in search of cardiovascular risk genes

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CONTENTS

GLOSSARY

Genetic epidemiology Studies the role of genetic factors and their interaction with environmental factors in the occurrence of disease

Heritability Proportion of population variance in a trait attributable to segregation of a gene or genes

Locus Physical location of a gene

Allele Alternative forms of a gene or marker due to changes at the DNA level

Genotype The observed alleles at a genetic locus of an individual

Phenotype Physical, biochemical or physiological appearance of a disorder

Haplotype The ordered arrangement of alleles on a chromosome

Genetic heterogeneity Several genes are associated with the same disease

Locus heterogeneity occurs when different genes lead to the same phenotype

Allelic heterogeneity occurs when variations in the same gene result in the same phenotype

Epistasis Two or more genes interacting with one another in a multiplicative fashion

Proband Individual in a pedigree that causes the pedigree to come to the attention of medical or research personnel

Polymorphism A piece of DNA that has more than one form (allele), each of which occurs with at least 1% frequency

Polygenic A trait is considered polygenic when caused by the combined effects of three or more loci

Multifactorial A trait is considered multifactorial when two or more genes, together with environmental factors, cause a phenotype

Genetic drift Random fluctuation of allele frequencies when genes are transmitted from one generation to the next

Founder effect Loss of genetic variation when a population is formed by a small number of individuals derived from a larger population (extreme example of genetic drift)

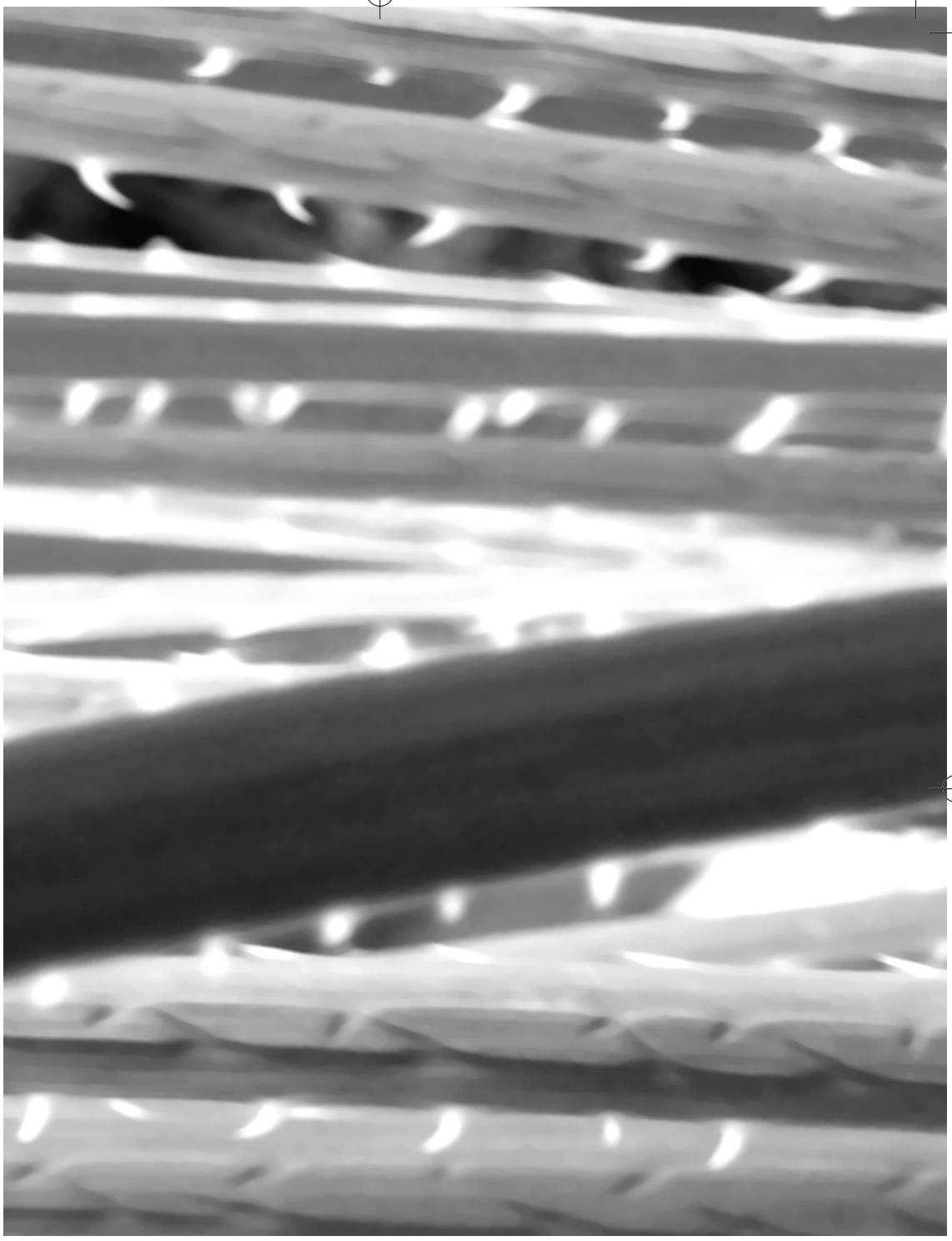
Linkage disequilibrium Two alleles at different loci that occur together within an individual more often than would be predicted by chance. Also called population allelic association

Recombination The process during meiosis by which two-paired chromosomes exchange genetic material

Recombination fraction The frequency of crossing over between two loci

Microsatellite A DNA variant due to tandem repetition of a short DNA sequence

Single Nucleotide Polymorphism (SNP) DNA sequence variation due to change in a single nucleotide



1.INTRODUCTION

1.1. HYPERTENSION IN A NUTSHELL

Introduction

Cardiac output x peripheral resistance = mean arterial blood pressure. Behind this apparently simple equation belies the very complex physiology of blood pressure regulation. Pathophysiological changes in blood pressure homeostasis may cause a chronic rise in arterial blood pressure. This condition is defined as hypertension and usually diagnosed when systolic blood pressure exceeds 140 mmHg and/or when diastolic blood pressure exceeds 90 mmHg (table 1).[1,2]

Epidemiology

Hypertension is the most prevalent cardiovascular risk factor in the industrialized world, affecting about 30 % of the adult population and up to 60 % of those aged above 70 years.[3] Epidemiological surveys have revealed many factors that are associated with blood pressure levels, including age, sex, race, socio-economic status, nutrition, alcohol intake, stress, smoking and physical activity.[4] Large population-based studies have shown a strong positive and continuous relationship between blood pressure levels and risk of cardiovascular morbidity and mortality.[5-10] Hypertension is a common and powerful contributor to the major cardiovascular diseases: coronary heart disease, stroke, peripheral artery disease, renal disease and heart failure.[11-13] Elevated blood pressure tends to cluster with other cardiovascular risk factors such as obesity, dyslipidemia, glucose intolerance, hyperinsulinemia and left ventricular hypertrophy.[10] Less than 20 % of hypertension occurs in the absence of any of the above-mentioned metabolically linked risk factors. Although hypertension independently contributes to risk of cardiovascular events, its impact is highly influenced by the presence of associated risk factors.

Despite the overwhelming number of known risk factors, in over 90 % of hypertensive patients no single identifiable cause can be established. Chronically elevated blood pressure without a known pathological cause is usually referred to as "primary" or "essential" hypertension. Essential hypertension is a very heterogeneous disorder, most likely the result of a complex interplay between inherited, behavioral and environmental factors that all influence physiological pathways involved in blood pressure regulation.

Pathophysiology

Essential hypertension may have its origin as early as the start of intrauterine life. Adverse environment during critical periods of development in fetal life and infancy may predispose to an unfavorable cardiovascular risk profile in later life.[14,15] Experimental and observational studies provide strong evidence for the importance of environmental and cultural effects on the development of hypertension.[16] These factors include obesity, high sodium intake, low potassium intake, excessive alcohol consumption and a

Table 1. Definition and classification of blood pressure levels according to the 1999 WHO/ISH criteria and the 2003 ESH guidelines

Category	SBP (mmHg)	DBP (mmHg)
Optimal	< 120	< 80
Normal	120-129	80-84
High-Normal	130-139	85-89
Grade 1 hypertension (mild)	140-159	90-99
Grade 2 hypertension (moderate)	160-179	100-109
Grade 3 hypertension (severe)	≥ 180	≥ 110
Isolated systolic hypertension	≥ 140	< 90

SBP, Systolic blood pressure; DBP, Diastolic blood pressure.

sedentary life style. Besides environmental factors, physiological and genetic factors have also been implicated in the development of hypertension.

The most important physiological factors that influence blood pressure homeostasis are sodium and fluid handling by the kidneys and the regulation of vasomotor tone. Both are under the influence of neuronal and endocrine systems. In hypertensive patients, the kidneys are not able to adequately excrete sodium and water at a normal arterial pressure.[17] The causes of this impaired renal-pressure natriuresis remain to be determined. Obesity has been suggested as one of the possible causes, as it induces an increase in renal tubular absorption and impairs pressure natriuresis through activation of the sympathetic and renin-angiotensin system as well as physical compression of the kidneys.[18] The kidney-fluid system is important in establishing long-term blood pressure regulation and impairment of renal-pressure natriuresis is likely to play a central role in the development of hypertension.[19]

Another hallmark of essential hypertension is an increased peripheral vascular resistance due to alterations in the structure, mechanical properties and function of small arteries, which is reflected in a reduced lumen and increased media:lumen ratio.[20] Whether this vascular remodeling process is a primary cause of hypertension or occurs in response to elevated blood pressure is subject of ongoing debate. Recently, endothelial dysfunction, a condition characterized by platelet aggregation, inflammation, impaired modulation of vascular growth, dysregulation of vascular remodeling and impaired endothelium-dependent vasorelaxation, has also been implicated in the etiology of hypertension.[21] Indeed, nitric oxide-related vasodilatation, a characteristic of healthy endothelium, is impaired in hypertensive patients.[22]

Two important endocrine regulatory systems are involved in the cardiovascular and renal changes observed in hypertensive patients: the sympathetic nervous system and the renin-angiotensin aldosteron system (RAAS). Increased sympathetic nervous system acti-

vity increases blood pressure and contributes to the development and maintenance of hypertension through stimulation of the heart, peripheral vasculature and renin release in the kidneys, causing increased cardiac output, increased vascular resistance and fluid retention.[23]

RAAS influences cardiovascular and renovascular homeostasis in various ways (figure 1). Renin is secreted by the kidney in response to reduced glomerular perfusion, reduced salt intake or stimulation of the sympathetic nervous system. Renin converts angiotensinogen into angiotensin I, which is in turn converted into angiotensin II by the angiotensin-converting enzyme (ACE). ACE also inactivates bradykinin, an important mediator in the release of potent vasodilators such as nitric oxide, prostacyclin and tissue plasminogen activator. Angiotensin II (ANG II) is the key peptide in the RAAS system. The effects of ANG II on blood pressure regulation are predominantly mediated through the angiotensin receptor I, located primarily in the adrenal glands, vascular smooth muscle cells, kidneys and the heart.[24] ANG II increases blood pressure via different pathways. First, it is a very potent systemic and renal vasoconstrictor and induces sodium and

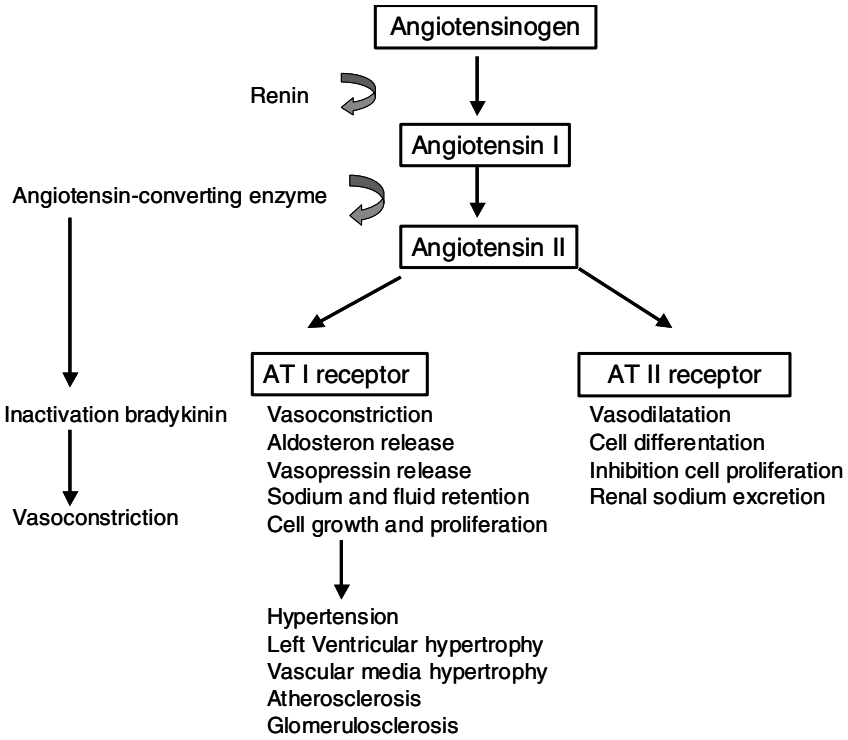


Figure 1. Schematic overview of the renin-angiotensin system and its effects on blood pressure regulation.

fluid retention in the kidneys.[25] Second, ANG II mediates cell growth and proliferation of vascular smooth muscle cells, cardiomyocytes and coronary endothelial cells by stimulating various cytokines and growth factors, such as insulin-like growth factor-I and platelet derived growth factor.[20,26] Furthermore, ANG II may induce endothelial dysfunction by reducing nitric oxide bioavailability.[27]

Besides ANG II and the catecholamines, several other hormones with growth inducing properties have been linked to hypertension, e.g. insulin, growth hormone and insulin-like growth factor I (IGF-I). IGF-I is thought to contribute to the vascular remodeling process observed in hypertension by stimulating vascular smooth muscle cell growth in response to humoral stimuli and elevation in vascular wall stress.[28] In addition to these mitogenic effects, IGF-I also acts on vascular tone via endothelial nitric oxide release.[29] Increased cardiac IGF-I expression has been implicated in the development of left ventricular hypertrophy associated with hypertension.[30]

In conclusion, altered renal and vascular function seem to form the corner stones of the pathophysiology of hypertension, cemented by various neuronal, endocrine, environmental and genetic stimuli.

