$). We assessed an independent statistically significant association between AGTR1 and the CF/CR ratio ($P=0.011$). It appeared that the CF/CR ratio decreased by 4%, if patients were carrier of the C allele of AGTR1. For all other polymorphisms, carriership of the rare allele was not statistically significant associated with the CF/CR ratio. Analyses based on the assessments of the CF-PWV showed virtually the same results (data not shown).

Table 6.2 Linear regression model for six polymorphisms (dominant model) and carotid-femoral to carotid-radial ratio for all patients.

Polymorphism	Genotype*	Univariate analysis (95% CI) [‡]		Multivariate analysis (95% CI) ^{‡†}	
		Overall	P	Overall	P
AGTR1 (A1166C)	C*	0.96 (0.93 – 0.99)	0.014	0.96 (0.93 – 0.99)	0.011
AGT (M235T)	T*	0.99 (0.96 – 1.03)	0.697	1.00 (0.97 – 1.03)	0.883
ACE (4656 _{rrpt})	G*	1.00 (0.96 – 1.04)	0.981	1.00 (0.96 – 1.04)	0.939
NOS3 (E298D)	T*	0.97 (0.93 – 1.02)	0.221	0.97 (0.92 – 1.01)	0.154
GNB3 (C825T)	T*	1.00 (0.97 – 1.04)	0.792	1.00 (0.97 – 1.04)	0.825
ADD1 (G460W)	T*	0.99 (0.96 – 1.03)	0.725	1.00 (0.96 – 1.03)	0.744

* The wild-type genotypes were used as reference category; PWV, pulse wave velocity; [‡] Data presented as unstandardized β coefficient (95% CI): the β represents the change in carotid-femoral to carotid-radial ratio for each unit change in continuous risk factors and for a change (absent or present) in dichotomous risk factors; CI denotes 95% confidence interval; [†] Multivariate adjustment was performed for age, gender, dyslipidemia, cardiovascular medication use and PP.

When comparing the CF/CR ratios for the whole study population, as well as for patients that were on cardiovascular medication, a statistically significant trend ($P=0.04$) was found for the ratio to decrease with the number of C alleles (codominant model, Figure 6.1). Furthermore, we performed an explorative analysis (dominant model) to evaluate the influence of cardiovascular medication use on the association between the AGTR1 (A1166C) polymorphism and the CF/CR ratio. The interaction between cardiovascular medication use and the AGTR1 (A1166C) polymorphism was statistically significant ($P=0.04$). Because the interaction term was statistically significant, we performed a stratified analysis to explore the direction of the effect. The results of these analyses are presented in Figure 6.2. This figure shows that in patients treated with cardiovascular medication the C allele of AGTR1 polymorphism is also significantly associated with a lower CF/CR ratio ($P=0.011$, overall F -test ($P=0.078$).), whereas no statistically significant association was observed in untreated patients. Post-hoc analyses of the different medication groups separately showed for all groups a protective association of the C allele of AGTR1 with the CF/CR ratio. However, only for the β -blockers statistical significance was reached ($P=0.006$), overall F -test ($P=0.03$).

Figure 6.1 Carotid-femoral to carotid-radial pulse wave velocity ratio (log-transformed values) in the whole study population, according to the AGTR1 (A1166C) polymorphism. The observed differences in CF/CR ratio are statistically significant ($P=0.04$). For each allele combination the number of patients is depicted. Values are unadjusted means $\pm$ standard error.

Figure 6.2 Carotid-femoral to carotid-radial pulse wave velocity ratio for carriership of the AGTR1 (A1166C) polymorphism. The observed difference in CF/CR ratio between patients treated with cardiovascular medication with AA genotype and treated carriers of the C allele is statistically significant ($P=0.011$). For each combination the number of patients is indicated. Values are unadjusted means $\pm$ standard error.

Discussion

In the present study, we found a statistically significant protective association of the C allele of AGTR1 (A1166C) with PWV ($P=0.011$) for patients in a primary care population. This effect was independent of age, gender, dyslipidemia, cardiovascular medication use and PP. Explorative analyses showed furthermore that patients treated with cardiovascular medication had a significantly lower CF/CR ratio in the presence of C alleles, of which the latter seemed to show a dose-allele effect.

Arterial stiffness, angiotensin II type I receptor polymorphism and treatment status

Angiotensin II plays a key role through the angiotensin II type I receptor in blood pressure regulation, cardiovascular cellular growth and remodelling. So, the AGTR1 polymorphism of the AGTR1 gene is, at least theoretically, a good candidate for an association with stiffening of the large arteries by inducing vascular remodelling. Indeed, our finding of an independent association of the AGTR1 (A1166C) polymorphism with CF-PWV is in accordance with the results of three previous studies.^{11,12,14} We found, like the study performed by Gardier *et al.*, but in contrast to Benetos *et al.*, that AA homozygous patients had a higher CF-PWV.¹⁴ Participants of our study were mainly overweight hypertensive patients from a primary care practice, whereas the patients in the study of Gardier *et al.* were retrieved from a hospital cardiology department with mainly severe or resistant hypertension. Benetos *et al.* included in his study never-treated hypertensives, retrieved from a hospital hypertension department. In our population, mainly antihypertensives were used. So, it can be argued that antihypertensive medication decreased arterial stiffness by a pressure-dependent effect, in particular in those patients with an increased CF-PWV in the untreated situation.³³ Our analyses seem to confirm this. Moreover, post-hoc analyses of the different medication groups separately pointed in the same direction for all regression coefficients, that is, a protective association of the C allele of AGTR1 with the CF/CR ratio. However, only for the β -blockers was statistical significance reached. One might argue that our results were confounded by disease severity: patients with most prominent risk factors were treated more intensively. First, our results were independent of dyslipidemia and PP. Second, the treatment intensity did not seem to play a role, as the number of drug classes was not significantly different among treated patients with the AA genotype versus those with the C allele carriership.

The assessed association of the AGTR1 (A1166C) polymorphism needs further explanation. In a recent report, left ventricular cardiac stiffness was increased in homozygous A allele carriers compared to C* (AC + CC) carriers in hypertensive patients, suggesting that chronic exposure to angiotensin II might induce, in

hypertensive patients with A allele, an imbalance between collagen synthesis and degradation.³⁴ An alternative explanation for the lower CF/CR ratio in the C allele carriers might be that the C allele is associated with an increased angiotensin II-induced nitric oxide (NO) release, mediated in endothelial cells by AGTR1 receptors and, thus, counterbalancing the direct vasoconstrictive effect of Ang II on the smooth muscle.^{35,36} However, such hypotheses require further confirmation in other studies.

Some limitations of the present study should be acknowledged. First, the AGTR1 (A1166C) polymorphism is probably not a functional one, because the AGTR1 polymorphisms is located in a noncoding region of the chromosome 3q22³⁷ and, therefore, the amino-acid sequence of the receptor is not altered. It is possible that the AGTR1 polymorphism is in linkage disequilibrium with an unidentified functional variant of the AGTR1 gene, or, alternatively, the polymorphism could be in linkage disequilibrium with a nearby gene, which is responsible for the association with the CF/CR ratio. Nevertheless, others and we have shown that this polymorphism, that is, the C allele, is associated with an increased sensitivity to angiotensin II.²⁸ Another limitation of this study is that we compared a recessive model of inheritance of the A allele in AA versus C* (AC + CC) carriers. This choice was made on the basis of results of other studies, though we realize this choice is arbitrary. Another approach would have been to analyse our data with a codominant model, therefore, we have shown the analysis presented in Figure 6.1. Finally, we realize that our design is cross-sectional. This implicates two major problems. First of all, the CF-PWV is influenced by cardiovascular medication, and secondly, channeling plays an important role, that is, the most severe cardiovascular patients are treated more intensively. In a cross-sectional design, the contribution of these two factors can not be distinguished and, therefore, our findings should be viewed as hypothesis generating and require further confirmation in prospective epidemiological studies. Nevertheless, our study has also a unique aspect. As compared to previous studies, we used a relatively unselected population from primary care. Results from studies among unselected populations may lead to better cardiovascular risk stratification.

In conclusion, this study shows that in this population a statistically significant relationship exists between the AGTR1 polymorphism, arterial stiffness and cardiovascular medication. Even though the underlying mechanisms have yet to be elucidated, these data suggest that the AGTR1 gene, or a yet unidentified gene locus in linkage disequilibrium with AGTR1 (A1166C) polymorphism, may be a genetic determinant of arterial stiffness.

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Chapter 7

FAMILY HISTORY OF COMMON DISEASES AS GENETIC TOOL IN PRIMARY CARE: WHAT IS THE EVIDENCE? A SYSTEMATIC REVIEW

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Submitted

Abstract

Objective

To summarize the evidence for the use of comprehensive family history taking for common, multifactorial diseases in primary health care.

Design

Systematic review of Medline (1966-2008), Embase (1986-2008) and Cochrane Library (until August 2008).

Methods

Search terms reflected 'primary care', 'family history' and 'genetics'. Included were original research papers on multifactorial disorders investigating family history taking in primary care. Given the heterogeneity of the studies, outcomes are presented in a qualitative way.

Results

Twenty-seven papers were identified: nine studies on primary care physicians' opinions, eight studies on current practice, seven studies on the development of family history tools, and three studies on the patient perspective. The general opinion that taking a family history is the task of the primary care physician was only partly confirmed by observational studies of consultations and analyses of medical records: wide variability, low regular update. Despite various efforts, the development of feasible, reproducible and valid family history tools is as yet unsatisfactory. Patient-doctor differences in perception of the meaning of a positive family history (for cancer, cardiovascular disease or diabetes) may cause miscommunication.

Conclusion

There is a need for research into feasible and high quality tools for detailed family history taking for multifactorial disorders in primary care.