Genetics

Many population-based studies as well as adoptions studies have indicated that familial aggregation of blood pressure is largely due to genetic factors.[31-33] A greater concordance rate of blood pressure in monozygotic twins versus dizygotic twins also indicates that genetic factors play an important role in blood pressure regulation. Given an affected first-degree relative, the risk of hypertension is increased 2-5 fold in family members compared to the population risk. Genetic factors may explain up to 30-40 % of the blood pressure variation in the population.[34]

So far, the elucidation of a number of mutations that contribute to Mendelian forms of salt-sensitive hypertension have been the bright spot in the area of molecular genetics of hypertension.[35] Although these single-gene mutations are extremely rare and unlikely to explain the blood pressure variation at the population level, understanding the pathophysiological mechanisms underlying these monogenic forms of hypertension may provide new insights into pathways involved in more common forms of hypertension. Despite the identification of several Mendelian forms of hypertension, genetic heterogeneity, unknown genetic models of inheritance and immeasurable environmental effects complicate the search for genetic factors that play a role in the development of hypertension. This may explain why, up to now, no genes with substantial effects on blood pressure variance in the general population have been identified.

Studies on the genetics of hypertension have largely focused on the analysis of candidate genes in biological systems known to affect blood pressure. Although some pro-

mising findings relate to genes of the renin-angiotensin system, these genetic variants seem to influence blood pressure only modestly and most susceptibility genes have not shown very consistent and reproducible associations with hypertension or blood pressure. An important limitation of the candidate gene approach is that it is restricted to known variants in susceptibility genes, which has led to interest in screening of the entire genome in search of new candidate regions explaining blood pressure variation or hypertension.[36] Although these genome wide scans have identified new promising loci on several chromosomes, these regions are still broad and in need of confirmation.

All in all, modest but hopeful results have emerged from both candidate gene studies and genome wide searches, however a major breakthrough in the search of susceptibility genes for hypertension is eagerly awaited.

References

1. 1999 World Health Organization-International Society of Hypertension Guidelines for the Management of Hypertension. Guidelines Subcommittee. *J Hypertens.* 1999;17:151-83.
2. 2003 European Society of Hypertension-European Society of Cardiology guidelines for the management of arterial hypertension. *J Hypertens.* 2003;21:1011-53.
3. Staessen JA, Wang J, Bianchi G, Birkenhager WH. Essential hypertension. *Lancet.* 2003;361:1629-41.
4. Whelton PK. Epidemiology of hypertension. *Lancet.* 1994;344:101-6.
5. Kannel WB, Castelli WP, McNamara PM, McKee PA, Feinleib M. Role of blood pressure in the development of congestive heart failure. The Framingham study. *N Engl J Med.* 1972;287:781-7.
6. Kagan A, Harris BR, Winkelstein W, Jr., Johnson KG, Kato H, Syme SL, Rhoads GG, Gay ML, Nichaman MZ, Hamilton HB, Tillotson J. Epidemiologic studies of coronary heart disease and stroke in Japanese men living in Japan, Hawaii and California: demographic, physical, dietary and biochemical characteristics. *J Chronic Dis.* 1974;27:345-64.
7. Stamler J, Neaton JD, Wentworth DN. Blood pressure (systolic and diastolic) and risk of fatal coronary heart disease. *Hypertension.* 1989;13:I2-12.
8. Blood pressure, cholesterol, and stroke in eastern Asia. Eastern Stroke and Coronary Heart Disease Collaborative Research Group. *Lancet.* 1998;352:1801-7.
9. Vasan RS, Larson MG, Leip EP, Evans JC, O'Donnell CJ, Kannel WB,

Levy D. Impact of high-normal blood pressure on the risk of cardiovascular disease. *N Engl J Med.* 2001;345:1291-7.
10. Kannel WB. Risk stratification in hypertension: new insights from the Framingham Study. *Am J Hypertens.* 2000;13:3S-10S.
11. Stamler J, Stamler R, Neaton JD. Blood pressure, systolic and diastolic, and cardiovascular risks. US population data. *Arch Intern Med.* 1993;153:598-615.
12. Kannel WB. Fifty years of Framingham Study contributions to understanding hypertension. *J Hum Hypertens.* 2000;14:83-90.
13. Kannel WB. Review of recent Framingham study hypertension research. *Curr Hypertens Rep.* 2000;2:239-40.
14. Barker DJ. The fetal and infant origins of adult disease. *BMJ.* 1990;301:1111.
15. Barker DJ, Bull AR, Osmond C, Simmonds SJ. Fetal and placental size and risk of hypertension in adult life. *BMJ.* 1990;301:259-62.
16. National High Blood Pressure Education Program Working Group report on primary prevention of hypertension. *Arch Intern Med.* 1993;153:186-208.
17. Guyton AC. Abnormal renal function and autoregulation in essential hypertension. *Hypertension.* 1991;18:III49-53.
18. Hall JE. The kidney, hypertension, and obesity. *Hypertension.* 2003;41:625-33.
19. Guyton AC. Blood pressure control--special role of the kidneys and body fluids. *Science.* 1991;252:1813-6.
20. Mulvany MJ. Small artery remodeling in hypertension. *Curr Hypertens Rep.* 2002;4:49-55.
21. Mulvany MJ. Vascular remodelling of resistance vessels: can we define this? *Cardiovasc Res.* 1999;41:9-13.
22. Arnal JF, Dinh-Xuan AT, Pueyo M, Darblade B, Rami J. Endothelium-derived nitric oxide and vascular physiology and pathology. *Cell Mol Life Sci.* 1999;55:1078-87.
23. Mark AL. The sympathetic nervous system in hypertension: a potential long-term regulator of arterial pressure. *J Hypertens Suppl.* 1996;14:S159-65.
24. Unger T. The role of the renin-angiotensin system in the development of cardiovascular disease. *Am J Cardiol.* 2002;89:3A-9A; discussion 10A.
25. Brewster UC, Perazella MA. The renin-angiotensin-aldosterone

system and the kidney: effects on kidney disease. *Am J Med.* 2004;116:263-72.
26. Carluccio M, Soccio M, De Caterina R. Aspects of gene polymorphisms in cardiovascular disease: the renin-angiotensin system. *Eur J Clin Invest.* 2001;31:476-88.
27. Rajagopalan S, Kurz S, Munzel T, Tarpey M, Freeman BA, Griendling KK, Harrison DG. Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *J Clin Invest.* 1996;97:1916-23.
28. Diez J. Insulin-like growth factor I in essential hypertension. *Kidney Int.* 1999;55:744-59.
29. Vecchione C, Colella S, Fratta L, Gentile MT, Selvetella G, Frati G, Trimarco B, Lembo G. Impaired insulin-like growth factor I vasorelaxant effects in hypertension. *Hypertension.* 2001;37:1480-5.
30. Wickman A, Isgaard J, Adams MA, Friberg P. Inhibition of nitric oxide in rats. Regulation of cardiovascular structure and expression of insulin-like growth factor I and its receptor messenger RNA. *J Hypertens.* 1997;15:751-9.
31. Biron P, Mongeau JG, Bertrand D. Familial aggregation of blood pressure in 558 adopted children. *Can Med Assoc J.* 1976;115:773-4.
32. Perusse L, Rice T, Bouchard C, Vogler GP, Rao DC. Cardiovascular risk factors in a French-Canadian population: resolution of genetic and familial environmental effects on blood pressure by using extensive information on environmental correlates. *Am J Hum Genet.* 1989;45:240-51.
33. Tambs K, Moum T, Holmen J, Eaves LJ, Neale MC, Lund-Larsen G, Naess S. Genetic and environmental effects on blood pressure in a Norwegian sample. *Genet Epidemiol.* 1992;9:11-26.
34. Ward R. Familial aggregation and genetic epidemiology of blood pressure. In: BM LJB, ed. *Hypertension: Pathophysiology, Diagnosis and Management.* New York: Raven Press; 1990:811-1000.
35. Luft FC. Molecular genetics of human hypertension. *Curr Opin Nephrol Hypertens.* 2000;9:259-66.
36. Samani NJ. Genome scans for hypertension and blood pressure regulation. *Am J Hypertens.* 2003;16:167-71.

1.2. GENETIC EPIDEMIOLOGY OF HYPERTENSION

Introduction

Unraveling the genetics of complex diseases has been and continues to be one of the major challenges in the field of biological science. Many areas of research have sought to find the ideal study design in order to shed a light on the genetic background of complex diseases. Although many disease genes have been identified, the majority of these genes are involved in monogenic Mendelian disorders with simple patterns of inheritance. The mapping of susceptibility genes involved in common complex diseases is a considerably more daunting task, asking for newer and more ingenious research approaches. Genetic epidemiology is a field of research that aims to identify the role of genetic factors, and their interaction with environmental factors, in the development of disease in the population. Recently, the focus of attention in genetic epidemiological research has shifted from family-based research on monogenic disorders to population-based research of complex, multifactorial disorders.

Human essential hypertension is a classic example of a complex, multifactorial trait. Blood pressure regulation is most likely the product of many genes, interacting with each other and with environmental factors. Although several Mendelian forms of hypertension have been identified in family-based studies, the contribution of these rare genes to blood pressure variation in the general population is likely to be very small. Large-scale population-based studies will be needed to identify susceptibility genes for hypertension that are relevant for the general population. In these studies close integration of genetic, physiological and epidemiological knowledge of hypertension will play a pivotal role for the future identification of genes influencing blood pressure homeostasis.

In this paper we will review the basic principles underlying genetic epidemiological research of complex traits. We will discuss the different approaches that are used in genetic epidemiological studies, and how these studies have been, and will be applied to disentangle the genetic basis of human hypertension.

Background

Hypertension is a very common disease and a major risk factor for cardiovascular morbidity and mortality.[1-3] Family, twin and adoption studies have clearly demonstrated that blood pressure is a heritable trait.[4-8] Up to 40 % of blood pressure variance in the general population may be explained by genetic factors.[9,10]

During the sixties a heated debate between Pickering and Platt arose over the question whether hypertension represents the upper tail of a continuous blood pressure distribution and is determined by a number of genes or is a qualitative trait due to an effect of a few major genes.[11,12] Platt assumed that the bimodality of blood pressure distribution in his study population reflected the effect of major genes causing hypertension. Pickering observed that the frequency distribution curves for blood pressure in

the relatives of patients with hypertension were similar in shape to those of a population-based sample but shifted upwards by about the same amount at all ages. So, blood pressure appeared to be inherited as a graded characteristic over the whole range of blood pressure levels.

At present, hypertension is assumed to represent the upper tail of a continuous blood pressure distribution, and considered a complex, multifactorial and polygenic trait, potentially influenced by the additive effects of many genes, interacting with various environmental factors. Despite significant progress in the development of genomic and statistical tools, the genetic dissection of hypertension remains a major challenge.

Definition of the phenotype

In studies on the genetics of (complex) diseases, identification and precise definition of the phenotype (trait) of interest is crucial. Although this may seem very straightforward, in hypertension research it is not always that easy. First of all, blood pressure is a highly variable trait. Blood pressure measurements can be very much influenced by the technical accuracy of the measurement device, biological variability of blood pressure (e.g. diurnal rhythm and posture) and the presence of a physician, so called "white-coat hypertension". These measurement errors may be minimized by careful standardization of measurement technique, training of observers and ambulatory or self-monitoring of blood pressure. Blood pressure levels should preferably be measured in exactly the same way in each participant, and the "threshold" for diagnosis of hypertension should be based on (internationally) generally accepted criteria in order to generate valid and reproducible results.[13]

Second, blood pressure is a polygenic trait, the result of many genes each with small additive effects. The continuous blood pressure distribution in the population suggests that blood pressure raising alleles will act in an additive or multiplicative manner to increase pressure at any level of blood pressure. So, genes that influence blood pressure variation are likely to also contribute to hypertension. Conversely, genes involved in hypertension are likely to influence blood pressure variation within the normotensive range. As a result, blood pressure can be studied both as a quantitative and qualitative trait (hypertension). Besides sampling families over the whole range of blood pressure distribution, selecting families from the top and bottom end of this distribution is assumed to increase the power of genetic analyses, as these families are likely to be more genetically distinct.[14-16] Sampling siblings with extreme concordant or discordant blood pressure levels may increase the power to detect low-frequency alleles which will increase or decrease blood pressure.[17-19] Another approach is to identify hypertensive subjects who are thought to have a more homogeneous genetic background for their high blood pressure. Stratification based on hypertension-related (intermediate) phenotypes

such as low plasma renin, obesity, dyslipidemia or insulin resistance could increase the homogeneity of the study population.[20,21] A fairly recent strategy to increase homogeneity, is to sample hypertensive subjects that originated from a genetically isolated population. They are assumed to share a more common genetic background because they are all descendants from a limited number of common ancestors. Studying hypertensive patients in this population may facilitate the identification of susceptibility genes, as they are thought to have hypertension due to the same reduced number of mutations inherited from a common ancestor.