Introduction

Advances in genetic knowledge and the development of genetic tests offer a great promise for health care. The promise is that individualized 'genetic risk profiles' will enable tailored pharmacotherapy, tailored life style modification (like smoking cessation, diet measures) and tailored prevention of chronic, multifactorial diseases like cardiovascular disease (CVD), cancer, asthma, COPD, dementia, depression or addiction disorders. However, current knowledge of specific associations of polymorphisms and multifactorial diseases ('genetic tests') still has limited added predictive value.¹

On the other hand, genetic information is hidden in the medical family history. Detailed knowledge of the medical family history could be most informative for detecting familial disease patterns, assessing the clinical significance of these patterns ('risk') and consequently use it in preventive and curative care. This hypothesis has led to new interest – as expressed in editorials and descriptive reviews - in the use of a comprehensive medical family history as a tool for primary and secondary prevention of chronic multifactorial diseases.²⁻⁹ Moreover, family history promises to serve as a critical element to identify candidates for genetic testing or targeted medical care. Given the community orientation of family physicians (primary care physicians, general practitioners), one can argue that they are in an ideal position to track subjects at risk and consequently to discuss possible preventive actions with them. In health care systems with a well developed primary care, family physicians like to see themselves as the 'case manager' of their patients and regard it their responsibility to keep an up to date comprehensive medical record of their patients. Furthermore, family history taking is supposed to be well established in primary care.^{4,7,11-12} Taking a detailed family history (for CVD and risk factors, cancer, dementia, COPD, asthma, mental disorders etc.) is regarded an inexpensive, non-invasive and reliable method for screening patients for their risk of developing one of these common multifactorial diseases.⁷ A few years ago, in a narrative review Rich *et al.* discussed various barriers that impede application of family history taking in primary care.¹³ In this systematic review we aim to summarize the evidence supporting the use of comprehensive family history taking for common, multifactorial diseases in primary health care.

Methods

Search strategy

Systematic search of the literature was conducted for references on primary care and family history in MEDLINE (1966-2008), EMBASE (1986-2008) and the Cochrane Library (search until August 2008). For the purposes of this review, the terms 'primary care' and 'family practice' will be treated synonymously, as will 'general practitioner'

and 'family physician'. Sets of terms relating to three specific elements were combined: (1) primary care, (2) family history, and (3) clinical genetics. When possible, the appropriate indexing term for each database was used.

Inclusion and exclusion criteria

Included were original research papers that investigated family history taking in primary care as a tool to identify individuals and families at increased risk for multifactorial disorders. Only papers in English were included. Titles and abstracts of papers identified by the electronic searches were screened by one reviewer (AWP) and obviously irrelevant papers were excluded. Full text copies of the remaining potentially relevant papers were screened and reviewed by two independent authors (AWP; HEJHS). When potentially relevant references were found in the bibliography of these papers, these were reviewed as well. Case reports, editorials, and papers concerning monogenetic disorders were excluded from this review. Therefore, studies on specific cancers were excluded from this review, whereas generic studies on cancer risk, or papers combining several cancers, were included.

Identification and selection of studies

Out of 109 titles, 64 were excluded on the basis of title and abstract, and of two papers no full text copies were available. After examination of the remaining 43 potentially relevant full text papers, eight papers were excluded from analysis (case report, editorials, reviews) and four potentially relevant titles were added for closer examination. After evaluation of the outcome, three papers were added for closer review and 15 papers on monogenetic disorders, psychological aspects, or a simple cardiovascular risk score were excluded. Finally, 27 original research papers were included in this review.

Data extraction and analysis

Data concerning study design, study population, methods, response rate and outcome were extracted according to the guidelines of the Cochrane Collaboration (Review Manager, version 4.2.8). On account of the heterogeneity of the papers reviewed, a qualitative analysis of the results was performed: the key findings from each paper were independently extracted by two authors (AWP; HEJHS), subsequently discussed and finally categorized and summarized in a table.

Results

Included papers

The included papers could be arranged in four categories: studies on primary care physicians' opinions on the application of family history as genetic tool (nine papers, Table 7.1)¹⁴⁻²², studies on the practice of family history taking (eight papers, Table 7.2)²³⁻³⁰, studies on developing tools for taking a family history (seven papers, Table 7.3)³¹⁻³⁷ and, finally, qualitative studies on the patient perspective and risk communication (three studies, Table 7.4).³⁸⁻⁴⁰

Physicians' opinions on taking a family history in the context of genetic medicine

Nine original research papers, of which two had a qualitative design, had investigated opinions on the application of family history in daily practice (Table 7.1).¹⁴⁻²² Both qualitative studies, executed last decade in the UK and Australia, reveal that general practitioners (GPs) considered 'a focus on common diseases' an important issue for genetics in daily practice. Exploring the 'family tree' was believed to be an important task of the GP. GPs said that taking a family history was a routine activity for them. However, they admitted it was often limited in detail and often done reactive to a patient's request. Also, the family history was not updated regularly, and considered not adequate for genetic advice. GPs also expressed lack of skill in taking a detailed family history or genogram.^{16,20} The results from quantitative questionnaire studies confirm these qualitative results.^{15,17,18,21} A minority of (UK) GPs said they often enquire about family history during routine consultations. In their view the number of patients with a question on risk for a common disease associated with disease in a family member was low.^{15,17} On first registration of a patient in their practice (UK) GPs reported to enquire on disease in family members, especially CVD, less on cancer. Knowledge of family history was said to influence decisions on diagnostic test ordering or referral.¹⁵ Only a minority of GPs reported confidence in their own ability to take a detailed family history.^{17,18} Calculating cancer risk was neither regarded a GP's task nor considered the GP's competence.^{18,22}

Table 7.1 General practitioners' views on family history as genetic tool.

Citation	Location	Methods	Participants	Response rate	Outcomes: opinions on the role of 'family history' in general practice; reported activities with regard to 'family history'; and perceived confidence in personal competence with regard to family history as a genetic tool.
Betha et al., 2008	Nottingham, UK	Cross-sectional questionnaire study	399 GPs and 316 PNs	425/715 (59%)	Confidence in personal competence (with regard to breast and bowel cancer): many health professionals reported confidence in advising on symptomatology but were much less confident in making a risk assessment or knowledge of relevant information to collect
Suther et al., 2004	Texas, USA	Cross-sectional questionnaire study	400 PCPs	30%	Opinion: genomic medicine could supplement family history: 91%. Reported activity: taking a detailed family history: 6%. Confidence in personal competence: taking a more detailed family history could easily be incorporated: 70%
Metcalf et al., 2002	Melbourne, Australia	Qualitative: focus group interviews; questionnaire survey	39 GPs / 160 GPs Survey	160/875 (18%)	Opinion: GPs consider the following genetic issues most important for daily practice: focus on common diseases; exploring the 'family tree'
Acton et al., 2000	Birmingham, UK	Cross-sectional questionnaire study (on cancer genetics)	1148 PCPs	254/1148 (22%)	Reported activities in cancer genetics: GPs report to obtain a family history of cancer: 63%; new patients are reported to be asked for a family history of cancer: 94%; updates are said to be made: 52%; Family history of cancer is said to be discussed in 46% of patients with new cancer; and in 52% of patients with a relative with cancer; Last year 21% said they referred at least 1 patient for genetic testing on the basis of a positive family history
Fry et al., 1999	Scotland, UK	Cross-sectional questionnaire study (on cancer genetics)	670 GPs	397/670 (59%)	Opinion: perceived as an important role of GP is 'taking a detailed family history' (61% agreed), but not 'calculating genetic cancer risk' (agreement 6.1%). Confidence in personal competence: taking a detailed family history 39%; doctors do NOT feel confident about calculating genetic risk for cancer: 95%
Suchard et al., 1999	Oxfordshire, UK	Cross-sectional questionnaire study	359 GPs	339/359 (94%)	Opinion: 61% reports to be willing to take a detailed family history/pedigree Reported activities: Mean number of patients/month with a question on risk for a common disease associated with disease in a family member is reported to be 2 (none: 20%); Estimated frequency of concerned patients: 4/1000 consultations; Confidence in personal competence: GPs feel sufficiently prepared to take family history/pedigree: 29%
Watson et al., 1999	Oxfordshire/d Nottingham, UK	Qualitative: focus groups, semi-structured interviews	26/15 GPs	Not applicable	Reported activities: Taking family history is routine, however often limited in detail, not updated regularly, not adequate for genetic advice; it is often reactive to patient demand, not pro-active; Confidence in personal competence: lack of skills e.g. in taking a detailed family history/pedigree
Summerton et al., 1997	Yorkshire, UK	Cross-sectional questionnaire study	291 GPs	193/291 (66%)	Reported activities: Family history in routine care: GPs say they (very) often enquire about family history during routine consultations: 29%; they report to suggest patients to tell a family member (very) often: 26%. On first registration of a patient in their practice, GPs report to enquire on disease in family members: CHD 94%, breast cancer 48%, colorectal cancer 31% Knowledge of family history is said to influence decisions on diagnostic test ordering or referral: 56-92% (rheumatoid disease, thyroid disease, change in bowel habits, breast lump under age 35, rectal bleeding, hypercholesterolemia)
Rohrbaugh et al., 1992	Williamsburg, USA	Cross sectional	6 FPs, 6 FTs, 58 genograms	Not applicable	Opinion: (In 1992) family physicians considered the following topics the most relevant information in genograms: medical condition, household composition, and medical condition of family member

PCPs: primary care physicians; FPs: family physicians; GPs: general practitioners; PNs: practice nurses; FTs: family therapists.

Table 7.2 The practice of family history taking and recording.