Finally, studying additional blood pressure phenotypes, besides systolic and diastolic blood pressure, such as the age-at-onset of hypertension, pulse pressure and mean arterial pressure, may be of interest because these phenotypes may lead to the identification of a different set of genes involved in blood pressure homeostasis. Indeed, Daw et al. analyzed age-at-onset of hypertension and SBP and they observed that age-at-onset was better at identifying "slope genes" whereas SBP might be more suitable to identify "baseline" genes.[22]

Evidence for genetic effects on blood pressure regulation

Clustering of a disease in families is suggestive for a genetic component in the development of this disease. Familial aggregation of a disease is present when the prevalence of the disease is more frequent in family members of an affected individual than in the general population. Familial aggregation of hypertension has been reported for the first time over 30 years ago and has since then been confirmed in numerous studies.[6,7,23,24] Given an affected first-degree relative, the risk of hypertension is found to be increased 2-5 fold in family members compared to the population risk. This risk measure is usually referred to as λ_S (lambda sib), which is the ratio of the risk of hypertension for siblings of a proband (affected family member) compared to the population risk.

Blood pressure levels within family members are highly correlated, with correlations ranging from 0.14-0.18 between parent-offspring and sibling pairs, up to 0.25-0.27 for dizygotic twins and up to 0.55-0.58 for monozygotic twins.[25] As families share genes and environment, these correlations are also the result of shared environment. However, relatively low blood pressure correlations between spouse pairs (0.06-0.08) and higher concordance rates of monozygotic versus dizygotic twins implicate a substantial influence of genetic factors in family blood pressure correlations. Furthermore, adoption studies indicate higher blood pressure correlations between parents and their biological children than between parents and their adopted children.[6]

The strength of a genetic component can be expressed in a quantity called heritability, which defines the proportion of phenotypic variance due to genetic factors over the total phenotypic variance observed. Heritability encompasses two genetic compo-

nents: additive and dominance genetic effects. Narrow-sense heritability (denoted as h^2) reflects the additive genetic effects and is a measure of the predictability of offspring trait values based on parental trait values. The magnitude of genetic effects in traits that are assumed to have a polygenic background (many genes each with a small additive effect) is usually reflected in the narrow-sense heritability. Broad-sense heritability estimates (denoted as H^2) also include dominance genetic effects, which explain part of the heritability due to the effects of major genes. However, they are not thought to contribute substantially to the heritability of complex quantitative traits in the general population. Large family-based studies have estimated narrow-sense heritabilities for DBP and SBP between 20-40 %.[26-29] In twin studies heritability estimates of blood pressure usually vary around 60 %.[8,30]

Segregation analysis: determination of the mode of inheritance

A segregation analysis aims to determine the transmission pattern of a trait within families and tests this pattern against predictions from specific genetic models. Various transmission models can be specified including major gene effects, Mendelian inheritance, all transmission due to shared environmental factors and more complicated mixed models with a single major gene and a polygenic component. In segregation analyses all parameters are estimated using maximum likelihood methods. These parameters consist, among other things, of allele frequencies, transmission patterns and susceptibility parameters. The likelihood represents the probability of observing the data given a certain set of parameters. The likelihood of different models can be compared using the likelihood ratio test or examining Akaike's information criterion. A more detailed description of the statistical methods in segregation analysis can be obtained in Khoury et al.[31]

Segregation analyses in 1050 pedigrees of the Framingham Heart Study for a quantitative and qualitative blood pressure trait (SBP and hypertension) both identified the same best fitting model: a mixed model allowing for two major genes and polygenic effects. However, the model parameter estimates were very different for both traits.[32] This underlines that the choice of blood pressure phenotype may have an important influence on the estimation of the parameters in the segregation model. Most segregation analyses for SBP and DBP have not been very consistent ranging from a no transmission, environmental model to a model with polygenic and/or major gene effects.[33-35] Heterogeneity of the study samples and the compound nature of blood pressure may be responsible for the inability to detect consistent segregation models.

Study Design

There are two general approaches for mapping genes in complex disorders. The first comprises a genome wide screen using a linkage analysis approach, which is limited to a

family-based study design. The second approach includes candidate gene studies based on association analyses, which can be conducted in family-based and population-based studies.

Genome wide screen

A genome wide screen comprises of scanning of the whole genome using markers approximately evenly spaced across the entire genome. These DNA markers can be mutations in single base pairs (Single Nucleotide Polymorphisms (SNPs)) or a variable number of repeats of two or more base pairs (microsatellites). A statistical method called linkage analysis is used to analyze the results of a genome screen. Linkage is tested using either a parametric or a non-parametric model.

The parametric linkage approach is a model-based method that requires that an assumption of the genetic model is specified a priori, including specification of certain parameters such as mode of inheritance, disease and marker allele frequencies and penetrance. This can be done in a segregation analysis. For each of the markers evidence for linkage to a possible disease mutation is tested using statistical procedures that identify the co-segregation of a trait or disease and a specific variant of the DNA marker within affected family members. The basic principle of this method is that loci physically close together on a chromosome are likely to be transmitted together. Patients who inherit a disease gene from a common ancestor, are expected to share also parts of the chromosome surrounding the disease gene. So, any marker located nearby the causal disease mutation is expected to be more often shared by affected relatives (cases) than by unaffected relatives (controls) (Figure 1). Evidence for linkage is usually presented in the form of a LOD-score function. The LOD-score stands for the logarithm of the likelihood that a locus is linked to the trait versus no linkage to the trait. This LOD-score is dependent on the recombination fraction (θ). This is the probability that two alleles at different loci on the same chromosome are derived from different parental chromosomes. When there is tight linkage between two alleles the recombination fraction is zero, when there is no linkage between two alleles the recombination fraction is 0.5. The closer two loci are on a chromosome the less likely that a recombination has occurred during meiosis, meaning alleles from both loci are always inherited together. This condition is known as linkage disequilibrium. The recombination fraction has evolved into a measure of genetic distance, defined in centiMorgan (cM). One cM corresponds approximately to a recombination fraction of 1 %. In general, a LOD score above 3.0 is considered substantial evidence for linkage and warrants additional fine mapping in the region of interest. This parametric linkage approach, also referred to as LOD score analysis, is very powerful but highly dependent on correct assumptions made about the genetic model.

A non-parametric linkage method does not require prior knowledge of the genetic model. This non-parametric method examines which parts of the genomes of a pair of

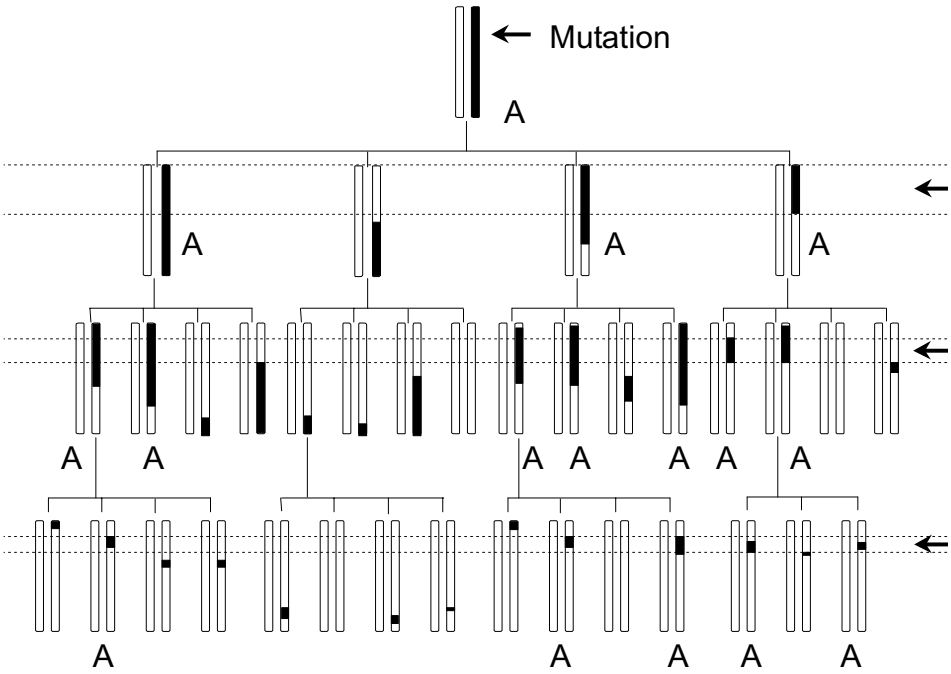


Figure1. Linkage analysis: segregation of disease mutation through a pedigree. Together with the mutation, DNA flanking the mutation is also transmitted. Due to recombination the part of shared DNA, including the mutation, becomes smaller over generations.

affected relatives are identical by descent (IBD).[36] The most familiar model probably is the sibpair method.[37-39] Siblings share about 50 % of their genetic material. The probability of siblings sharing no, one or two alleles with each other is respectively 25, 50 and 25 %. Linkage is supported if two affected or two unaffected siblings are significantly more alike (more allele sharing) at a marker locus than sibling pairs with just one affected member. Affected sibs are expected to share more alleles for markers located close to the disease mutation than expected under the null hypothesis (one allele shared). Non-parametric linkage was originally used in siblings, but has been extended to pedigrees. A locus that might harbor genes that influence quantitative (complex) traits is called a quantitative trait locus (QTL) and can be detected using non-parametric or model free linkage.

Candidate gene studies

In contrast to linkage studies, which are usually genome wide, association studies are usually restricted to candidate genes or candidate regions in the genome. There are two types of candidate gene association studies. The concept of *population-based* genetic

association studies is very similar to that of classic case-control studies in epidemiology. In these studies, the frequency of (functional) allelic variants is compared between subjects with a specific phenotype (hypertension) and subjects without this phenotype (normotension). A quantitative trait value, e.g. systolic blood pressure, may also be compared between subjects with different allelic variants. This allelic association can be explained either by a direct biological action of the polymorphism or by linkage disequilibrium with a nearby susceptibility gene. Attractive candidate genes are those that may, either by influencing protein levels or protein functionality, alter the function of a protein that may play a role in the development of the disease of interest. Failure to replicate *population-based* genetic association studies is a very common problem and has often been ascribed to population stratification. Population stratification exists when the population under study consists of a mixture of two or more subpopulations that have different allele frequencies and disease risks. An approach to overcome this problem is the careful matching of cases and controls in a population-based study design, or the use of family-based controls. The latter forms the basis of the transmission-disequilibrium test (TDT), a *family-based* association method.[40,41] This test assesses the difference between the frequency of marker alleles transmitted from heterozygous parents to the affected offspring and the frequency of markers not transmitted. TDT involves establishment of a pseudo case-control study, in which cases are the parental alleles transmitted to the affected proband, and controls are those that were not transmitted. So, as case-control pairs are matched within a family, allele frequency differences at the population level become irrelevant. A drawback of the TDT design is the need of parental DNA, who may be deceased when studying late-onset disorders.

As the phenotype of complex diseases is polygenic in nature, association studies considering only a small fraction of the genetic variants may not suffice to find relevant candidate genes. The creation of the Single Nucleotide Polymorphism (SNP) consortium has led to the identification of 1.5 million SNPs, the most common form of human genetic variation.[42-44] Between 50.000 and 100.000 of these SNPs lie within coding and adjacent regions and may alter gene function and protein expression.[45] With the development of high throughput genotyping technologies, large-scale testing of these SNPs in whole-genome association studies is likely to become feasible in the next few years.

Finding genes for hypertension and blood pressure regulation

Studies on the genetics of hypertension and blood pressure regulation have developed into a major field of research over the last decade. The bright spot of this research area has been the identification of several Mendelian forms of hypertension. However, these forms are rare and in the vast majority of patients the genetic basis of their hypertension is likely to be the result of many genes, each with small additive effects.

So far, genetic studies on hypertension have largely sought to associate polymorphic variations in candidate genes with the risk of hypertension or variation in blood pressure levels. However, these association studies are restricted to known variants in genes of which we a priori expect that they may possibly be involved in the physiological pathways of blood pressure regulation. This limitation has led to interest in conducting genome scans, aimed at identifying new susceptibility genes for hypertension.

In the following section, a brief overview will be given regarding the findings on monogenic forms of hypertension, candidate genes and genome scans for hypertension and blood pressure regulation.

Mendelian forms of hypertension

In favor of Platt, who argued that major genes must be involved in the development of hypertension, several monogenic forms of hypertension have been identified. Some of these Mendelian forms of hypertension are associated with defects in steroid metabolism, e.g. the syndrome of apparent mineralcorticoid excess (AME), an autosomal form of hypertension due to inactivating mutations in the 11β-hydroxysteroid dehydrogenase.[46] Liddle's and Gordon's syndrome are monogenic disorders associated with abnormalities in

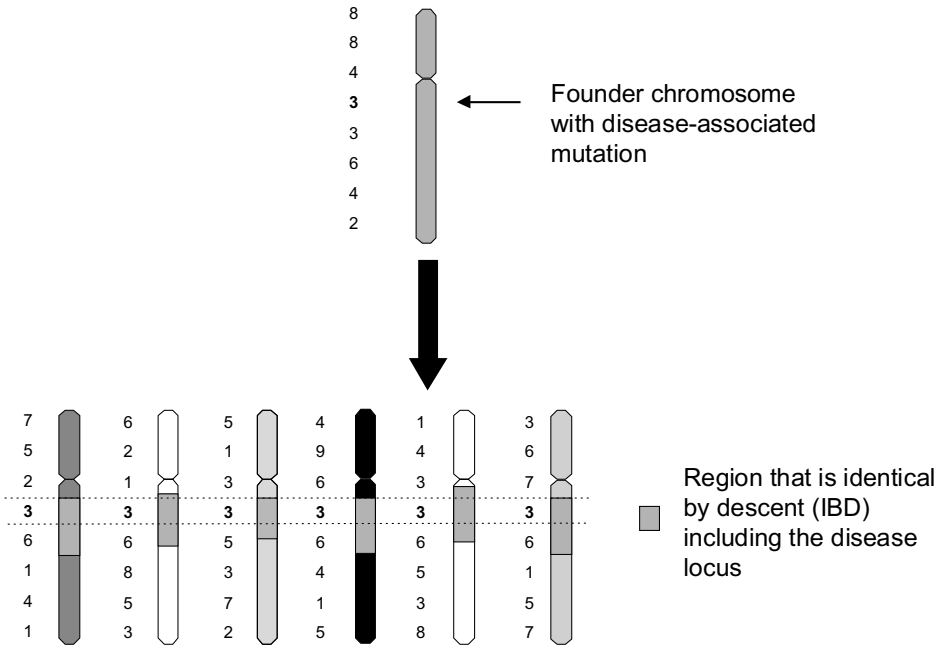


Figure 2. Allele sharing in a genetically isolated population.
A disease-associated mutation is introduced into the population by a founder chromosome (above). This mutation, together with surrounding DNA, is shared by patients with the same phenotype (IBD). Although they may not be aware, they all descend from the same founder.