Citation	Location	Methods	Data from	Response rate	Outcomes
Klein Woolthuis et al., 2007	The Netherlands	EMR analysis	13581 EMRs of patients aged 45–75 years without known diabetes	Not applicable	Family history of diabetes and obesity: in 9723 patients (72%) no risk for diabetes could be estimated. In a selected group of patients in whom screening was discussed and approved (n=1643), in 51% family history of diabetes and in 59% family history of obesity was lacking
Tyler et al., 2006	Cleveland, USA	Retrospective chart analysis	Random sample of 734 patients of academic family medicine center	Not applicable	Family history of cancer in medical record: any information on familial cancer 98%; a positive family history for cancer 36.5%, of which 63% was considered inadequate (no details on relative, type of cancer or age at diagnosis)
Murff et al., 2004 (1)	Boston, USA	Retrospective chart analysis	Charts of 995 new patient visits	Not applicable	Family history in medical record: any family history 93%; Family history of cancer (breast, colon) in medical record: any information on familial cancer 68% (of whom 35% negative); in case of a positive family history age of diagnosis was collected in only 39% (first-degree relative) and 16% (second-degree relative); specific information on which relative and type of cancer was collected in 61%
Murff et al., 2004 (2)	Boston, USA	Retrospective chart analysis	Charts of 516 new adult patients without diabetes, visiting for a routine or gynaecological examination or preventative consultation	Not applicable	Family history of diabetes mellitus in medical record: 15% of new patients visiting primary care appeared to have a family member with diabetes mellitus (DM). If a positive family history of DM was known, physicians ordered a glucose test almost 3 times as often: 52% versus 19%. A known family history for DM had no impact on lifestyle advice offered by the physician
Sifri et al., 2002	Philadelphia, USA	Retrospective chart audit	Charts of 500 randomly chosen patients aged 40–60 years	Not applicable	Family history in medical record: 89% had some family history; 63% had a genogram; 57% supplied family history information at 1 st visit; of these 59% (34% of all) was not updated; update over three years was 20%. Family history of cancer in medical record: Family history of cancer (positive or negative) was recorded in 55% of the records; 43% had a positive family history for cancer. Details: site of cancer was listed in 88%; specific relative was listed in 92% (51% 1 st degree, 37% 2 nd degree); age at diagnosis was listed in 8%; age at death was listed in 19%
Acheson et al., 2000	Ohio, USA	Cross-sectional study, direct observation of consultations	4,454 consultations (138 community family physicians; consecutive patients, 2 observation days)	89% of all patients seen on observation days	Family history during consultations Family history was discussed in 24% of the consultations; physicians varied between 0–81%: Updating is a major problem Younger physicians with fewer years at practice more often discuss the family history
Rose et al., 1999	Oxfordshire, UK	Cross-sectional study, recorded consultations	124 patients aged 20–34 years, invited to a new primary care clinic on 'genetic and preconception' issues'	19% of the registered target population	Family history as 'reason for encounter': Among the main reasons why patients attended the clinic were: planning a pregnancy (35%), cancer in family (22%), heart disease in family (17%). Most prevalent positive family history: multifactorial disorder presenting later in life: cardiovascular disease 9.7%, cancer 8.1%, atopy 6.5%; single gene or chromosome disorder 3.2%; multifactorial disease presenting at birth 2.4%
Crouch et al., 1986	Missouri, USA	Cross-sectional analysis of medical patients with family history records of practice in problem list, and (2) a systematic sample of 375 patients; detailed audit of questionnaires	Medical records of (1) 375 patients with family history records of practice in problem list, and (2) a systematic sample of 375 patients; detailed audit of 2x75 records.	Not applicable	Family history in medical record: 4.4% of patient records contained family history information in Problem List. Commonly recorded: high blood pressure, diabetes mellitus, coronary artery disease and cancer Patients questionnaires revealed more family history information than physicians' worksheets

Table 7.3 Developing tools for taking a detailed family history.

Citation	Location	Design & outcome measures	Participants	Response rate	Results
McNeill et al., 2008	Texas, USA	Descriptive study of cardiac health assessment, incl. a web-based tool, 'My Family Health Portrait' Outcome: completeness of cardiovascular family history profile	85 ethnically diverse staff 'My members of an elder-care facility	44/85 (52%)	A full three-generation family history could be provided by only 18% of the participants; knowledge of parents and siblings was complete in 52%. First-degree relatives with cardiac disease or diabetes were not known in 25% and 11% respectively; age at diagnosis (above or below 60) was not known in 27% and 14% respectively.
Murff et al., 2007	Boston, USA	Comparison of charts with patient survey data	Survey of family history (genogram tables) among 995 new patients (see also Murff 2004 (1)	310/995 (31%)	The survey identified 128 additional relatives with cancer; age was given in 81% (vs. 40% in chart review); of 48 individuals at risk, 29 (60%) were discovered by the survey instrument.
Qureshi et al., 2005	London & Nottinghamshire, UK	Comparative study between self administered FHQ and a structured pedigree interview; Outcomes: agreement between methods and validity of FHQ	326 newly registered patients aged 18-65 (205 FHQ only, 121 FHQ + pedigree interview)	>40% (326/ max.800)	Agreement between FHQ and interview (n=121): 77% (kappa 0.52) Many but not all patients with genetic risks in primary care were identified by FHQ: those warranting specialist referral: 4/6; those who might be offered carrier testing: 10/22; those with a family history of a polygenic disorder: 10/16 Conclusion: The FHQ needs further modification and validation
Qureshi et al., 2001	Nottingham, UK	Randomized controlled trial: single practice; questionnaire study (health check and FHQ versus health check only); Primary outcome: anxiety in patients	2x50 patients, not having received a health check during the last 2 years, aged 18-60	76%	Anxiety after filling in a FHQ: anxiety increased in first 2 weeks after filling in FHQ, whereas in the health check only group anxiety levels decreased (significant difference); after 3 months there was no difference between both groups
Rogers et al., 1990	Houston, USA	Cross-sectional; self administered genogram (3 generations) filled out at office, repeated 3 months later (mailed, filled out at home) Outcomes: completion time, test-retest reliability	11 women, aged 23-69	Not applicable	Median completion time (1 st form): 45 minutes (range 30-90 minutes). Median completion rate: 78% (1 st form) and 69% (2 nd form). Test-retest agreement: 100% with regard to patients themselves and children; relatively low: age (parents, grandparents), date of death (of grand parents); reliability was lower for more distant generations. Conclusion: 'the apparently simple issue of obtaining complete and reliable family information is quite complex'
Ebell et al., 1988	Michigan, USA	Cross-sectional; computer generated genogram by patients (4 generations, special focus on cancer, cardiovascular diseases, hypertension and diabetes) Outcomes: completion time, acceptability by patients	48 patients, aged 16-60, newly registered or without genogram in chart	48/50 (96%)	Mean completion time: 19 minutes (range 9-55 minutes). Every participant succeeded in generating at least a partial genogram; the average number of family members was 11, the average number of medical conditions was 7.4 Acceptability was high (pleasant/very pleasant: 94%)* Conclusion: in primary care an interactive computer program can be used to generate a standardized genogram, containing important family history information; reliability and accuracy need to be assessed further. * in this population computer experience was 40%, a home computer was owned by 15%
Jolly et al., 1980	New York, USA	Cross sectional; genograms recorded and interpreted by family physicians Outcomes: completion time, completeness, ability of family physicians to interpret genograms	6 physicians interviewed (identical) patients; 6 other physicians interpreted 3 complete standardized genograms	3 Not applicable	Mean completion time : 16 minutes (range 9-30 minutes). Completeness varied between 66-93% (average score: 83%). Variation in interpretation of genograms was low: 91-96% (average score: 94.5%)

Table 7.4 Patient perspective: risk communication.

Citation	Location	Design & outcome measures	Participants	Response rate	Results
Hall et al., 2007	Exeter, UK	Qualitative study: patients completed a family history questionnaire (FHQ) on heart disease; videotaped consultations; patients interviewed 2 weeks later	21 patients without heart disease, diabetes or familial hyper-cholesterolemia from 4 general practices	Not applicable	Agreement between patients and professionals on level of cardiovascular risk and how to reduce it. Three problematic issues arose, that were a cause of miscommunication: neglect of the professionals of patient's explanations of relative's disease; not addressing interaction of family history and other risk factors; problematic understanding of overall risk, incl. a positive family history
Walter et al., 2006	Cambridge, UK	Qualitative study, semi-structured interviews	30 patients with a family history of cancer, heart disease or diabetes	30/179 (17%); saturated dataset.	Patients' ideas on controllability of a disease that runs in the family are influenced by: their perception of consequences ('serious threat' or not), timeline ('long suffering' vs. 'inconvenience') and cause ('inherited' vs. 'lifestyle'). This influences individual's motivation for preventive actions.
Walter et al., 2005	Cambridge, UK	Qualitative study, semi-structured interviews	30 patients with a family history of cancer, heart disease or diabetes	30/179 (17%); saturated dataset.	Differences exist between patients perceptions and professional assessment of family history of common diseases (cancer, coronary artery disease and diabetes mellitus) Personal experiences and emotional impact influence personal risk perception of 'family disease' by patients

The practice of family history taking and recording

Eight empirical studies explored the practice of integrating family history in primary care (Table 7.2).²³⁻³⁰ Analyses of the presence of 'family history of common diseases' in the medical records showed a very low percentage (4.4%) in a (US) study from 1986 and a much higher percentage in a (US) study from 2002 (89%).^{23,26} In another (US) study, 'family history' appeared to be discussed in a quarter of all consultations, however there was a large variability among GPs (0-81%).²⁵ Updating was observed to be a problem.^{25,26} A recent Dutch study on diabetes screening showed that in 72% of patients the risk for diabetes could not be estimated from the electronic medical record (EMR) because data on family history were missing.³⁰ Two studies from the US showed that data in the medical record often were insufficient to estimate cancer risk.^{28,29} One study confirmed the opinion that knowing the family history of a patient influences clinical decision making: if a positive family history of diabetes was known, physicians ordered a glucose test almost three times as often. However, this knowledge had no impact on lifestyle advice offered by the physician.²⁷ One study revealed that patients considered more family information important than doctors.²³

Developing tools for taking family history

Seven original research papers had explored the use of tools for taking family history in primary care. Various tools were studied: genograms recorded by doctors, self-administered computer generated genograms (three or four generations), self-administered paper genograms (three generations), a family history questionnaire, a structured pedigree interview and a web-based tool (Table 7.3).³¹⁻³⁷ Three older (US) studies showed that it is possible to complete a genogram by a patient or a doctor (completeness on average 80%), but it takes time (range 9-90 minutes).³¹⁻³³ A recent small study in the US however, showed that a full three-generation family history could only be provided by a minority of the participants.³⁷ In a small study, the test-retest reliability of self-administered genograms appeared to be high for patients themselves and their children, but low for more distant generations and for details like age of onset (of parents and grandparents) and date of death (of grandparents).³³ A recent (UK) study showed a moderate agreement between a self-administered family history questionnaire and a structured pedigree interview (Cohen's kappa 0.52). In this study the sensitivity of the family history questionnaire appeared to be suboptimal (Table 7.3).³⁵ Another study from the US showed that by sending patients a questionnaire with genogram tables, much more detail in familial cancer risk could be established than was available from the medical record.³⁶ A similar result was achieved in the Dutch study on diabetes risk mentioned here above, where additional risk assessment showed that in 51.2% of patients without an EMR-derived risk a positive family history of diabetes was present.³⁰

Patient perspective: risk communication

Three recent qualitative studies from the UK revealed interesting facts on the patient perspective and communicating familial risk in cancer, diabetes and CVD (Table 7.4).³⁸⁻⁴⁰ Although there was agreement between doctors and patients on family risk, patient-doctor communication was hampered by differences in opinion on explanations for and implications of a higher family risk. Doctors often seemed not to know how to integrate patients' experiences and opinions into shared decision making on management (Table 7.4).