Table 1. Mendelian forms of hypertension

Syndrome	MOI	Chromosome/gene	Gene function/ disease mechanism
Defects in ion transport			
Liddle's syndrome	ADI	16p12-p13 SCNN1B/SCNN1G	Mutations in either β or γ subunit of epithelial sodium channel (EnaC)
Pseudo hypoaldosteronism type II (Gordon's syndrome)	ADI	1q13-q42,17p11-p21, 12p13 WKN1/WKN4	Gain of function mutations WKN kinases 1 en 4 resulting in increased salt reabsorption and intravascular volume
Defects in steroid metabolism			
Glucocorticoid-remediable hyperaldosteronism (GRA)	ADI	8q22 CYP11B2/CYP11B1	Chimeric gene consisting of 11β-hydroxylase gene and aldosterone synthase gene places aldosterone production under control of ACTH
Apparent mineralocorticoid excess (AME)	ARI	16q22 HSD11R2	Inactivating mutation in 11ß-hydroxysteroid dehydrogenase gene exposes mineralocorticoid receptor to effects of cortisol
Hypertension exacerbated in pregnancy	ADI	4q31.1	Activating missense mutation in mineralocorticoid receptor
Male pseudo-hermaphroditism	ARI	10q24 CYP17A	Inactivation of 17α-hydroxylase gene
Female pseudo-hermaphroditism	ARI	8q22 CYP11B1	Inactivation of 11β-hydroxylase gene
Overproduction of catecholamines			
Isolated pheochromocytoma	ADI	1p	?
MEN , type II A	ADI	10q11.2	RET proto-oncogene
MEN, type II B	ADI	10q11.2	RET proto-oncogene
Von Hippel-Lindau syndrome	ADI	3p26-25	VHL tumor suppressor gene
Neurofibromatosis, type I	ADI	17q11.2	NF-1 gene
Others			
Hypertension and brachydactyly	ADI	12p12.2-11.2	?
Syndrome of insulin resistance, diabetes mellitus and hypertension	ADI	3p25 PPARγ	Inactivating missense mutation in peroxisome proliferator-activated receptor gamma

MOI: Mode of inheritance, ADI: autosomal dominant inheritance, ARI: autosomal recessive inheritance.

renal ion transport and cause a salt-sensitive form of hypertension.[47,48] Pheochromocytomas cause an episodic form of hypertension and are the consequence of mutations in proto-oncogenes or tumor suppressor genes.[49-51] Monogenic forms of hypertension are summarized in table 1. Although these forms are rare and do not explain blood pressure variability in the general population, they might help in better understanding the pathophysiology of hypertension and offer clues towards identifying genes involved in more common forms of hypertension.

Candidate genes for hypertension and blood pressure regulation

Blood pressure homeostasis is the result of a complex integrated physiological network, including renal, neuronal, endocrine and vascular systems. Within this network most likely multiple genes are expressed, influencing blood pressure in various ways. Consequently, there are many genes that may potentially serve as candidate genes for hypertension. Several candidate genes in the renin-angiotensin aldosteron system (RAAS) have been identified as genetic determinants of blood pressure. The most prominent one is the insertion(I)/ deletion(D) polymorphism in the angiotensin-converting enzyme (ACE). Subjects carrying two copies of the D-allele have 50 % higher plasma and tissue ACE levels.[52,53] Higher ACE levels may account for higher angiotensin II generation, an important vasoconstrictor, and increased bradykinin inactivation, a potent vasodilator. Both may increase vascular resistance causing a rise in blood pressure. Although many association studies on the ACE I/D polymorphism and hypertension have been performed, findings remain inconsistent.[54-57] Differences in the effects of gene-gene and gene-environment interactions in the various study populations may explain part of the controversial role of this gene in the development of cardiovascular disease.

Single nucleotide polymorphisms in the angiotensinogen (AGT) gene have been consistently associated with elevated angiotensinogen levels and hypertension in many studies.[58-60] The 235T variant of the M235T polymorphism was more frequently observed in hypertensive cases than controls in a study by Jeunemaitre et al.[59] This polymorphism appeared to be in tight linkage disequilibrium with a promoter mutation –6 bp upstream of the initial transcription site, resulting in a higher basal transcription rate and, consequently higher AGT levels.[61] Other genetic variations in the RAAS that have been associated with hypertension include those observed in the angiotensin II type 1 receptor gene and aldosterone synthase gene.[62,63]

Candidate genes that may influence the effects of the sympathetic nervous system on blood pressure, include genetic variations in the α- and β-adrenoreceptors.[64-66] Genetic mutations that alter renal sodium reabsorption have also been implied in the genetics of hypertension. A polymorphism (G460T) in the α-adducin gene has been associated with increased salt-sensitivity in hypertensive patients.[67] A common polymorphism in exon 10 of the β3-subunit of the G-protein gene has been associated with

adipositas and hypertension.[68,69] A polymorphism in the endothelial nitric synthase gene has been associated with endothelial function during pregnancy and blood pressure response to exercise.[70]

Genome wide scans for hypertension and blood pressure

In the last few years, several genome wide scans for hypertension and blood pressure have been reported.[71] These genome wide scans have identified numerous chromosomal regions that might be close to QTLs influencing blood pressure. Most data was based either on (affected) sibling pairs or (nuclear) families. Although these genome wide screens are very diverse in phenotype definition, ethnicity of the study population, selection criteria and number and structure of family data used, a number of regions have been consistently reported in multiple studies. Regions on chromosome 2p[72-75], 6q[72,76,77] and 15q[72,76,78] have been identified in at least three different studies. Although several studies reported chromosomal regions nominal or suggestive of linkage, significant "genome-wide" linkage was reported only for a few regions located on chromosomes 2, 4, 6, 17 and 18.[77,79-82]

Most regions identified so far are broad and may represent false positive linkage due to lack of statistical power. The shift from identifying regions of interest in linkage studies to identifying susceptibility genes for hypertension therefore remains one of the largest challenges in hypertensive research for the upcoming years.

Genetic research in isolated populations

Although many loci for common diseases have been mapped in outbred populations, results have been very inconsistent and few loci have proven to be reproducible. Genetic heterogeneity in outbred populations is likely to play an important role in the disappointing results. Recently, population isolates have been identified as a powerful tool to study the genetics of complex diseases.[83,84] They show considerably less genetic diversity than outbred populations as by definition these isolates originate from a small number of founders. Many of these populations have experienced population bottlenecks, characterized by a marked reduction in population sample size followed by the survival and expansion of a small random sample of the original population. Consequently, the occurrence of genetic drift and inbreeding will reduce genetic diversity in these populations. It is expected that fewer susceptibility genes for common diseases segregate in these populations due to a more restricted gene pool.[84-86] So, founder effects in combination with genetic drift increase the genetic homogeneity and make it more likely that patients in genetic isolates have developed disease due to the same mutation, inherited from a common ancestor. The availability of accurate genealogical records allows for the analysis of extended pedigrees, increasing the power of genetic analyses.[87] Patients in isolated populations are expected to share the same disease gene and considerable parts

of DNA surrounding the disease gene (haplotype) originating from a common ancestor (figure 2). The extent of linkage disequilibrium (LD) determines the size of the shared ancestral haplotypes: the more time has elapsed since a mutation was introduced into a population, the more likely an ancestral haplotype has been disrupted by a recombination event, reducing the extent of LD and the size of the shared haplotype. Linkage disequilibrium mapping also known as Identity by Descent (IBD) or haplotype mapping aims at identifying shared haplotypes in the vicinity of disease genes in affected persons more often than in healthy controls This can be done in specific candidate regions of the genome or by scanning of the whole genome (genome screen).[88-90]

Finland and Iceland are well known North European isolated populations, which have identified several candidate regions for complex disorders.[91,92] So far, two genome wide screens for hypertension have been preformed in isolated communities. Kristjansson et al. found significant linkage to chromosome 18q in 490 Icelandic hypertensive patients.[81] A genome wide scan in 35 related individuals with severe hypertension, all inhabitants of an isolated Sardinian village, revealed significant linkage to chromosome 2p.[79] However, others have not yet replicated these findings.

A drawback of genetic studies in isolated populations could be that only a few susceptibility genes segregate within this population and that the effects of these genes are limited to the specific population that was studied and thus results may not be extendable to the general population. Studies in more recently isolated populations may overcome this problem as they are expected to more closely resemble the general population.

Conclusions

The completion of the sequence of the human genome has nourished high expectations about the discovery of genes involved in human diseases. Although major progress has been made in identifying genes involved in a large number of familial disorders, at the population level, the genetic background of common complex disorders remains to be elucidated. The genetic analysis of hypertension has revealed many complex and inconsistent results. Blood pressure regulation appears to be the result of many genes, which act and interact in an additive and/or multiplicative fashion with other genes and environmental factors. This severely complicates genetic research strategies as different combinations of genes may result in hypertension in different individuals. Study populations may be admixed with different subpopulations, each with their own specific set of genes and environmental risk factors causing hypertension. Given this high level of genetic heterogeneity in hypertension, selection of etiologically distinct subgroups of patients, identified based on their ethnic background, exposure to environmental risk factors or co morbidity, may be warranted in order to increase the power of genetic studies. A promising study design in this respect is the inclusion of hypertensive patients living in gene-

tic isolates, as they are expected to be genetically as well as environmentally more homogeneous than patients of outbred populations. However, whether susceptibility genes identified in isolated populations also contribute to disease in the general population remains to be determined.

In conclusion, studies on the genetics of hypertension have developed into a refined area of research. The continuing discovery of variants of the human genome that contribute to the genetic diversity of the human population and the development of more powerful genetic analytical methods will enable future research to identify susceptibility genes as well as determine the importance of gene-gene and gene-environment interactions in the regulation of blood pressure.

References

1. Staessen JA, Wang J, Bianchi G, Birkenhager WH. Essential hypertension. *Lancet*. 2003;361:1629-41.
2. Vasan RS, Larson MG, Leip EP, Evans JC, O'Donnell CJ, Kannel WB, Levy D. Impact of high-normal blood pressure on the risk of cardiovascular disease. *N Engl J Med*. 2001;345:1291-7.
3. Kannel WB. Risk stratification in hypertension: new insights from the Framingham Study. *Am J Hypertens*. 2000;13:3S-10S.
4. Livshits G, Gerber LM. Familial factors of blood pressure and adiposity covariation. *Hypertension. 2001*;37:928-35.
5. Havlik RJ, Garrison RJ, Feinleib M, Kannel WB, Castelli WP, McNamara PM. Blood pressure aggregation in families. *Am J Epidemiol.* 1979;110:304-12.
6. Biron P, Mongeau JG, Bertrand D. Familial aggregation of blood pressure in 558 adopted children. *Can Med Assoc J.* 1976;115:773-4.
7. An P, Rice T, Gagnon J, Borecki IB, Perusse L, Leon AS, Skinner JS, Wilmore JH, Bouchard C, Rao DC. Familial aggregation of resting blood pressure and heart rate in a sedentary population: the HERITAGE Family Study. Health, Risk Factors, Exercise Training, and Genetics. *Am J Hypertens.* 1999;12:264-70.
8. Williams RR, Hunt SC, Hasstedt SJ, Hopkins PN, Wu LL, Berry TD, Stults BM, Barlow GK, Schumacher MC, Lifton RP, et al. Are there interactions and relations between genetic and environmental factors predisposing to high blood pressure? *Hypertension.* 1991;18:I29-37.
9. Gu C, Borecki I, Gagnon J, Bouchard C, Leon AS, Skinner JS, Wilmore JH, Rao DC. Familial resemblance for resting blood pressure with par-

ticular reference to racial differences: preliminary analyses from the HERITAGE Family Study. *Hum Biol.* 1998;70:77-90.
10. Ward R. Familial aggregation and genetic epidemiology of blood pressure. In: BM LJB, ed. *Hypertension: Pathophysiology, Diagnosis and Management*. New York: Raven Press; 1990:811-1000.
11. Pickering G. The inheritance of arterial pressure. In: Stamler J SR, Pullman TN, ed. *The epidemiology of hypertension.* New York: Grune & Stratton; 1967:18-27.
12. Platt R. The influence of heredity. In: Stamler J SR, Pullman TN, ed. *The epidemiology of hypertension.* New York: Grune & Stratton; 1967:9-17.
13. 1999 World Health Organization-International Society of Hypertension Guidelines for the Management of Hypertension. Guidelines Subcommittee. *J Hypertens.* 1999;17:151-83.
14. Watt GC, Foy CJ, Holton DW, Edwards HE. Prediction of high blood pressure in young people: the limited usefulness of parental blood pressure data. *J Hypertens.* 1991;9:55-8.
15. Watt G. Design and interpretation of studies comparing individuals with and without a family history of high blood pressure. *J Hypertens.* 1986;4:1-7.
16. Watt GC. Strengths and weaknesses of family studies of high blood pressure. *J Hum Hypertens.* 1994;8:327-8.
17. Zhang H, Risch N. Mapping quantitative-trait loci in humans by use of extreme concordant sib pairs: selected sampling by parental phenotypes. *Am J Hum Genet.* 1996;59:951-7.
18. Risch NJ, Zhang H. Mapping quantitative trait loci with extreme discordant sib pairs: sampling considerations. *Am J Hum Genet.* 1996;58:836-43.
19. Risch N, Zhang H. Extreme discordant sib pairs for mapping quantitative trait loci in humans. *Science.* 1995;268:1584-9.
20. Williams GH. Genetic factors associated with volume-sensitive hypertension. *Mol Cell Endocrinol.* 2004;217:41-4.
21. Kotchen TA, Kotchen JM, Grim CE, George V, Kaldunski ML, Cowley AW, Hamet P, Chelius TH. Genetic determinants of hypertension: identification of candidate phenotypes. *Hypertension.* 2000;36:7-13.
22. Daw EW, Liu X, Wu CC. Age-of-onset of hypertension vs. a single measurement of systolic blood pressure in a combined linkage and segregation analysis. *BMC Genet.* 2003;4 Suppl 1:S80.