Discussion

This systematic review tried to summarize available evidence on comprehensive family history taking for common multifactorial diseases - like CVD, cancer or diabetes - in primary care. Twenty-seven papers were analyzed. Four categories of studies were identified: studies on physicians' opinions on the application of family history as genetic tool, empirical studies on the daily practice of family history taking, studies on the development of tools for taking a family history, and finally, studies on the patient perspective and patient-doctor risk communication.

Main findings

Fifteen out of 27 studies were published more than five years ago. Many studies were small or suffered from a low response rate and the objectives and outcomes of the studies were heterogeneous. One third of the studies reviewed were on doctors' opinions. Studies on specific diseases were mostly on cancer, a few on diabetes and CVD. Still, a general picture can be derived from our analysis. Firstly, primary care physicians considered taking a detailed family history to be their role in genetic medicine and regarded it to be a routine activity. The first registration or visit of a patient was mentioned as suitable occasion to collect family history data. However, these opinions were only partly confirmed by empirical studies of consultations in which medical family issues were addressed (24% of observed consultations), as well as analyses of medical records (4-89% contained 'some family history'). Chart analyses and observations of consultations also showed that family history data were not regularly updated (estimated at 20%/3 years).

Secondly, primary care physicians expressed doubts on their ability to gather detailed information to estimate genetic risk, e.g. for cancer. This opinion was confirmed by several empirical studies, that is, on cancer risk and diabetes, revealing insufficient data in the medical records to make reliable estimations.

Thirdly, taking a detailed family history or genogram appeared to be time-consuming (9-90 minutes). Reproducibility and validity of self-administered genograms or a family history questionnaire have been scarcely studied and the results are as yet

unsatisfactory: test-retest appeared to be low for more distant generations as well as for dates of disease onset and death; agreement between self-administered questionnaire and structured interview was only moderate. Results for completeness were variable between studies and between doctors, but completeness decreased with genetic distance. One study, using a web-based family history tool, showed less than 20% complete three-generation family histories.

Finally, quantitative and qualitative studies showed discrepancies in perceptions of the meaning of a positive family history (for cancer, CVD or diabetes) between patients and physicians, with consequences for patient-doctor communication and (preventive) management.

Interpretation

A limitation of this review might be publication bias. It is well documented that studies with non-significant or negative results are substantially less likely to be submitted for publication.^{41,42} Additionally, generalizability to various primary care populations is a problem of the studies presented in this review. Although we only included studies from primary care, almost all papers we reviewed were from the US, the UK or Australia.

From our review it is evident that more research is needed to strengthen the feasibility, validity and added value of comprehensive family history taking for prevention and management of multifactorial disorders in general practice. Our review supports the view of Rich *et al.*, who in their review demonstrated the barriers for application in primary care – lack of time, poor reimbursement, limited knowledge and skills - and the limited evidence for improved outcome.¹³

Furthermore, recent qualitative studies from the UK on family risk perception of patients and family risk communication between patients and doctors showed that integrating family history in diagnosis and management is problematic in many consultations.³⁸⁻⁴⁰ These results further specify the findings of an earlier systematic review of qualitative research on the understanding of familial risk by patients.⁴³

Added value of comprehensive medical family data

The conviction that knowing a patient's detailed family history has added value should be proven in long-term prognostic studies. Primary care seems to be a likely setting for this kind of research. Inspiring developments come from the field of preventive medicine, that is, from a collaborative group associated with the Centers for Disease Control in the US.^{44,45} They developed a family history tool for CVD. It was demonstrated that a family history expanded beyond early-onset CVD in a first-degree relative (that is, the traditional question) was significantly associated with prevalent CVD.⁴⁶ Also, 'familial risk' added significantly to a risk model for identifying people with early-onset CVD. Finally, it was shown that patients with a strong familial risk

possibly could derive great benefit from preventive life style interventions that reduce cardiovascular risk.⁴⁷

Another example comes from the field of cancer research. In the GRAIDS trial, software was used that linked a pedigree-drawing tool to patient-specific management advice regarding breast/ovarian cancer and colorectal cancer, based on regional guidelines for these diseases. General practice teams (UK) that used this support system not only had higher referral rates to regional genetics clinics for familial breast and colorectal cancer, but their referrals also were more appropriate as compared to teams that only had received a short training on familial cancer risk.⁴⁸ Similar software is developed in Australian primary care for heart disease and diabetes.⁹

Collection, interpretation and application of medical family data

Only comprehensive and reliable medical family data can be the basis for the weighing of familial disease information within the context of each individual patient ('risk assessment'). As this review shows, high quality medical family information is not easily available from medical records. Furthermore, medical family data provided by patients also often are insufficient, whether provided on paper or collected with a web-based tool. On the other hand, patients can provide a simple family tree, that is, without morbidity data, quite easily. In many health care systems it is the responsibility of the family physician to keep an up to date comprehensive medical record of his patients, including a list of important diagnoses, such as chronic diseases. These diagnoses often are based on clear criteria or specialist examinations. Furthermore, they often are available in coded format.

Therefore, we propose the following idea. We suggest a development in which the family pedigree provided by the patient can be combined - through a secure web based interface - with the medical information kept in the electronic medical record management systems of the family physicians of his relatives. Developing informed consent procedures on both sides of the family line - 'I (do not) agree to share this medical information with my relatives if requested' and 'I (do not) agree that medical information of my relatives is made known to me if possibly clinically relevant' - should be an integral part of such a technology. The aim would be to be able to generate genograms with up-to-date, complete and best quality medical diagnoses. Current information technology is able to facilitate this point-of-care delivery of detailed and high-quality medical family information. Consequently, the family physician - after having received adequate education in genetics and family risk communication - could take up his role of professional advisor on familial risk for common chronic disorders. Our group is planning literature reviews, pilot studies and qualitative research among GPs and patients to pursue the feasibility of this approach.

In conclusion, although our review revealed that taking and recording detailed family history data in primary care as yet is insufficient for rational medical decision making, there also is a growing conceptual, methodological and technological basis for further research and development.

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Chapter 8

GENERAL DISCUSSION

Introduction

The general aim of the work described in this thesis was to determine in a non-selected patient population the relationship between polymorphisms and biological (risk factors) and clinical (intermediate endpoints) cardiovascular outcome parameters. Based on the literature available at the start of the project, we restricted ourselves to the analysis of six polymorphisms. Three of the polymorphisms - angiotensinogen [AGT (M235T)], angiotensin-converting enzyme [ACE (4656_{rpt})] and the angiotensin II type 1 receptor [AGTR1 (A1166C)] - were analyzed since these genes are involved in the generation and action of angiotensin II.¹ In addition, we assessed the role of two polymorphisms that might be related to an enhanced reabsorption of sodium: the G-protein $\beta 3$ subunit [GNB3 (C825T)] and α -adducin [ADD1 (G460W)] polymorphisms.^{2,3} Finally, we looked at the endothelial nitric oxide (NO) synthase [NOS3 (E298D)] polymorphism due to its possible association with altered NO availability.⁴ We reasoned that these six polymorphisms could be relevant individually or in combination as additional risk factors for cardiovascular disease (CVD). We hypothesized that the presence of one or more rare alleles of these polymorphisms would improve cardiovascular risk stratification, not only for an individual but probably also for all (first-degree) family members with a comparable allele pattern, and that this approach might yield genetically tailored treatments. This resulted in the **Hypertension: Interaction and Prevalence of POLymorphisms related to Cardiovascular Risk and the Association to Treatment Efficacy Study (HIPPOCRATES)**, of which baseline results have been described in this thesis. The different studies that formed part of work focused on (1) the burden of CVD in the south of the Netherlands, (2) the association between CVD and the six polymorphisms, (3) the association between intermediate endpoints and the six polymorphisms and (4) a systematic review of the role of family history. This last focus of our work addressed the practice and outcomes of family history-taking, since family history is closely related to the genetic background. This chapter presents and discusses the main findings of these studies, and addresses the possible implications for clinical practice and perspectives for future research.

Main findings

Cardiovascular disease in the south of the Netherlands

The HIPPOCRATES project involved the population of a general practice centre (Medisch Centrum West Kerkrade) located in the southeast of Limburg, which constitutes an urbanized area around the cities of Heerlen and Kerkrade. From a population genetics point of view, this region is interesting for two reasons: (1) a relatively high cardiovascular mortality had been described in unpublished Dutch

reports and (2) migration (both into and out of the region) is relatively low. In Chapter 2 of this thesis we compare the burden of CVD in the Heerlen/Kerkrade region relative to those in a nearby region (Maastricht) and the average Dutch population. That study showed that the burden of CVD is higher in the Heerlen/Kerkrade region than in the Maastricht region, and this was not fully explained by a higher prevalence of risk factors. We conclude in that chapter that a better characterization of the interactions between environmental and genetic factors could be the key to understanding the pathogenesis of CVD. Consequently, we studied both genetics and classic risk factors at the individual level in the HIPPOCRATES project.

Cardiovascular disease and polymorphisms of the renin-angiotensin system

Chapter 3 describes the results of our baseline cross-sectional data analyses. We included a total of 803 study subjects (48% males, 52% females) who were mainly overweight hypertensives with dyslipidemia. There were no significant differences in allele frequencies of the six polymorphisms between patients with CVD and the control group. Although some alleles showed a protective effect after Bonferroni correction for multiple testing, these findings were of little clinical significance to the group as a whole. Receiver operating characteristic (ROC) analyses showed that adding the six polymorphisms to a multivariate model including all the main traditional risk factors improved the predictive value of traditional cardiovascular risk factors by only 1%. It should be emphasized that ROC analyses were not the objective of this study, and hence the ROC results should be interpreted as indicative (and hence not quantitatively accurate) of the contribution of genetic information. However, our data suggested that the studied polymorphisms are unlikely to make a major contribution to cardiovascular risk prediction in this primary-care population, although our findings could still be relevant to an individual patient.