23. Rose RJ, Miller JZ, Grim CE, Christian JC. Aggregation of blood pressure in the families of identical twins. *Am J Epidemiol.* 1979;109:503-11.
24. Grim CE, Wilson TW, Nicholson GD, Hassell TA, Fraser HS, Grim CM, Wilson DM. Blood pressure in blacks. Twin studies in Barbados. *Hypertension.* 1990;15:803-9.
25. Williams RR, Hunt SC, Hasstedt SJ, Hopkins PN, Wu LL, Berry TD, Stults BM, Barlow GK, Kuida H. Genetics of hypertension: what we know and don't know. *Clin Exp Hypertens A.* 1990;12:865-76.
26. Hsueh WC, Mitchell BD, Aburomia R, Pollin T, Sakul H, Gelder Ehm M, Michelsen BK, Wagner MJ, St Jean PL, Knowler WC, Burns DK, Bell CJ, Shuldiner AR. Diabetes in the Old Order Amish: characterization and heritability analysis of the Amish Family Diabetes Study. *Diabetes Care.* 2000;23:595-601.
27. Knuiman MW, Divitini ML, Welborn TA, Bartholomew HC. Familial correlations, cohabitation effects, and heritability for cardiovascular risk factors. *Ann Epidemiol.* 1996;6:188-94.
28. Mitchell BD, Kammerer CM, Blangero J, Mahaney MC, Rainwater DL, Dyke B, Hixson JE, Henkel RD, Sharp RM, Comuzzie AG, VandeBerg JL, Stern MP, MacCluer JW. Genetic and environmental contributions to cardiovascular risk factors in Mexican Americans. The San Antonio Family Heart Study. *Circulation.* 1996;94:2159-70.
29. North KE, Howard BV, Welty TK, Best LG, Lee ET, Yeh JL, Fabsitz RR, Roman MJ, MacCluer JW. Genetic and environmental contributions to cardiovascular disease risk in American Indians: the strong heart family study. *Am J Epidemiol.* 2003;157:303-14.
30. Snieder H, Harshfield GA, Treiber FA. Heritability of blood pressure and hemodynamics in African- and European-American youth. *Hypertension.* 2003;41:1196-201.
31. Khoury MJ CB, Beaty TH. *Fundamentals of Genetic Epidemiology.* New York: Oxford University Press; 1993.
32. Crockford GP, Bishop DT, Barrett JH. Segregation analysis comparing liability and quantitative trait models for hypertension using the Genetic Analysis Workshop 13 simulated data. *BMC Genet.* 2003;4 Suppl 1:S79.
33. Rice T, Bouchard C, Borecki IB, Rao DC. Commingling and segregation analysis of blood pressure in a French-Canadian population. *Am J Hum Genet.* 1990;46:37-44.

34. Perusse L, Moll PP, Sing CF. Evidence that a single gene with gender- and age-dependent effects influences systolic blood pressure determination in a population-based sample. *Am J Hum Genet.* 1991;49:94-105.
35. Cheng LS, Livshits G, Carmelli D, Wahrendorf J, Brunner D. Segregation analysis reveals a major gene effect controlling systolic blood pressure and BMI in an Israeli population. *Hum Biol.* 1998;70:59-75.
36. Penrose L. The detection of autosomal linkage in data which consist of pairs of brothers and sisters of unspecified parentage. *Ann Eugen.* 1935;6:133-138.
37. Risch N. Linkage strategies for genetically complex traits. I. Multilocus models. *Am J Hum Genet.* 1990;46:222-8.
38. Risch N. Linkage strategies for genetically complex traits. II. The power of affected relative pairs. *Am J Hum Genet.* 1990;46:229-41.
39. Risch N. Linkage strategies for genetically complex traits. III. The effect of marker polymorphism on analysis of affected relative pairs. *Am J Hum Genet.* 1990;46:242-53.
40. Ewens WJ, Spielman RS. The transmission/disequilibrium test: history, subdivision, and admixture. *Am J Hum Genet.* 1995;57:455-64.
41. Spielman RS, McGinnis RE, Ewens WJ. Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM). *Am J Hum Genet.* 1993;52:506-16.
42. Venter JC, et al. The sequence of the human genome. *Science.* 2001;291:1304-51.
43. Sachidanandam R, Weissman D, Schmidt SC, Kakol JM, Stein LD, Marth G, Sherry S, Mullikin JC, Mortimore BJ, Willey DL, Hunt SE, Cole CG, Coggill PC, Rice CM, Ning Z, Rogers J, Bentley DR, Kwok PY, Mardis ER, Yeh RT, Schultz B, Cook L, Davenport R, Dante M, Fulton L, Hillier L, Waterston RH, McPherson JD, Gilman B, Schaffner S, Van Etten WJ, Reich D, Higgins J, Daly MJ, Blumenstiel B, Baldwin J, Stange-Thomann N, Zody MC, Linton L, Lander ES, Altshuler D. A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. *Nature.* 2001;409:928-33.
44. Collins FS, Guyer MS, Charkravarti A. Variations on a theme: cataloging human DNA sequence variation. *Science.* 1997;278:1580-1.
45. Botstein D, Risch N. Discovering genotypes underlying human phenotypes: past successes for mendelian disease, future approaches for

complex disease. *Nat Genet.* 2003;33 Suppl:228-37.
46. Mune T, Rogerson FM, Nikkila H, Agarwal AK, White PC. Human hypertension caused by mutations in the kidney isozyme of 11 beta-hydroxysteroid dehydrogenase. *Nat Genet.* 1995;10:394-9.
47. Shimkets RA, Warnock DG, Bositis CM, Nelson-Williams C, Hansson JH, Schambelan M, Gill JR, Jr., Ulick S, Milora RV, Findling JW, et al. Liddle's syndrome: heritable human hypertension caused by mutations in the beta subunit of the epithelial sodium channel. *Cell.* 1994;79:407-14.
48. Mansfield TA, Simon DB, Farfel Z, Bia M, Tucci JR, Lebel M, Gutkin M, Vialettes B, Christofilis MA, Kauppinen-Makelin R, Mayan H, Risch N, Lifton RP. Multilocus linkage of familial hyperkalaemia and hypertension, pseudohypoaldosteronism type II, to chromosomes 1q31-42 and 17p11-q21. *Nat Genet.* 1997;16:202-5.
49. Hofstra RM, Landsvater RM, Ceccherini I, Stulp RP, Stelwagen T, Luo Y, Pasini B, Hoppener JW, van Amstel HK, Romeo G, et al. A mutation in the RET proto-oncogene associated with multiple endocrine neoplasia type 2B and sporadic medullary thyroid carcinoma. *Nature.* 1994;367:375-6.
50. Moley JF, Brother MB, Fong CT, White PS, Baylin SB, Nelkin B, Wells SA, Brodeur GM. Consistent association of 1p loss of heterozygosity with pheochromocytomas from patients with multiple endocrine neoplasia type 2 syndromes. *Cancer Res.* 1992;52:770-4.
51. Mulligan LM, Kwok JB, Healey CS, Elsdon MJ, Eng C, Gardner E, Love DR, Mole SE, Moore JK, Papi L, et al. Germ-line mutations of the RET proto-oncogene in multiple endocrine neoplasia type 2A. *Nature.* 1993;363:458-60.
52. Rigat B, Hubert C, Alhenc-Gelas F, Cambien F, Corvol P, Soubrier F. An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. *J Clin Invest.* 1990;86:1343-6.
53. Danser AH, Schalekamp MA, Bax WA, van den Brink AM, Saxena PR, Riegger GA, Schunkert H. Angiotensin-converting enzyme in the human heart. Effect of the deletion/insertion polymorphism. *Circulation.* 1995;92:1387-8.
54. O'Donnell CJ, Lindpaintner K, Larson MG, Rao VS, Ordovas JM, Schaefer EJ, Myers RH, Levy D. Evidence for association and genetic linkage of the angiotensin- converting enzyme locus with hyperten-

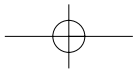
sion and blood pressure in men but not women in the Framingham Heart Study. *Circulation.* 1998;97:1766-72.
55. Jeunemaitre X, Lifton RP, Hunt SC, Williams RR, Lalouel JM. Absence of linkage between the angiotensin converting enzyme locus and human essential hypertension. *Nat Genet.* 1992;1:72-5.
56. Fornage M, Amos CI, Kardia S, Sing CF, Turner ST, Boerwinkle E. Variation in the region of the angiotensin-converting enzyme gene influences interindividual differences in blood pressure levels in young white males. *Circulation.* 1998;97:1773-9.
57. Schmidt S, van Hooft IM, Grobbee DE, Ganten D, Ritz E. Polymorphism of the angiotensin I converting enzyme gene is apparently not related to high blood pressure: Dutch Hypertension and Offspring Study. *J Hypertens.* 1993;11:345-8.
58. Jeunemaitre X, Inoue I, Williams C, Charru A, Tichet J, Powers M, Sharma AM, Gimenez-Roqueplo AP, Hata A, Corvol P, Lalouel JM. Haplotypes of angiotensinogen in essential hypertension. *Am J Hum Genet.* 1997;60:1448-60.
59. Jeunemaitre X, Soubrier F, Kotelevtsev YV, Lifton RP, Williams CS, Charru A, Hunt SC, Hopkins PN, Williams RR, Lalouel JM, et al. Molecular basis of human hypertension: role of angiotensinogen. *Cell.* 1992;71:169-80.
60. Sethi AA, Nordestgaard BG, Agerholm-Larsen B, Frandsen E, Jensen G, Tybjaerg-Hansen A. Angiotensinogen polymorphisms and elevated blood pressure in the general population: the Copenhagen City Heart Study. *Hypertension.* 2001;37:875-81.
61. Inoue I, Nakajima T, Williams CS, Quackenbush J, Puryear R, Powers M, Cheng T, Ludwig EH, Sharma AM, Hata A, Jeunemaitre X, Lalouel JM. A nucleotide substitution in the promoter of human angiotensinogen is associated with essential hypertension and affects basal transcription in vitro. *J Clin Invest.* 1997;99:1786-97.
62. Agachan B, Isbir T, Yilmaz H, Akoglu E. Angiotensin converting enzyme I/D, angiotensinogen T174M-M235T and angiotensin II type 1 receptor A1166C gene polymorphisms in Turkish hypertensive patients. *Exp Mol Med.* 2003;35:545-9.
63. Kumar NN, Benjafield AV, Lin RC, Wang WY, Stowasser M, Morris BJ. Haplotype analysis of aldosterone synthase gene (CYP11B2) polymorphisms shows association with essential hypertension. *J Hypertens.* 2003;21:1331-7.

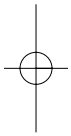
64. Bray MS, Krushkal J, Li L, Ferrell R, Kardia S, Sing CF, Turner ST, Boerwinkle E. Positional genomic analysis identifies the beta(2)-adrenergic receptor gene as a susceptibility locus for human hypertension. *Circulation.* 2000;101:2877-82.
65. Svetkey LP, Timmons PZ, Emovon O, Anderson NB, Preis L, Chen YT. Association of hypertension with beta2- and alpha2c10-adrenergic receptor genotype. *Hypertension.* 1996;27:1210-5.
66. Tomaszewski M, Brain NJ, Charchar FJ, Wang WY, Lacka B, Padmanabahn S, Clark JS, Anderson NH, Edwards HV, Zukowska-Szczechowska E, Grzeszczak W, Dominiczak AF. Essential hypertension and beta2-adrenergic receptor gene: linkage and association analysis. *Hypertension.* 2002;40:286-91.
67. Cusi D, Barlassina C, Azzani T, Casari G, Citterio L, Devoto M, Glorioso N, Lanzani C, Manunta P, Righetti M, Rivera R, Stella P, Troffa C, Zagato L, Bianchi G. Polymorphisms of alpha-adducin and salt sensitivity in patients with essential hypertension. *Lancet.* 1997;349:1353-7.
68. Sartori M, Semplicini A, Siffert W, Mormino P, Mazzer A, Pegoraro F, Mos L, Winnicki M, Palatini P. G-protein beta3-subunit gene 825T allele and hypertension: a longitudinal study in young grade I hypertensives. *Hypertension.* 2003;42:909-14.
69. Brand E, Wang JG, Herrmann SM, Staessen JA. An epidemiological study of blood pressure and metabolic phenotypes in relation to the Gbeta3 C825T polymorphism. *J Hypertens.* 2003;21:729-37.
70. Hingorani AD. Endothelial nitric oxide synthase polymorphisms and hypertension. *Curr Hypertens Rep.* 2003;5:19-25.
71. Samani NJ. Genome scans for hypertension and blood pressure regulation. *Am J Hypertens.* 2003;16:167-71.
72. Krushkal J, Ferrell R, Mockrin SC, Turner ST, Sing CF, Boerwinkle E. Genome-wide linkage analyses of systolic blood pressure using highly discordant siblings. *Circulation.* 1999;99:1407-10.
73. Rice T, Rankinen T, Province MA, Chagnon YC, Perusse L, Borecki IB, Bouchard C, Rao DC. Genome-wide linkage analysis of systolic and diastolic blood pressure: the Quebec Family Study. *Circulation.* 2000;102:1956-63.
74. Rice T, Rankinen T, Chagnon YC, Province MA, Perusse L, Leon AS, Skinner JS, Wilmore JH, Bouchard C, Rao DC. Genomewide linkage scan of resting blood pressure: HERITAGE Family Study. Health, Risk

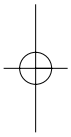
Factors, Exercise Training, and Genetics. *Hypertension.* 2002;39:1037-43.