The data presented in Chapter 3 raise the question of whether variability in cardiovascular endpoints influenced our results. We, therefore, performed a study in which ischemic heart disease (IHD) was the only outcome measure. The selection criterion for IHD was narrowed down to a first event of IHD. The variability in the association with the first event of IHD was further reduced by specifying the results according to gender and age. The main finding of that study, described in Chapter 4, was that the rare T allele of the AGT (M235T) polymorphism is protective for males with a first event of IHD, even after applying Bonferroni correction for multiple testing. A possible explanation for this unexpected observation is a low-renin state, which can be enhanced by the GNB3 (C825T) polymorphism and being older.^{2,5,6} A protective association for the T allele of AGT could be related to lower angiotensin levels under these conditions. Xu *et al.* recently performed a meta-analysis of the AGT

(M235T) polymorphism, studying 43 association studies on coronary artery disease that included 13,478 cases and 17,024 controls.⁷ Their meta-analysis showed that the overall effect of this polymorphism is weak, showing minor effects that could be either protective or harmful.⁷

As described in Chapter 4, most findings of gender-specific associations of cardiovascular phenotypes with candidate genes are rather unexpected. Such findings are often categorized as type I errors that are attributed to sample stratification with multiple comparisons and in some cases to the small number of individuals in a subgroup.⁸ However, adjustment for gender during the analyses and interpretation of data can actually hide a significant portion of genome-driven physiology unique to males and females.^{9–12} This is illustrated in Chapter 4 by sliding-mean analyses showing that the risks of a first IHD event are completely different for men and women at different ages. Together these findings underscore the need for gender-specific evaluations.

Intermediate endpoints and polymorphisms of the renin-angiotensin system

In the subsequent two studies, described in Chapters 5 and 6, we were interested in the association of the six polymorphisms with so-called intermediate vascular endpoints: carotid intima media thickness (CIMT) and arterial stiffness as assessed by pulse wave velocity (PWV) measurements. These new non-invasive technologies might help in applying preventive measures correctly at a relatively early stage,¹³ a strategy that might be particularly helpful in a primary care setting.

The study described in Chapter 5 investigated the association between CIMT as an intermediate endpoint for preclinical atherosclerosis and our six polymorphisms. The results show that CIMT was significantly associated with age, gender, waist circumference, dyslipidemia and use of cardiovascular medication. Multiple linear regression analyses with correction for these covariates showed that the C allele of AGTR1 was associated with a thicker CIMT in females only, and that the T allele of ADD1 coincided with a smaller CIMT.

The described association between carriership of the C allele of AGTR1 and thicker CIMT has been reported by other authors.^{14,15} The AGTR1 (A1166C) polymorphism is located in the 3'-untranslated region (3'-UTR) of the gene, which is where mRNA stability is regulated. The C allele of AGTR1 has previously been reported to be non-functional and is presumed to be in linkage disequilibrium with a variant that alters receptor sensitivity, resulting in an increased response to angiotensin II.^{16,17} The 3'-UTR site has recently been shown to be involved in microRNA gene silencing.^{18,19} MicroRNAs are single-stranded RNA molecules - typically 21–23 nucleotides in length -

that regulate gene expression. They are processed from primary transcripts and their main function is to down-regulate gene expression (gene silencing). C allele carriers have impaired microRNA binding and a consequent impairment of translation attenuation, resulting in increased receptor density. Such AGTR1 receptor alterations could exert several possible effects that would influence CIMT (see Chapter 5), eventually together initiating the inflammation process in vascular cells.²⁰ It is therefore not unreasonable to assume that this explains the observed thicker CIMT in C allele carriers of the AGTR1 polymorphism. There is no evidence in the literature that these mechanisms differ with gender.

Several studies have found an association between the ADD1 (G460W) polymorphism, salt sensitivity or high sodium intake, and an increased risk of cardiovascular events and mortality.²¹ In these studies, the CIMT and cardiovascular morbidity and mortality were increased in T allele carriers. However, in our study we observed that this T allele coincided with a reduced CIMT. Tobin *et al.* also found such a protective association for ADD1 and suggested that this was attributable to the premature death of T allele carriers or to a protective effect of this allele through a cellular mechanism that differs from its effect leading to hypertension.²² However, as described in Chapter 5, we suggest that this is related to the treatment status of the study subjects. More specifically, as indicated by other authors, a longer duration of pharmacological treatment reduces the increase in CIMT.^{23,24} Our cross-sectional study indicates that this could indeed be an alternative explanation for the observed protective effect of the T allele of ADD1. Carriers of the rare T allele of the ADD1 (G460W) polymorphism are probably medicated at an earlier age and consequently for longer, leading to a reduced CIMT.²⁵ We checked for the effect of treatment duration in our database, and found no statistically significant difference in treatment duration between carriers with the normal genotype (GG) and carriers of the rare T allele of the ADD1 (G460W) polymorphism. However, there are two major problems with our study design being cross-sectional: (1) CIMT is influenced by cardiovascular medication and (2) channelling plays an important role; that is, the most severe cardiovascular patients receive more intensive treatment. A cross-sectional study cannot distinguish the contribution of these two factors. However, these types of associations are observed in patients treated in daily clinical practice. Therefore, future prospective studies are needed to establish the role of treatment.

The finding of the protective association of the T allele of ADD1 raised the question of whether the same sort of association could be assessed using another intermediate vascular endpoint, that is, increased arterial stiffness as measured by carotid-femoral PWV measurements. In the study presented in Chapter 6, we found a significant protective association for the AGTR1 (A1166C) polymorphism with arterial stiffness. This association was significantly influenced by the use of cardiovascular medication and showed a dose-allele effect, with the carotid-femoral/carotid-radial ratio

(CF/CR ratio) decreasing with the number of C alleles. However, the results found in that study were not consistent with the results presented in Chapter 5. More specifically, Chapter 5 describes how the AGTR1 (A1166C) polymorphism is associated with a thicker CIMT, whereas Chapter 6 states that the same polymorphism is associated with a lower PWV. Various hypotheses to explain these results are presented in Chapter 6, and testing them again requires prospective epidemiological studies. Although the findings presented in Chapters 5 and 6 are important for future research in the field of functional genomics, they are currently of little clinical significance.

Strengths and limitations

Most of the studies presented in this thesis compared a recessive model of inheritance of the homozygous wild-type with heterozygous and homozygous rare allele carriers. This choice was made based on the results of other studies, though we realize this choice is somewhat arbitrary. Furthermore, we only studied the effect of one polymorphism per gene, which does not exclude the role of other polymorphic alleles that are not in linkage disequilibrium.

Our work also had some strong aspects, such as the inclusion of a primary care population in which the entire range of low-to-high-risk patients was available. Additionally, our population was selected using strict inclusion criteria from a rather small geographical area and hence had similar social living conditions. Although our work had an exploratory character, each study generally had a clear a-priori hypothesis, which reduced multiple hypothesis testing.

Family history of common diseases in primary care

Family history is closely related to the genetic background. We can hypothesize that the presence of certain genetic variants is more important in subjects with a positive family history for CVD. This can only be studied in the presence of high-quality family history-taking. In Chapter 7 we present the results from a systematic literature search of studies that have dealt with family history-taking in primary care as a genetic tool to identify individuals and families at increased risk of multifactorial disorders. We found that it is useful to know a patient's detailed family history, but this has not been shown in long-term prognostic studies. Although our review revealed that taking and recording detailed family-history data in primary care are currently insufficient for rational medical decision-making, there is also a growing conceptual, methodological and technological basis for further research and development.

What the present work adds

It could be concluded retrospectively that we were slightly naive at the start of HIPPOCRATES, in that we expected that genetics would clarify major causes of all kinds of multifactorial diseases, including CVD. The results from the association studies presented in this thesis show that the contribution of the six polymorphisms to the 'prediction' of cardiovascular risk is of minor clinical significance for this primary care population as a whole. Estimations of the cardiovascular risk based on the traditional risk factors in this population are already excellent. On the other hand, this thesis presents several results that might be important for individual patients. Firstly, the rather unexpected protective associations of the AGT (M235T) polymorphism (Chapters 3 and 4) and the ADD1 (G460W) polymorphism (Chapter 5) need to be evaluated in future research. Secondly, the gender-specific association of the AGT (M235T) polymorphism (Chapter 4) needs to be further investigated. Thirdly, our investigation of the influence of medication use yielded possible new insights into and hypotheses for the associations between the ADD1 (G460W) polymorphism and CIMT (Chapter 5) and between the AGTR1 (A1166C) polymorphism and PWV (Chapter 6). Again these findings need to be evaluated in long-term follow-up studies. Together these findings are important for future research in the field of functional genomics. However, the results of this thesis also question whether it is useful to proceed with this type of population-based genetic research (that is, association studies of genetic polymorphisms with intermediate or hard endpoints in primary care), or whether other options would be better.

Perspectives for future research

Assessment of more polymorphisms

Jeunemaitre recently reviewed the evidence from the most recent studies on the genetics of the human renin-angiotensin system (RAS).¹ His main conclusion was that although there is still major interest in elucidating the role of these polymorphisms, it is currently too early to propose recommendations and/or specific drug treatments for patients with particular RAS genotypes.¹ The results of our work described in this thesis are consistent with this conclusion. It should be noted that a novel and promising approach for unravelling the genetic basis of complex genetic diseases has recently been introduced.²⁶ These so-called genome-wide association studies analyze several hundreds of thousands of polymorphisms simultaneously, posing substantial biostatistical and computational challenges. The expectation is that applying this new methodology to large and comprehensively phenotyped patient cohorts will readily reveal the most clinically relevant disease-modifying and drug-responsive genes.²⁷ This will hopefully reveal the exact value of RAS polymorphisms for individual

patients. HIPPOCRATES is currently too small to perform such an investigation. On the other hand, what would be the impact on the daily clinical practice of the general practitioner (GP) of being able to interpret the results of several hundreds of thousands of polymorphisms per patient? For this specific population, we consider that it would be better to apply the time and effort involved in such genetic assessments to risk management based on the traditional cardiovascular risk factors.

Add CIMT or PWV?

Primary prevention of CVD is based only on the most important traditional risk factors. The literature indicates that exposure to every risk factor substantially influences atherosclerosis.¹³ For example, it was recently shown that adding the results of an ankle-brachial index measurement to the Framingham risk score improved risk prediction.²⁸ Assessment over time by non-invasive CIMT or PWV might reveal the progression of preclinical atherosclerosis in patients who are not yet diagnosed with manifest CVD. Combining these measurements with traditional cardiovascular risk-factor management appears to be relevant to clinical prevention, since this might improve cardiovascular risk stratification. However, as indicated by Ter Avest *et al.*, before the clinical application of CIMT and PWV is considered in individual CVD risk prediction, the additive value of these measurements in predicting the CVD risk in an individual above and beyond measurements of traditional clinical and biochemical parameters has to be demonstrated in long-term follow-up studies.¹³ Furthermore, appropriate cut-off values have to be derived across populations exhibiting various risk levels, including those at high risk. This could be a challenging objective for a HIPPOCRATES follow-up study.

Assessment of comprehensive family history

Since current knowledge of specific associations of polymorphisms and multifactorial diseases has limited added predictive value,²⁹ another approach could be to use the family medical history. Detailed knowledge of the family medical history could detect familial disease patterns, assess the clinical significance of these patterns (that is, the 'risk') and consequently use this knowledge in preventive and curative care. This hypothesis has led to new interest—as expressed in editorials and descriptive reviews—in the use of a comprehensive family medical history as a tool for the primary and secondary prevention of chronic multifactorial diseases.^{30–37} Moreover, the family history would allow candidates for genetic testing or targeted medical care to be identified. The community orientation of family physicians (primary care physicians and GPs) puts them in an ideal position to track subjects at risk and consequently to discuss possible preventive actions with them. However, only comprehensive and reliable family medical data should be used for weighting the importance of familial disease information within the context of each patient ('risk assessment'). Our review showed that high-quality family medical information is not

readily obtainable from medical records. Furthermore, the family medical data provided by patients are also often insufficient, whether provided on paper or collected with a web-based tool. On the other hand, patients can provide a simple family tree (that is, without morbidity data) quite easily. Therefore, in Chapter 7 we propose the development of an information and communication technology tool that combines pedigree information provided by patients - through a secure web-based interface - with medical information kept in the electronic-medical-record management systems of family physicians of the patients' relatives. The aim will be to provide genograms with up-to-date, complete, detailed and correct medical diagnoses (and results of genetic tests). Our group is currently planning pilot studies and qualitative research involving GPs and patients to assess the feasibility of this approach. As part of the HIPPOCRATES project we could perform additional exploratory association analyses in subjects with a positive family history of CVD in first-degree family members, either men or women, versus those without this condition. These options illustrate that a large field of research remains to be explored. This could eventually lead to new insights into the pathophysiology of CVD, changes in the approach to CVD diagnosis and to more appropriate therapeutic strategies for managing CVD patients in primary care.

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SUMMARY

Summary

Atherosclerotic cardiovascular disease (CVD), involving the heart, brain and peripheral arteries of the legs, is a major health problem around the world. The aetiology of CVD is multifactorial. Both genetic and environmental factors play an important role. The most common forms of CVD are believed to be multifactorial and the result of interaction of several genes, each with a relatively small effect, working alone or in combination with other modifiers, genes and/or environmental factors. Research, predominantly epidemiologic studies, is continuing at high speed to elucidate which (combination of) genes or genetic factors play an active role in the aetiology of CVD. In this thesis we determined biological (risk factors) and clinical (intermediate endpoints) cardiovascular outcome parameters in a non-selected patient population and set out to establish the association of certain polymorphisms and these parameters. Based on the literature available at the start of the project, we restricted ourselves to the analysis of six polymorphisms. Three of these polymorphisms - angiotensinogen [AGT (M235T)], angiotensin-converting enzyme [ACE (4656_{rpt})] and the angiotensin II type 1 receptor [AGTR1 (A1166C)] - were analyzed since these genes are involved in the generation and action of angiotensin II. In addition, we assessed the role of two polymorphisms that might be related to an enhanced reabsorption of sodium: the G-protein $\beta 3$ subunit [GNB3 (C825T)] and α -adducin [ADD1 (G460W)] polymorphisms. Finally, we looked at the endothelial nitric oxide (NO) synthase [NOS3 (E298D)] polymorphism due to its possible association with altered NO availability. We reasoned that these six polymorphisms could be relevant individually or in combination as additional risk factors for CVD, given their role in the regulation of blood pressure, water and salt balance and endothelial function. We hypothesized that the presence of one or more rare alleles of these polymorphisms would improve cardiovascular risk stratification, not only for an individual but probably also for all (first-degree) family members with a comparable allele pattern, and that this approach might yield genetically tailored treatments. This resulted in the **Hypertension: Interaction and Prevalence of POLymorphisms related to Cardiovascular Risk and the Association to Treatment Efficacy Study (HIPPOCRATES)**, of which baseline results have been described in this thesis. The different studies that were performed as part of this project focused on (1) the burden of CVD in the south of the Netherlands, (2) the association between CVD and six polymorphisms, (3) the cross-sectional association between intermediate endpoints and six polymorphisms and (4) a systematic review of the role of family history as a tool in genetic studies in primary care.

Cardiovascular disease in the south of the Netherlands

After a general introduction in *Chapter 1*, *Chapter 2* focuses on the comparison of the burden of CVD in the Heerlen/Kerkrade region relative to those in a nearby region

(Maastricht) and the average Dutch population. That study showed that the burden of CVD is higher in the Heerlen/Kerkrade region than in the Maastricht region, and this was not fully explained by a higher prevalence of classical risk factors. At that time, we concluded that a better characterization of the interactions between environmental and genetic factors could be the key to understanding the pathogenesis of CVD. Consequently, we continued studying both genetics and classical risk factors at the individual level in the HIPPOCRATES project.

Cardiovascular disease and six polymorphisms

Chapter 3 describes the results of our baseline cross-sectional data analyses. We included a total of 803 study subjects (48% males, 52% females) who were mainly overweight hypertensives with dyslipidemia. There were no significant differences in allele frequencies of the six polymorphisms between patients with CVD and event-free controls. Although some alleles showed a protective effect after Bonferroni correction for multiple testing, these findings were of little clinical significance to the group as a whole. Our data suggested that the studied polymorphisms are unlikely to make a major contribution to cardiovascular risk prediction in this primary care population, although our findings could still be relevant to an individual patient.

In *Chapter 4*, we investigated whether the mix of cardiovascular endpoints influenced the results described in *Chapter 3*. We therefore performed a study in which ischemic heart disease (IHD) was the only outcome measure. The selection criterion for IHD was narrowed down to a first event of IHD. The variability in the association with the first event of IHD was further reduced by specifying the results according to gender and age. The main finding of that study was that the rare T allele of the AGT (M235T) polymorphism is protective for males with regard to a first event of IHD. A possible explanation for this unexpected observation is a low-renin state, which can be enhanced by the presence of the mutant allele of the GNB3 (C825T) polymorphism and older age. Consequently, the protective association for the T allele of AGT could be related to lower angiotensin II levels under these conditions.

Intermediate endpoints and six polymorphisms

The study described in *Chapter 5* investigated the association between carotid intima media thickness (CIMT) as an intermediate endpoint for preclinical atherosclerosis and the six polymorphisms. The results show that CIMT was significantly associated with age, gender, waist circumference, dyslipidemia and use of cardiovascular medication. Multiple linear regression analyses with correction for these covariates showed that the C allele of AGTR1 was associated with a thicker CIMT in females only, and that the T allele of ADD1 coincided with a smaller CIMT. The described association between carriership of the C allele of AGTR1 and thicker CIMT could be explained by the fact that C allele carriers have probably impaired microRNA binding. The main function of microRNA is to down-regulate gene expression (gene silencing). Such AGTR1 receptor

alterations could exert several possible effects that, in combination with the other risk factors, can influence CIMT eventually resulting in the observed thicker thickness. However, evidence for gender differences with respect to these mechanisms was not present in the literature. While in most studies male gender is associated with a thicker CIMT and females have often the thinnest CIMT, we found in the study described in *Chapter 5* the opposite. A possible explanation might be that males are treated more than females with cardiovascular risk-reducing therapies.

The finding that the T allele of ADD1 coincided with a smaller CIMT was more difficult to explain. We suggested in *Chapter 5* that this may be related to the duration of pharmacological treatment of the study subjects, that is, carriers of the rare T allele of the ADD1 (G460W) polymorphism are probably medicated at an earlier age and consequently treated for a longer period, leading to a reduced CIMT. However, in the present database we were unable to adequately investigate this hypothesis due to two major problems: (1) CIMT is influenced by cardiovascular medication and (2) channelling plays an important role; that is, the most severe cardiovascular patients receive more intensive treatment. Our cross-sectional study cannot distinguish the contribution of these two factors. However, these types of associations are observed in patients treated in daily clinical practice. Therefore, future prospective studies are needed to establish the role of treatment.

The arterial system gradually stiffens because of the shared effects of ageing, high blood pressure, genetic factors, and other risk factors. Arterial stiffness, and especially a stiffened aorta, has been associated with an excess of cardiovascular morbidity and mortality. In the study presented in *Chapter 6* we found a significant protective association for the AGTR1 (A1166C) polymorphism with arterial stiffness as assessed by pulse wave velocity (PWV) measurements. This association was significantly influenced by the use of cardiovascular medication and showed a dose-allele effect, with the carotid-femoral/carotid-radial ratio (CF/CR ratio) decreasing with the number of C alleles. Various hypotheses to explain these results are presented in this chapter, but testing them, again, would require prospective epidemiological studies. Although the findings presented in the *Chapters 5* and *6* are important for future research in the field of functional genomics, they should be appreciated as hypothesis generating, and are currently of little clinical significance.

Family history of common diseases in primary care

Family history is closely related to the genetic background. Given their community orientation, general practitioners (family physicians, primary care physicians) seem to be in the ideal position to integrate medical family data in their primary and secondary preventive activities. In *Chapter 7* we presented the results of a systematic literature search of studies that have dealt with family history-taking in primary care as a genetic tool to identify individuals and families at increased risk of multifactorial

disorders. We found that it is useful to know a patient's detailed family history, but this has, unfortunately, not (yet) been shown in long-term prognostic studies. Although our review revealed that taking and recording detailed family history data in primary care are currently insufficient for rational medical decision-making, there is also a growing conceptual, methodological and technological basis for further research and development.