75. Atwood LD, Samollow PB, Hixson JE, Stern MP, MacCluer JW. Genome-wide linkage analysis of blood pressure in Mexican Americans. *Genet Epidemiol.* 2001;20:373-82.
76. Hunt SC, Ellison RC, Atwood LD, Pankow JS, Province MA, Leppert MF. Genome scans for blood pressure and hypertension: the National Heart, Lung, and Blood Institute Family Heart Study. *Hypertension.* 2002;40:1-6.
77. Allayee H, de Bruin TW, Michelle Dominguez K, Cheng LS, Ipp E, Cantor RM, Krass KL, Keulen ET, Aouizerat BE, Lusis AJ, Rotter JI. Genome scan for blood pressure in Dutch dyslipidemic families reveals linkage to a locus on chromosome 4p. *Hypertension.* 2001;38:773-8.
78. Xu X, Rogus JJ, Terwedow HA, Yang J, Wang Z, Chen C, Niu T, Wang B, Xu H, Weiss S, Schork NJ, Fang Z. An extreme-sib-pair genome scan for genes regulating blood pressure. *Am J Hum Genet.* 1999;64:1694-701.
79. Angius A, Petretto E, Maestrale GB, Forabosco P, Casu G, Piras D, Fanciulli M, Falchi M, Melis PM, Palermo M, Pirastu M. A new essential hypertension susceptibility locus on chromosome 2p24-p25, detected by genomewide search. *Am J Hum Genet.* 2002;71:893-905.
80. Levy D, DeStefano AL, Larson MG, O'Donnell CJ, Lifton RP, Gavras H, Cupples LA, Myers RH. Evidence for a gene influencing blood pressure on chromosome 17. Genome scan linkage results for longitudinal blood pressure phenotypes in subjects from the framingham heart study. *Hypertension.* 2000;36:477-83.
81. Kristjansson K, Manolescu A, Kristinsson A, Hardarson T, Knudsen H, Ingason S, Thorleifsson G, Frigge ML, Kong A, Gulcher JR, Stefansson K. Linkage of essential hypertension to chromosome 18q. *Hypertension.* 2002;39:1044-9.
82. Caulfield M, Munroe P, Pembroke J, Samani N, Dominiczak A, Brown M, Benjamin N, Webster J, Ratcliffe P, O'Shea S, Papp J, Taylor E, Dobson R, Knight J, Newhouse S, Hooper J, Lee W, Brain N, Clayton D, Lathrop GM, Farrall M, Connell J. Genome-wide mapping of human loci for essential hypertension. *Lancet.* 2003;361:2118-23.
83. Lander ES, Schork NJ. Genetic dissection of complex traits. *Science.* 1994;265:2037-48.

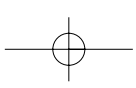
84. Peltonen L, Palotie A, Lange K. Use of population isolates for mapping complex traits. *Nat Rev Genet.* 2000;1:182-90.
85. Kruglyak L. Prospects for whole-genome linkage disequilibrium mapping of common disease genes. *Nat Genet.* 1999;22:139-44.
86. Terwilliger JD, Weiss KM. Linkage disequilibrium mapping of complex disease: fantasy or reality? *Curr Opin Biotechnol.* 1998;9:578-94.
87. Williams JT, Duggirala R, Blangero J. Statistical properties of a variance components method for quantitative trait linkage analysis in nuclear families and extended pedigrees. *Genet Epidemiol.* 1997;14:1065-70.
88. Service SK, Lang DW, Freimer NB, Sandkuijl LA. Linkage-disequilibrium mapping of disease genes by reconstruction of ancestral haplotypes in founder populations. *Am J Hum Genet.* 1999;64:1728-38.
89. Te Meerman GJ, Van der Meulen MA, Sandkuijl LA. Perspectives of identity by descent (IBD) mapping in founder populations. *Clin Exp Allergy.* 1995;25 Suppl 2:97-102.
90. Bourgain C, Genin E, Holopainen P, Mustalahti K, Maki M, Partanen J, Clerget-Darpoux F. Use of closely related affected individuals for the genetic study of complex diseases in founder populations. *Am J Hum Genet.* 2001;68:154-159.
91. Pajukanta P, Terwilliger JD, Perola M, Hiekkalinna T, Nuotio I, Ellonen P, Parkkonen M, Hartiala J, Ylitalo K, Pihlajamaki J, Porkka K, Laakso M, Viikari J, Ehnholm C, Taskinen MR, Peltonen L. Genomewide scan for familial combined hyperlipidemia genes in finnish families, suggesting multiple susceptibility loci influencing triglyceride, cholesterol, and apolipoprotein B levels. *Am J Hum Genet.* 1999;64:1453-63.
92. Moises HW, Yang L, Kristbjarnarson H, Wiese C, Byerley W, Macciardi F, Arolt V, Blackwood D, Liu X, Sjogren B, et al. An international two-stage genome-wide search for schizophrenia susceptibility genes. *Nat Genet.* 1995;11:321-4.





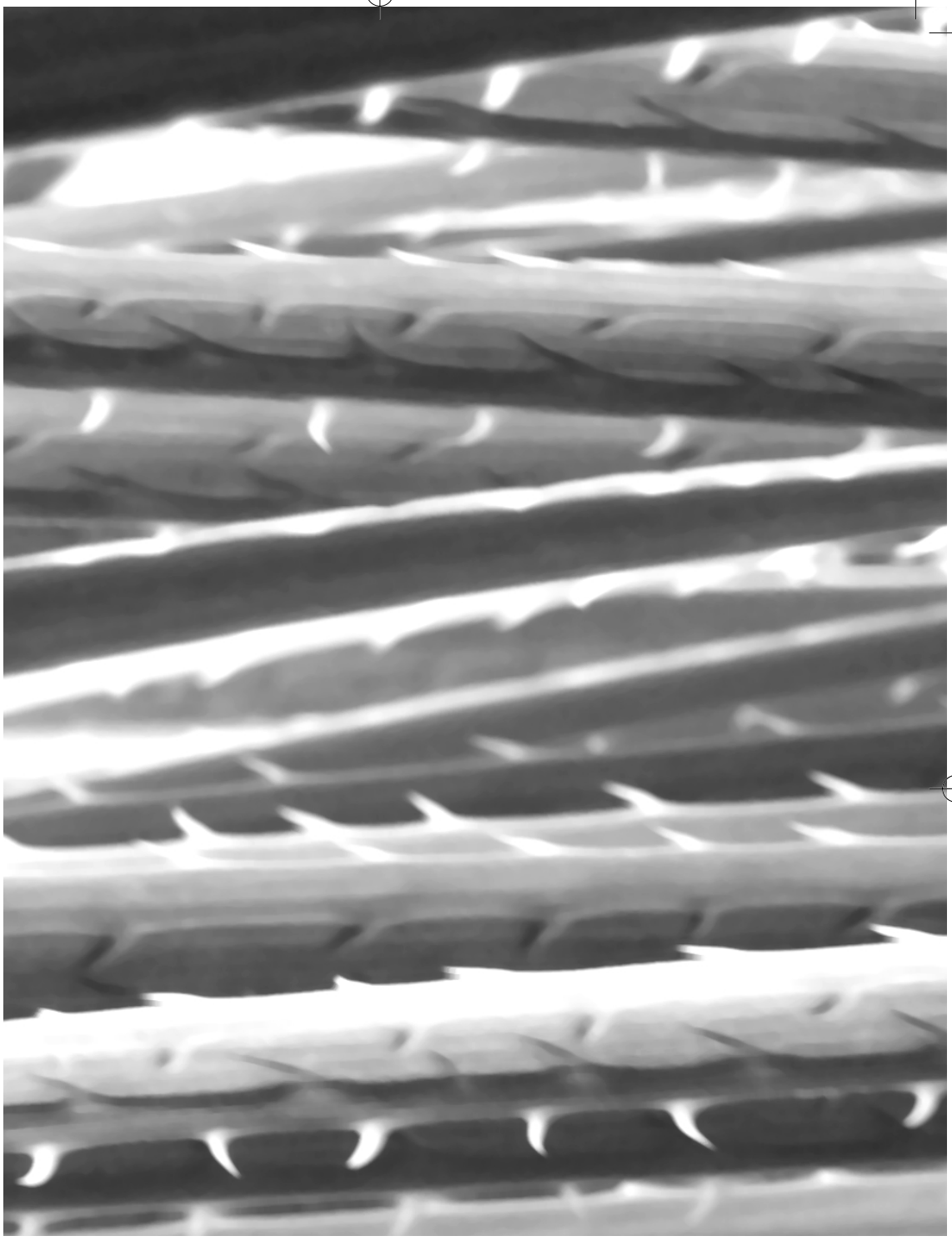


1.3. SCOPE OF THE THESIS



A better understanding of the genetic basis of hypertension may provide more insight into the pathophysiology of this condition, and consequently lead to more effective preventive and treatment strategies. Unraveling the complex genetics of hypertension has proven difficult, nevertheless in this thesis a careful attempt was made. We conducted a genetic epidemiological search for the identification and quantification of genetic risk factors in the development of hypertension and its cardiovascular sequelae. First, two candidate genes were studied in relation to hypertension, atherosclerosis and cardiac disease in a population-based cohort study. Second, heritability of blood pressure and familial aggregation of hypertension was studied in a Dutch genetically isolated community.

Chapter 2 describes the results of two candidate gene studies in the Rotterdam study. A polymorphism in the angiotensin-converting enzyme (ACE) gene is studied in relation to blood pressure, carotid artery atherosclerosis and heart failure. In Chapter 3 the results of two association studies regarding a genetic polymorphism in the promoter region of the insulin-like growth factor-I (IGF-I) gene are presented. This polymorphism is studied in relation to early signs of atherosclerosis in the carotid arteries, arterial stiffness and left ventricular hypertrophy. Chapter 4 encompasses the results of two studies performed in a genetically isolated population in the Southwest part of the Netherlands. In the first study heritability estimates of blood pressure are presented. In the second study we describe the familial aggregation of hypertension in this population. Finally, in Chapter 5 the work described in this thesis is concluded with a discussion on the role of candidate gene studies and the value of genetically isolated populations in the search of susceptibility genes for hypertension and its cardiovascular sequelae.



2. CANDIDATE GENE STUDIES

ANGIOTENSIN CONVERTING ENZYME INSERTION/DELETION POLYMORPHISM

2.1. SMOKING-DEPENDENT EFFECTS OF THE ANGIOTENSIN-CONVERTING ENZYME GENE INSERTION/DELETION POLYMORPHISM ON BLOOD PRESSURE

Objective: Studies on the role of the Angiotensin-Converting-Enzyme (ACE) gene in the development of hypertension have yielded conflicting results. Recent studies suggested that the ACE gene might have smoking-dependent effects on the development of cardiovascular disease. We studied the relation between the ACE insertion/deletion polymorphism, blood pressure and risk of hypertension in current, former and non-smokers in a population-based cohort.

Methods: We included 2414 non-smokers, 2794 former smokers and 1508 current smokers, all participants of the Rotterdam Study. In each group, we assessed the relation between the ACE I/D polymorphism, systolic (SBP) and diastolic blood pressure (DBP) and risk of hypertension. Mean blood pressure levels and prevalence of hypertension were compared between carriers and non-carriers of the D-allele. All analyses were adjusted for age, sex, BMI, diabetes mellitus, HDL- cholesterol, total cholesterol and anti-hypertensive medication use.

Results: In non- and former smokers, blood pressure and the risk of hypertension did not differ significantly between genotypes. In smokers, we found a significant increase in SBP in DD-carriers (139.6 ± 22.8 mmHg) compared to II-carriers (136.0 ± 22.7 mmHg)(p=0.04). No effect of ACE genotype was observed for DBP. The risk of hypertension was significantly increased in smokers who carried one (OR: 1.4; 95%CI: 1.0-1.9; p=0.05) or two copies of the D-allele (OR: 1.5; 95%CI: 1.1-2.2; p=0.02).

Conclusion: The D-allele of the ACE polymorphism is associated with a significantly increased SBP and risk of hypertension in smokers. Our study underlines the importance of gene-environment interactions in the study of candidate genes for hypertension.

Introduction

Angiotensin converting enzyme (ACE), also known as kinase II (EC 3.4.15.1), is involved in the generation of angiotensin II (ANGII) and the degradation of bradykinin.[1] Since both vasoactive peptides mediate a great variety of cardiovascular effects, it is thought that ACE plays a prominent role in cardiovascular physiology. The major therapeutic advances made by ACE-inhibitors in the treatment of hypertension and heart failure emphasize the important role of ACE in the cardiovascular system. An insertion/deletion (I/D) polymorphism in the gene coding for ACE has been found to account for about half of the variance in serum ACE levels.[2] The D-allele, associated with increased ACE-activity, has been put forward as a genetic risk factor for cardiovascular disease (CVD).[3] Although hypertension is an important risk factor for CVD, findings regarding the association between the I/D polymorphism and blood pressure remain inconclusive.[4-7]

Determining the relative contribution of genetic and environmental factors in the development of complex diseases is difficult. In fact, many of the conflicting findings on the ACE polymorphism and blood pressure might arise from differences in gene-gene and gene-environmental interactions between the populations studied.[8] Recently, the D-allele has been associated with endothelial dysfunction, especially in smokers.[9-11] Both smoking and the D-allele have been associated with increased generation of ANGII, which in turn has been shown to increase the formation of super oxide anions and the degradation of nitric oxide (NO), causing endothelial dysfunction.[12] In a large population-based cohort, we assessed the effect of the ACE polymorphism on blood pressure and risk of hypertension. To study whether there are smoking-dependent effects of the ACE polymorphism, non-smokers, former smokers and current smokers were analyzed separately.