Discussion and conclusion

In *Chapter 8* the main findings of the studies in this thesis are discussed in light of the current literature. In addition, the clinical relevance of the findings and recommendations for future research are given. We concluded that the contribution of the six polymorphisms to the 'prediction' of cardiovascular risk is of minor clinical significance for this primary care population as a whole. On the other hand, this thesis presents several results that might be important for individual patients. Firstly, the rather unexpected protective associations of the AGT (M235T) polymorphism (*Chapters 3 and 4*) and the ADD1 (G460W) polymorphism (*Chapter 5*). Secondly, the gender-specific association of the AGT (M235T) polymorphism (*Chapter 4*). Thirdly, our investigation of the influence of medication use yielded possible new insights into and hypotheses for the associations between the ADD1 (G460W) polymorphism and CIMT (*Chapter 5*) and between the AGTR1 (A1166C) polymorphism and PWV (*Chapter 6*). Together these findings are important for future research in the field of functional genomics. Finally we provide suggestions for future research. We would like to emphasize that determination of more polymorphisms in the HIPPOCRATES population is not useful. Indeed, what would be the impact on the daily clinical practice of the general practitioner of being able to interpret the results of several hundreds of thousands of polymorphisms per patient? Secondly, the results of this thesis indicate that combining CIMT and PWV measurements with traditional risk-factor management might be relevant in clinical prevention, since this might improve cardiovascular risk stratification. However, before the clinical application of CIMT and PWV is considered in individual CVD risk prediction, the additive value of these measurements in predicting the CVD risk in an individual above and beyond measurements of traditional clinical and biochemical parameters has to be demonstrated in long-term follow-up studies. This could be a challenging objective for a HIPPOCRATES follow-up study. Finally we indicate that since current knowledge of specific associations of polymorphisms and multifactorial diseases has limited added predictive value another approach could be to use the family medical history. Currently, our group is planning pilot studies and qualitative research among GPs and patients to pursue the feasibility of this approach.

SAMENVATTING

Samenvatting

Hart-en vaatziekten (HVZ) behoren tot de belangrijkste doodsoorzaken in de westerse wereld. Het ontstaan van HVZ is multifactorieel. Omgevingsfactoren en meer of minder bekende risicofactoren, waaronder ook genetische factoren, spelen hierbij een belangrijke rol. Bij de meeste vormen van HVZ geldt dat de genetische predispositie het resultaat is van interactie tussen verschillende genen, ieder met een relatief klein effect, die in sommige gevallen samenwerken met andere genen of (omgevings)factoren. Deze complexe interacties zijn bij lange na nog niet opgehelderd. Daarom vindt er continu onderzoek plaats, met name epidemiologisch onderzoek, om op te helderen welke combinaties van genen of genetische factoren een rol spelen in het ontstaan van HVZ.

Voor dit proefschrift hebben we biologische (risicofactoren) en klinische (intermediaire) cardiovasculaire eindpunten bepaald in een eerstelijns patiëntenpopulatie. In deze populatie zijn we vervolgens associaties tussen deze eindpunten en bepaalde polymorfismen gaan bestuderen. Op basis van de literatuur die bij het begin van dit promotieonderzoek beschikbaar was, hebben we ons beperkt tot de analyse van zes polymorfismen. Drie van deze polymorfismen - angiotensinogeen [AGT (M235T)], angiotensine I converterend enzym [ACE (4656rpt)] en de angiotensine II type 1 receptor [AGTR1 (A1166C)] - werden geanalyseerd omdat deze genen betrokken zijn bij de vorming en effecten van angiotensine II. Daarnaast bepaalden we de rol van twee polymorfismen die gerelateerd zijn aan verhoogde zoutgevoeligheid, namelijk de G-protein $\beta 3$ subunit [GNB3 (C825T)] en het α -adducine [ADD1 (G460W)] polymorfisme. Als laatste werd het endotheliaal stikstofoxide synthase NOS3 (E298D)] polymorfisme bestudeerd, een polymorfisme dat betrokken is bij de gewijzigde beschikbaarheid van endotheelafhankelijk stikstofoxide (NO). Onze redenering was dat deze zes polymorfismen, individueel of in combinatie, relevant zouden kunnen zijn als additionele risicofactoren voor HVZ. Onze hypothese was dan ook dat de aanwezigheid van één of meer allelen van deze polymorfismen de cardiovasculaire risicoclassificatie zou verbeteren en mogelijk niet alleen voor een individuele patiënt maar hoogstwaarschijnlijk ook voor alle (eerstegraads) familieleden met een vergelijkbaar patroon van allelen. Deze benadering zou er toe kunnen leiden dat patiënten op basis van hun genetisch profiel specifieke (preventieve) behandelingen zouden kunnen krijgen. Dit resulteerde in het “Hypertension: Interaction and Prevalence of **P**olymorphisms related to **C**ardiovascular **R**isk and the **A**ssociation to **T**reatment **E**fficacy **S**tudy (HIPPOCRATES)” project waarvan de baseline resultaten in dit proefschrift beschreven staan. De verschillende studies die als onderdeel van dit project werden uitgevoerd, gaan over (1) de prevalentie van HVZ in het zuiden van Nederland, (2) de associatie tussen HVZ en de zes polymorfismen, (3) de cross-sectionele associatie tussen intermediaire eindpunten en de zes polymorfismen en (4)

de rol van familie-anamnese in de eerstelijns geneeskunde in kaart gebracht in een systematische review.

Hart-en vaatziekten in het zuiden van Nederland

Na een algemene introductie in *Hoofdstuk 1*, worden in *Hoofdstuk 2* de prevalenties van HVZ beschreven en met elkaar vergeleken voor de regio's Heerlen/Kerkrade en Maastricht. Bovendien wordt de prevalentie van HVZ in de regio Heerlen/Kerkrade vergeleken met die van de Nederlandse bevolking. Deze studie liet zien dat er meer HVZ voorkomen in Heerlen/Kerkrade en dat deze hogere prevalentie van HVZ niet volledig kan worden verklaard door een hogere prevalentie van traditionele cardiovasculaire risicofactoren. Toentertijd concludeerden we dan ook dat een betere karakterisering van de interacties tussen gen- en omgevingsfactoren essentieel zou zijn om de pathogenese van HVZ verder te ontrafelen. Daarom gingen we in HIPPOCRATES verder met de bestudering van zowel de genetische als de klassieke cardiovasculaire risicofactoren op een individueel niveau.

Hart-en vaatziekten en zes polymorfismen

Hoofdstuk 3 beschrijft de basale resultaten van ons cross-sectionele onderzoek. We includeerden 803 personen (48% mannen, 52% vrouwen) die voor het merendeel gekarakteriseerd konden worden als hypertensieve patiënten met overgewicht en dislipidemie. Er werden tussen de patiënten met HVZ en de controlepersonen zonder HVZ geen statistisch significante verschillen in allelfrequenties van de zes polymorfismen gevonden. Hoewel sommige allelen een beschermend effect lieten zien, leken deze bevindingen op grond van ROC-analyses van slechts gering klinisch belang voor de populatie als geheel. De resultaten in dit hoofdstuk laten zien dat het onwaarschijnlijk is dat de bestudeerde polymorfismen een belangrijke bijdrage leveren aan de voorspelling van het cardiovasculaire risico in deze eerstelijns populatie als geheel. Dat sluit niet uit dat onze bevindingen nog steeds relevant kunnen zijn voor individuele patiënten.

We vroegen ons af of het feit dat we cardiovasculaire eindpunten gecombineerd hadden geanalyseerd, invloed zou kunnen hebben op de resultaten in *Hoofdstuk 3*. Daarom werd in *Hoofdstuk 4* ischemische hartziekte (IHZ) als enigste uitkomstmaat gekozen. De selectie criteria werden beperkt tot een eerste optreden van IHZ. De diversiteit van de associatie tussen een eerste optreden van IHZ en de zes polymorfismen werd verder gereduceerd door de resultaten te stratificeren naar geslacht en leeftijd. De belangrijkste bevinding van dit onderzoek was dat het T allel van het AGT (M235T) polymorfisme beschermend is voor mannen met een eerste optreden van IHZ. Een mogelijke verklaring voor deze onverwachte waarneming zou te maken kunnen hebben met een laag, niet-geactiveerd renine, waarvan het effect kan worden versterkt door de aanwezigheid van het GNB3 (C825T) polymorfisme en hogere leeftijd. Beide gevallen worden namelijk gekenmerkt door een niet

geactiveerd renine-angiotensine systeem. Onder deze omstandigheden kan een beschermend effect van het T allel van AGT gerelateerd zijn aan lagere angiotensine II spiegels, zoals die worden gevonden bij dragers van het T allel van het AGT polymorfisme.

Intermediaire eindpunten en zes polymorfismen

In de studie beschreven in *Hoofdstuk 5* onderzochten we de associatie tussen carotis intima media dikte (CIMD) als een intermediair eindpunt voor preklinische atherosclerose en de zes polymorfismen. We vonden dat er een statistisch significante associatie was tussen CIMD en leeftijd, geslacht, middelomtrek, dislipidemie en het gebruik van cardiovasculaire medicatie. Lineaire regressie-analyse met correctie voor deze covariabelen liet zien dat alleen bij vrouwen het C allel van het AGTR1 (A1166C) polymorfisme geassocieerd was met een dikkere CIMD en dat het T allel van het ADD1 (G460W) polymorfisme gerelateerd is aan een dunnere CIMD. De beschreven associatie tussen dragerschap van het C allel van het AGTR1 polymorfisme en een dikkere CIMD kan worden verklaard door het feit dat dragers van het C allel een verminderde binding hebben van het zogenaamde microRNA. Dit microRNA speelt een belangrijke rol in het down-reguleren van de gen-expressie. Als dit down-reguleren niet of minder gebeurt, leidt dit tot een toegenomen receptor dichtheid. Dergelijke veranderingen in de relatie tussen angiotensine II en zijn receptor kunnen verschillende effecten induceren die, in combinatie met andere risicofactoren, de CIMD beïnvloeden. Dit zou uiteindelijk kunnen leiden tot de waargenomen dikkere CIMD. Echter er zijn nog geen studies beschikbaar die een verschil tussen mannen en vrouwen laten zien aangaande deze mechanismen. Eerdere algemene studies naar CIMD hebben laten zien dat juist mannen een dikkere CIMD hebben dan vrouwen. Een mogelijke verklaring voor de dikkere CIMD bij vrouwen in onze populatie zou kunnen zijn dat het cardiovasculair risico management bij mannen intensiever is dan bij vrouwen.

De bevinding dat bij dragers van het T allel van het ADD1 (G460W) polymorfisme vaker een dunnere CIMD werd gevonden, is moeilijker te verklaren. We suggereerden dat deze bevinding weleens verklaard zou kunnen worden door de duur van medicamenteuze behandeling van de geïnccludeerde patiënten. Met andere woorden, dragers van het T allel van het ADD1 (G460W) polymorfisme beginnen waarschijnlijk op jongere leeftijd met cardiovasculaire medicatie, worden daardoor langer medicamenteus behandeld en dit leidt uiteindelijk tot een dunnere CIMD. Helaas konden we in de huidige database deze hypothese niet meer afdoende onderzoeken, omdat de cross-sectionele opzet van onze onderzoek de volgende twee belangrijke problemen veroorzaakt: (1) CIMD wordt beïnvloed door het gebruik van cardiovasculaire medicatie en (2) 'channelling' speelt een belangrijke rol, dat wil zeggen dat patiënten met de meest ernstige uitingen van HVZ de meest krachtige

behandelingen krijgen. Onze studie kan door zijn cross-sectionele karakter geen onderscheid maken tussen de bijdragen van deze twee factoren. Dit type associatie wordt echter wel waargenomen bij patiënten die behandeld zijn in de dagelijkse klinische praktijk. Het is daarom van belang dat toekomstige prospectieve studies de rol van behandeling verder in kaart gaan brengen.

De gevolgen van veroudering, hoge bloeddruk, genetisch factoren en andere risicofactoren op de circulatie, leiden tot een geleidelijke verstijving van de bloedvatwand, met name van de arteriën. Personen met een toegenomen arteriële stijfheid, in het bijzonder van de aorta, hebben een verhoogde kans op het krijgen van cardiovasculaire morbiditeit en mortaliteit. In de studie beschreven in *Hoofdstuk 6* vonden we een statistisch significante, beschermende associatie tussen het AGTR1 (A1166C) polymorfisme en door middel van pulse wave velocity (PWV) bepalingen vastgestelde stijfheid van de aortawand. Deze associatie werd significant beïnvloed door het gebruik van cardiovasculaire medicatie en liet zien dat de carotis-femoralis/carotis-radialis ratio afnam met het aantal C allelen. In dit hoofdstuk geven we verschillende hypothesen om deze resultaten te verklaren, maar om die te toetsen is prospectief epidemiologisch onderzoek nodig. Alhoewel de bevindingen in *Hoofdstuk 5* en *6* belangrijk zijn voor toekomstig ‘functional genomics’ onderzoek en dientengevolge ook moeten worden gezien als hypothese genererend, zijn deze bevindingen op dit moment van minder klinisch belang.

Familie-anamnese van veel voorkomende ziekten in de eerstelijns geneeskunde

Familie-anamnese is nauw verbonden met het genetisch profiel van patiënten. Omdat de huisarts dichtbij de patiënt staat en vaak de familie kent, lijken huisartsen in de ideale positie te verkeren om de familie-anamnese in hun primaire en secundaire preventieve activiteiten te integreren. In *Hoofdstuk 7* staan de resultaten beschreven van een systematische review over studies die gingen over het afnemen van de familie-anamnese in de eerstelijns geneeskunde, als instrument om individuen en families te identificeren met een verhoogd risico op multifactoriële chronische aandoeningen, zoals HVZ en kanker. De resultaten lieten zien dat het belangrijk is om een gedetailleerde familie-anamnese te kennen, maar dat het voordeel hiervan op de lange termijn nog niet is aangetoond. Daarnaast bleek uit deze review dat de huidige mogelijkheden voor het afnemen en vastleggen van een uitgebreide familie-anamnese in de eerstelijns beperkt en onvoldoende zijn om verstandige medische besluitvorming op te baseren. We geven aan welke conceptuele, methodologische en technische basis aanwezig is om het gebruik van een uitgebreide familie-anamnese verder te ontwikkelen.

Beschouwing en conclusie

Tenslotte worden in *Hoofdstuk 8* de belangrijkste resultaten van dit proefschrift besproken in het licht van de huidige literatuur. Daarnaast worden de klinische relevantie en de aanbevelingen voor toekomstig onderzoek gegeven. Wij concludeerden dat het onwaarschijnlijk is dat de bestudeerde polymorfismen een belangrijke bijdrage leveren aan de voorspelling van het cardiovasculair risico in deze eerstelijns populatie als geheel. Aan de andere kant laat dit proefschrift een aantal bevindingen zien die nog steeds relevant kunnen zijn voor individuele patiënten. Voorbeelden hiervan zijn allereerst de onverwachte bevinding van het beschermend effect van het AGT (M235T) polymorfisme (*Hoofdstuk 3 en 4*) en het ADD1 (G460W) polymorfisme (*Hoofdstuk 5*). Ten tweede de rol van geslacht bij het AGT (M235T) polymorfisme (*Hoofdstuk 4*). Ten derde leverden de studies naar de invloed van medicatiegebruik op de associaties tussen het ADD1 (G460W) polymorfisme en CIMT (*Hoofdstuk 5*) en tussen het AGTR1 (A1166C) polymorfisme en PWV (*Hoofdstuk 6*) nieuwe inzichten en hypothesen op. Deze bevindingen zijn belangrijk voor toekomstig onderzoek specifiek op het gebied van de functional genomics. Tot slot geven we nog een aantal extra aanbevelingen voor toekomstig onderzoek. Wij stellen dat bepaling van nog veel meer polymorfismen in de HIPPOCRATES populatie niet zinvol zal zijn. Immers, wat zal het effect zijn op de dagelijkse klinische praktijk van de huisarts als deze de resultaten moet interpreteren van een paar honderdduizend polymorfismen per patiënt? Ten tweede vonden wij aanwijzingen dat het combineren van CIMD of PWV met traditioneel risicofactor management weleens relevant zou kunnen zijn voor klinische preventie, omdat patiënten beter kunnen worden geclassificeerd. Voordat IMD en PWV echter kunnen worden toegepast in de voorspelling van het cardiovasculair risico, zal eerst moeten worden gekeken naar de toegevoegde waarde van deze metingen ten opzichte van bepaling en management van de traditionele cardiovasculaire en biochemische parameters in langdurige follow-up studies. Dit zou een uitdagend onderwerp zijn voor een vervolgonderzoek binnen het HIPPOCRATES project. Als laatste doen we de suggestie dat, gezien het feit dat polymorfismen slechts een beperkte voorspellende waarde blijken te hebben bij het schatten van het cardiovasculaire risico, zou kunnen worden overwogen om na te gaan of een (positieve) familie-anamnese voldoende bijdraagt aan de risicoclassificatie. Op dit moment worden pilot studies en kwalitatief onderzoek onder huisartsen en patiënten voorbereid om de haalbaarheid van deze benadering in kaart te brengen.

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DANKWOORD

Dankwoord

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CURRICULUM VITAE

Curriculum vitae

Arian Plat werd geboren op 1 maart 1975 te Steenwijk. In 1992 behaalde hij het HAVO diploma aan de Katholieke Scholengemeenschap (KSG) Gelderingen te Steenwijkerwold en in 1994 behaalde hij het VWO diploma aan de Regionale Scholengemeenschap (RSG) te Steenwijk. Aansluitend startte hij met de studie Farmacie aan de Rijksuniversiteit Groningen (RuG), waar hij in 2001 zijn doctoraalexamen in de farmaco-epidemiologie behaalde. Het onderwerp van zijn doctoraalscriptie was het bepalen van de mate van inbreng van de apotheek bij het voorkomen van interacties met cumarines in de regio Groningen. Deze studie werd uitgevoerd in nauwe samenwerking met de Stichting Trombosedienst Groningen (thans Labnoord; Margriet Piersma - Wichers, internist), apotheek 't Hooge Zand (Anne de Vries - Bots, apotheker) en de afdeling Sociale Farmacie, Farmaco-epidemiologie en Farmacotherapie (prof. dr. Lolkje de Jong - van den Berg) waar onder enthousiaste begeleiding zijn interesse in wetenschappelijk onderzoek ontstond. Alle resultaten van deze studie zijn zowel nationaal als internationaal gepubliceerd.

Van november 2001 tot en met juni 2007 werkte hij als promovendus bij de afdeling Huisartsgeneeskunde, School for Public Health and Primary Care (CAPHRI), Maastricht Universitair Medisch Centrum en de afdeling Interne Geneeskunde, Cardiovascular Research Institute Maastricht (CARIM), Maastricht Universitair Medisch Centrum, onder leiding van dr. Jelle Stoffers, dr. Bram Kroon, prof. dr. Onno van Schayck en prof. dr. Peter de Leeuw. Het onderzoek dat tijdens deze periode werd uitgevoerd, heeft geleid tot dit proefschrift. Gedurende het promotietraject voltooide hij ook een opleidingsprogramma voor wetenschappelijk onderzoeker epidemioloog (Epidemioloog B) onder begeleiding van prof. dr. Martin Prins.

Sinds juli 2007 werkt hij bij het PHARMO Institute for Drug Outcomes Research in Utrecht. De eerste zes maanden werkte hij als onderzoeker (research associate) en daarna werd hij onderzoeksmanager (research manager). In deze functie verzorgt hij het management van onderzoeksprojecten op het gebied van hart-en vaatziekten en diabetes. Vanaf mei 2008 is hij tevens verantwoordelijk voor de acquisitie van deze onderzoeksprojecten binnen de afdeling business development and global accounts.

Hij is lid van de Koninklijke Nederlandse Maatschappij ter bevordering der Pharmacie (KNMP), de Vereniging voor Epidemiologie (VvE) en de European Association for the Study of Diabetes (EASD).

Hij woont samen met zijn vriendin Leen Liefsoens in hun spiksplinternieuwe appartement in Blokhoeve Buiten te Nieuwegein.