Methods

Study Population

The study was conducted within the Rotterdam Study, a single-center prospective follow-up study in which all residents aged 55 years and over of the Rotterdam suburb Ommoord were invited to take part. The baseline examination of the Rotterdam Study was conducted between 1990 and 1993. The Medical Ethics Committee of Erasmus Medical Center Rotterdam approved the study. Written informed consent was obtained from all participants. The design of the study has been described previously.[13] 7983 participants were examined (response 78%). In 6869 subjects, the ACE insertion/deletion polymorphism was genotyped successfully (86%). In the remaining 1114 subjects, no genotypes were available. We included 2414 non-smokers, 2794 former smokers and 1508 current smokers in our study. We excluded 155 subjects because no information on smoking status was available.

Measurements

At baseline, information concerning medical history, medication use and smoking behavior was obtained with a computerized questionnaire.[13] Height and weight were measured and body mass index (BMI in kg/m2) was calculated. Blood pressure was measured twice, after a minimum of 5 minutes rest, in the sitting position at the right upper arm using a random zero sphygmomanometer. Participants were asked to abstain from smoking and drinking alcoholic or caffeine-containing beverages at least two hours before blood pressure measurements were taken. The average of two measurements, obtained at a single visit, was used for analysis. Hypertension was defined as a diastolic blood pressure (DBP) of 100 mmHg or higher and/or a systolic blood pressure (SBP) of 160 mmHg or higher and/or use of anti-hypertensive medication indicated for treatment of hypertension (grade 2 and 3 of the 1999 WHO criteria)[14]. Diabetes mellitus was defined as the use of blood glucose-lowering medication and/or random serum glucose level ≥ 11.1 mmol/l. History of myocardial infarction (MI) was defined as self-reported MI confirmed by a physician or MI on ECG. Total serum cholesterol and HDL-cholesterol were determined with an automated enzymatic procedure.[15]

Genotyping

The II, ID and DD genotypes were detected using the polymerase chain reaction technique (PCR) according to the method of Lindpaintner et al. with some modifications.[16] The insertion and deletion alleles of the ACE gene were identified using a set of oligonucleotide primers flanking the polymorphic site in intron 16 (sense primer, 5'GCC CTG CAG GTG TCT GCA GCA TGT3' and antisense primer, 5'GGA TGG CTC TCC CCG CCT TGT CTC3'). The final volume of the PCR mix was 20 ml, containing 50 ng DNA, PCR buffer (Invitrogen), 1.3 mM MgCl2, 200 mmol dNTPS, 20 pmol primer mix and 0.35 units *Taq* polymerase. The thermo cycling procedure was identical to the method of Lindpaintner et al. The result of amplification was a 319-bp amplicon for the D-allele and a 597-bp amplicon for the I-allele. Because the D-allele in heterozygous subjects is preferentially amplified, there is a tendency of misclassification of ID-genotypes into DD-genotypes (4-5%). In order to avoid this misclassification, a second independent PCR was performed with a primer pair that recognizes an insertion specific sequence (5'TGG GAC CAC AGC GCC CGC CAC TAC3' and 5'TCG CCA GCC CTC CCA TGC CCA TAA3'). To optimize the second PCR, 10% DMSO, 0.35 units Ampli*Taq* Gold DNA polymerase and GeneAmp PCR Gold buffer (Applied Biosystems) were used in the PCR mix. This reaction yielded a 335-bp amplicon only if the I-allele was present. All reactions were performed in 96-well plates with the help of a robot (Beckman Biomek® 2000). Fragments were separated and visualized using 3% Agarose gels, Ethidium Bromide staining and UV trans-illumination. Two independent investigators read pictures from each gel. All ambiguous samples were analyzed a second time.

Data Analysis

Hardy-Weinberg equilibrium proportions of the ACE insertion/deletion polymorphism were tested using the GENEPOP-package (Raymond M. & Rousset F., 1995. GENEPOP version). General characteristics, stratified by smoking status and ACE genotype, were compared using the unpaired T-test for continuous variables and χ^2- statistics for dichotomous variables. Mean systolic and diastolic blood pressure in non-, former and current smokers were stratified by ACE genotype and analyzed using multivariate analysis of variance. To examine the effect of the ACE genotype on the risk of hypertension, logistic regression analysis was performed within the non-, former and current smokers. In these analyses, subjects carrying the II-genotype were used as the reference group. The analyses on hypertension, SBP and DBP were adjusted for possible confounders age, sex, BMI, total cholesterol, HDL-cholesterol and diabetes mellitus. The analyses on SBP and DBP were additionally adjusted for anti-hypertensive medication use. For all statistical analyses we used SPSS for Windows, version 11.0.

Results

Genotype frequencies were in Hardy-Weinberg equilibrium in all three smoking groups. Table 1 presents baseline characteristics of the overall study population and stratified by smoking status. About 50-60% of the current and former smokers were male, compared to only 9% in non-smokers. Current smokers were younger (66.7 ± 8.2 yrs) compared to former smokers (68.5 ± 8.2 yrs) and non-smokers (71.9 ± 10.0 yrs) ($p<0.01$). SBP was significantly lower in current and former smokers (136.6 ± 22.5 mmHg and 138.6 ± 21.8 mmHg) compared to non-smokers (142.4 ± 22.8 mmHg) ($p<0.05$). DBP was lowest in current smokers and highest in former smokers (73.2 ± 11.8 mmHg vs. 74.0 ± 11.6 mmHg). The prevalence of hypertension was lowest in current smokers (27.9%) and highest in non–smokers (39.6%) ($p<0.01$). The use of anti-hypertensive medication was also lowest in current smokers (23.9% compared to 33.1% for former and 37.1% for non-smokers) ($p<0.05$). BMI, total and HDL-cholesterol were significantly lower in current and former smokers compared to non-smokers ($p<0.05$). The prevalence of myocardial infarction was significantly higher in current and former smokers than in non-smokers (12.1 % and 13.6 % vs. 7.9 %) ($p<0.01$). The prevalence of diabetes mellitus did not differ significantly between the three groups.

In table 2a, 2b and 2c, general characteristics of non-smokers, formers smokers and current smokers, stratified by ACE genotype, are presented. Mean packyears (mean number of cigarettes smoked per day times the total years of smoking ± SD) for current smokers was 30.4 ± 19.8 for II-carriers, 33.3 ± 22.2 for ID-carriers and 30.7 ± 20.1 for DD-carriers. Mean packyears for former smokers was 26.0 ± 26.7 for II-carriers, 24.7 ± 24.5 for ID-carriers and 26.0 ± 26.5 for DD-carriers. Both in current and former smokers mean

Table 1. General characteristics of the total study population and stratified by smoking status

	Total study population	Non-smokers	Former smokers	Current smokers
Number - no (%)	6869	2412	2794	1508
Sex - % men	40.1	9.4	60.3**	53.2**
Age - yrs	69.5 ± 9.2	71.9 ± 10.0	68.5 ± 8.2*	66.7 ± 8.2*
SBP - mmHg	139.3 ± 22.4	142.4 ± 22.8	138.6 ± 21.8*	136.6 ± 22.5*
DBP - mmHg	73.7 ± 11.6	73.6 ± 11.6	74.0 ± 11.6	73.2 ± 11.8
Hypertension - %	34.3	39.6	33.7*	27.9**
Anti-hypertensive medication - %	32.6	37.1	33.1*	23.9**
BMI - kg/m^2	26.3 ± 3.7	26.8 ± 3.9	26.4 ± 3.4*	25.4 ± 3.8**
Total cholesterol - mmol/l	6.6 ± 1.2	6.7 ± 1.2	6.5 ± 1.2*	6.6 ± 1.3*
HDL-cholesterol - mmol/l	1.3 ± 0.4	1.4 ± 0.4	1.3 ± 0.4*	1.3 ± 0.4*
Myocardial Infarction - %	10.9	7.9	13.6**	12.1**
Diabetes mellitus - %	10.3	11.4	9.3	10.5

*All values are presented as percentage or mean ± standard deviation. *Significantly different from non-smokers, p<0.05. **Significantly different from non-smokers, p<0.01.*

Table 2a. General characteristics non-smokers stratified by ACE genotype

ACE genotype	II	ID	DD
Number - no (%)	512 (21.2)	1199 (49.7)	701 (29.1)
Sex - % men	9.0	9.7	9.1
Age - yrs	71.7 ± 10.2	72.0 ± 9.8	72.0 ± 10.1
SBP - mmHg	141.7 ± 23.2	142.1 ± 23.0	142.6 ± 21.8
DBP - mmHg	73.0 ± 11.7	73.9 ± 11.6	74.0 ± 11.2
Hypertension - %	36.9	40.5	40.1
Anti-hypertensive medication - %	34.7	36.6	39.6
- β-blocker	16.1	15.5	14.2
- diuretic	17.5	19.7	21.0
- other	12.4	13.0	15.2
BMI - kg/m^2	26.9 ± 4.0	26.6 ± 3.8	26.9 ± 4.0
Total cholesterol - mmol/l	6.7 ± 1.3	6.7 ± 1.2	6.7 ± 1.2
HDL-cholesterol - mmol/l	1.4 ± 0.4	1.4 ± 0.4	1.4 ± 0.4
Myocardial Infarction - %	6.6	10.1	7.6
Diabetes mellitus - %	12.3	12.1	9.4

All values are presented as percentage or mean ± standard deviation.

Table 2b. General characteristics former smokers stratified by ACE genotype

ACE genotype	II	ID	DD
Number - no (%)	625 (22.4)	1381 (49.4)	788 (28.2)
Packyears smoking	26.0 ± 26.7	24.7 ± 24.5	26.0 ± 26.5
Sex - % men	63.0	59.4	59.6
Age - yrs	67.9 ± 8.1	68.7 ± 8.1	68.7 ± 8.3
SBP- mmHg	137.8 ± 21.5	138.4 ± 22.0	139.2 ± 21.5
DBP- mmHg	74.4 ± 11.8	73.8 ± 11.7	74.1 ± 11.1
Hypertension - %	32.6	34.6	32.8
Anti-hypertensive medication - %	32.3	33.9	32.1
- β-blocker	17.0	16.5	15.5
- diuretic	14.1	14.1	13.8
- other	13.6	14.8	14.6
BMI - kg/m^2	26.3 ± 3.3	26.4 ± 3.5	26.5 ± 3.3
Total cholesterol - mmol/l	6.5 ± 1.3	6.6 ± 1.2	6.5 ± 1.2
HDL-cholesterol - mmol/l	1.3 ± 0.4	1.3 ± 0.4	1.3 ± 0.4
Myocardial Infarction - %	14.9	14.5	14.1
Diabetes mellitus - %	9.1	10.2	8.0

All values are presented as percentage or mean ± standard deviation. Packyears is defined as mean number of cigarettes smoked per day times the total years of smoking.

packyears did not significantly differ between genotypes. Within the non- and former smokers, no significant differences in general characteristics were seen between the genotype strata. In current smokers however, SBP was significantly higher in DD-subjects (137.8 ± 23.8 mmHg) compared to II-subjects (134.3 ± 20.7 mmHg) ($p<0.05$). The prevalence of hypertension was significantly higher in DD-and ID-subjects (29%) than in II-subjects (23%) ($p<0.05$). The use of anti-hypertensive medication was also significantly higher in ID- and DD-subjects (26.3 and 23.4%) compared to II-subjects (19.2%) ($p<0.01$). Use of diuretics and other forms of anti-hypertensive medication was highest in ID-subjects. Mean age, percentage of males, BMI, total and HDL-cholesterol, and the prevalence of MI and diabetes mellitus did not differ significantly between the three genotypes.

Figure 1 shows mean adjusted SBP, stratified by ACE genotype in non-, former and current smokers. SBP was significantly lower in smokers compared to non- and former smokers, independent of genotype status ($p<0.05$). Within the smokers group, SBP significantly increased with the number of D-alleles present (p-trend=0.05). Smokers carrying the DD-genotype had a mean SBP of 139.6 ± 22.8 mmHg compared to 136.0 ± 22.7 mmHg for smokers carrying the II-genotype. The difference in SBP between II and DD-

Table 2c. General characteristics current smokers stratified by ACE genotype

ACE genotype	II	ID	DD
Number - no (%)	336 (22.3)	778 (51.6)	394 (26.1)
Packyears smoking	30.4 ± 19.8	33.3 ± 22.2	30.7 ± 20.1
Sex - % men	49.1	54.8	53.6
Age - yrs	66.0 ± 8.0	67.0 ± 8.1	66.7 ± 8.4
SBP - mmHg	134.3 ± 20.7	136.8 ± 22.8	137.8 ± 23.8*
DBP - mmHg	73.0 ± 11.8	73.2 ± 11.7	73.7 ± 11.9
Hypertension	23.2	29.0*	29.6*
Anti-hypertensive medication - %	19.2	26.3**	23.4*
- β-blocker	9.9	11.1	10.5
- diuretic	7.1	12.0*	8.7
- other	6.8	12.6*	9.7
BMI - kg/m^2	25.7 ± 4.4	25.4 ± 3.7	25.2 ± 3.5
Total cholesterol - mmol/l	6.7 ± 1.3	6.6 ± 1.2	6.6 ± 1.2
HDL-cholesterol - mmol/l	1.3 ± 0.4	1.3 ± 0.3	1.3 ± 0.4
Myocardial Infarction - %	9.5	12.9	13.7
Diabetes mellitus - %	8.6	11.3	10.4

*All values are presented as percentage or mean ± standard deviation. *Significantly different from II-subjects, p<0.05. **Significantly different from II-subjects, p<0.01. Packyears is defined as mean number of cigarettes smoked per day times the total years of smoking.*

carriers in smokers was significant (3.6 mmHg; 95 %CI: 0.3-6.9, p=0.04). In non-smokers, SBP did not differ significantly between genotypes (II: 140.3 ± 23.3 mmHg, ID: 140.7 ± 23.0 mmHg, DD: 141.3 ± 21.5 mmHg). Findings in former smokers were similar to those of non-smokers; no effect of the ACE polymorphism on SBP was observed in this group (II: 138.3 ± 21.6 mmHg, ID: 138.2 ± 22.1 mmHg, DD 139.1 ± 23.3 mmHg).

In figure 2, mean adjusted DBP in non-, former and current smokers, stratified by ACE genotype, is presented. Mean DBP did not differ significantly between II, ID and DD subjects within the three smoking groups.

Figure 3 shows the risk of hypertension in non-, former and current smokers for each genotype. Subjects homozygous for the I-allele were used as the reference group and their risk of hypertension defined as one. In non- and former smokers, subjects carrying one or two copies of the D-allele did not have a significantly increased risk of hypertension. In smokers, the risk of hypertension was significantly increased both in ID-subjects and DD-subjects. Smokers who carry one or two copies of the D-allele had an increased risk of hypertension of 1.4 (95% CI: 1.0-1.9, p=0.05) and 1.5 (95%CI: 1.1-2.2, p=0.02) respectively.

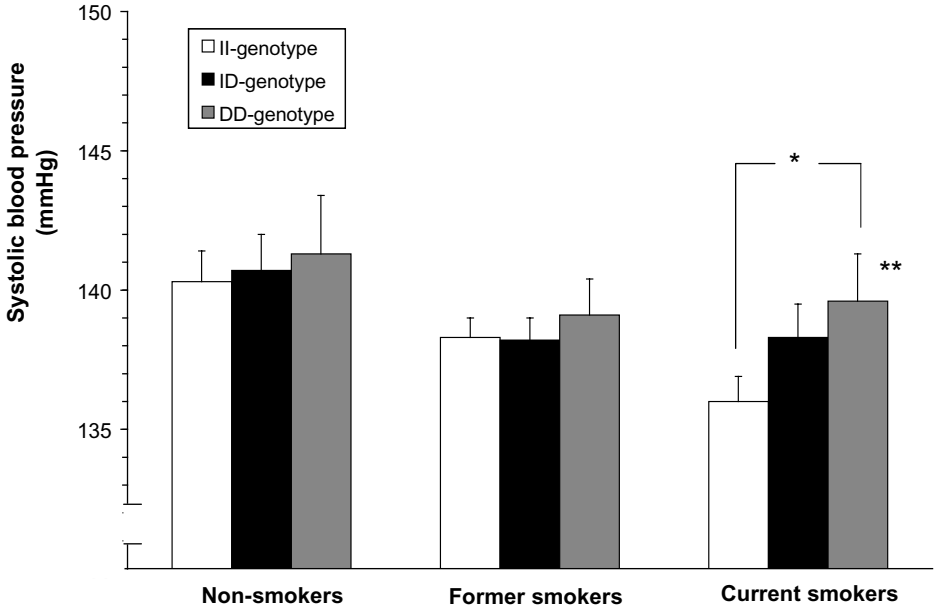


Figure 1. Systolic blood pressure (mmHg) in non-smokers, former smokers and current smokers, stratified by ACE genotype: II (white bars), ID (black bars) and DD (gray bars). *p for trend=0.05. **Significantly different between II and DD-subjects, p<0.05. All values were adjusted for age, sex, BMI, total cholesterol, HDL-cholesterol, diabetes mellitus and anti-hypertensive medication.

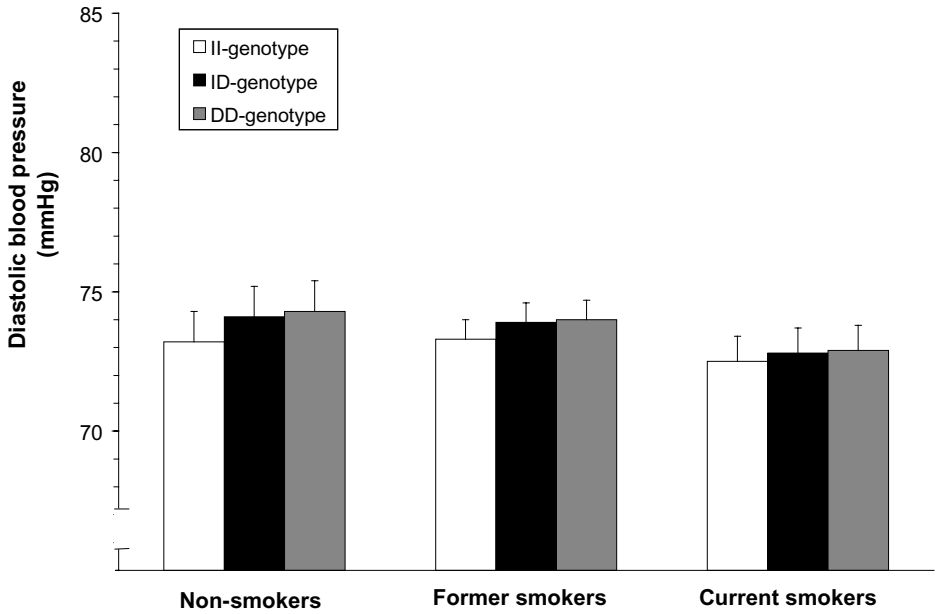


Figure 2. Diastolic blood pressure (mmHg) in non-smokers, former smokers and current smokers, stratified by ACE genotype: II (white bars), ID (black bars) and DD (gray bars). All values were adjusted for age, sex, BMI, total cholesterol, HDL-cholesterol, diabetes mellitus and anti-hypertensive medication.

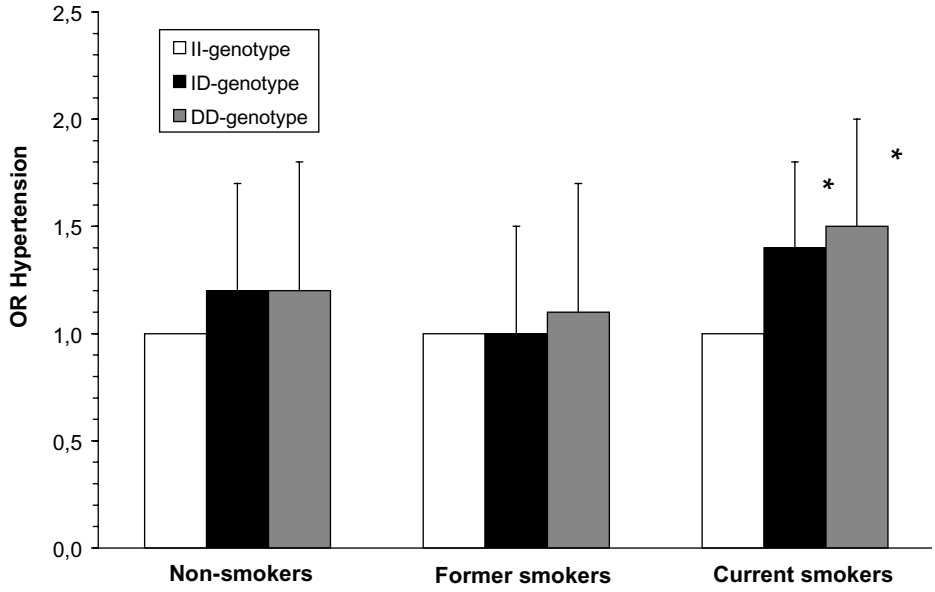


Figure 3. Risk of hypertension in non-smokers, former smokers and current smokers, stratified by ACE genotype: II (white bars), ID (black bars) and DD (gray bars). Subjects carrying two copies of the I-allele were used as a reference in each smoking group. *Significantly different from reference group (II), $p<0.05$. All values were adjusted for age, sex, BMI, total cholesterol, HDL-cholesterol and diabetes mellitus.

Discussion

In this population-based study, we found a significant association between the ACE I/D polymorphism, systolic blood pressure and the risk of hypertension in smokers. Subjects who smoke and carry the D-allele have significantly increased systolic blood pressure and an increased risk of hypertension compared to subjects who smoke and carry the II-genotype. We also found that in smokers, who carry one or two copies of the D-allele, even though they have higher systolic blood pressure, a higher percentage uses anti-hypertensive medication than in smokers carrying the II-genotype. In non-smokers and former smokers, no relation between the ACE genotype and blood pressure or risk of hypertension was observed.

The ACE I/D polymorphism has been studied extensively in relation to hypertension but findings remain controversial. Since regulation of blood pressure most likely results from a complex of interactions between gene-gene and gene-environmental factors, part of the conflicting findings on candidate genes for hypertension may be explained by differences in genetic make-up and environmental exposure status of the populations studied.[8] The large diversity in geographical, racial and medical background of the study populations may have yielded false positive as well as false negative associations. This

context-dependency may also account for the conflicting findings on the ACE gene and cardiovascular disease. Three large population-based studies found evidence for an effect of the ACE polymorphism on blood pressure in males only, however many others could not confirm any association between the ACE gene and blood pressure in neither men nor women.[7,17-22] Although this male-specificity of the ACE gene remains to be fully investigated it could have accounted for our (false) negative findings in the non-smokers group, since in this group only 9% was male. Nevertheless, male-specificity is unlikely to have caused the negative findings in the former smokers group, since about 60% was male in this group. Our findings suggest that male-specific findings on the ACE gene may in fact be confounded by differences in environmental exposure status, e.g. smoking-habits, between males and females.

Recently, a number of studies have indeed raised evidence for smoking-associated effects of the ACE I/D polymorphism on cardiovascular disease. Hibi et al.[23] reported an increased severity of coronary atherosclerosis in subjects who smoke and carry two copies of the D-allele. In a study by Butler et al.[10], significant blunting of endothelial function was observed in DD-carriers, especially when they smoked. So far, these studies on smoking-associated effects of the ACE gene have been performed in small, selected groups of patients and, although endothelial dysfunction is one of the key features of hypertension, studies on the smoking-associated effect of the ACE-gene in the development of high blood pressure have not been performed.

In order to appreciate our results in the right context, several issues require discussion. We have found that carrying the D-allele is associated with increased systolic blood pressure in smokers, but, overall, smokers had significantly lower systolic blood pressure and were less often hypertensive than non- and former smokers. This observation has been reported consistently in other large epidemiological studies.[24] A great number of possible explanations for this observation have been discussed, but the underlying mechanism remains essentially unexplained. Our findings suggest that, besides the effect of the ACE genotype in smokers, there must be other smoking-associated factors that cause smokers to have lower blood pressure than non-smokers. Another issue that needs to be addressed is that the effect of the D-allele and smoking seems to be restricted to systolic blood pressure only. In accordance with our findings, smoking has frequently been associated with increased arterial stiffness, as expressed by an increase in systolic rather than diastolic blood pressure.[25-27] Findings regarding the ACE I/D polymorphism and systolic blood pressure or isolated systolic hypertension have not, however, been very conclusive so far.[28,29]

Our findings suggest that there is a direct effect of smoking together with the D-allele on blood pressure. This observation may be explained by the fact that both smoking and ACE increase the degradation of NO and production of free radicals, causing endo-

thelial damage and impaired vasodilatation.[11,30] There is also evidence that suggests that smoking causes enhanced endothelial ACE activity through increased ACE expression.[31]

We are the first to assess the systemic smoking-associated effects of the ACE gene in the general elderly population. Although the smoking-dependent effects of the ACE gene may be different or even absent in a younger population we believe our study provides additional evidence to the observation that the ACE gene may have smoking-dependent effects on the vasculature. Several studies have already reported that smoking status may influence the effects of the ACE-genotype on vasomotor tone and endothelial function. We hypothesize that synergistic effects on the modulation of vascular tone may explain the effects of the D-allele and smoking on systolic blood pressure. Although our findings may not have direct implications for the treatment of hypertension, we think our observation may contribute to a better understanding of the pathophysiological mechanism of gene-environment interactions in the development of high blood pressure.

In conclusion, we found a modest but significant effect of the ACE I/D polymorphism on systolic blood pressure and risk of hypertension in smokers. Although the exact pathophysiological pathway of the effects of smoking together with the presence of the D-allele on the development of high blood pressure remains to be solved, our study underlines the importance of gene-environment interactions in the study of complex diseases such as hypertension.

References

1. Butler R, Morris AD, Struthers AD. Angiotensin-converting enzyme gene polymorphism and cardiovascular disease. *Clin Sci (Lond).* 1997;93:391-400.
2. Rigat B, Hubert C, Alhenc-Gelas F, Cambien F, Corvol P, Soubrier F. An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. *J Clin Invest.* 1990;86:1343-6.
3. Butler R. The DD-ACE genotype and cardiovascular disease. *Pharmacogenomics.* 2000;1:153-67.
4. Morise T, Takeuchi Y, Takeda R. Angiotensin-converting enzyme polymorphism and essential hypertension. *Lancet.* 1994;343:125.
5. Barley J, Blackwood A, Miller M, Markandu ND, Carter ND, Jeffery S, Cappuccio FP, MacGregor GA, Sagnella GA. Angiotensin converting enzyme gene I/D polymorphism, blood pressure and the renin-angiotensin system in Caucasian and Afro-Caribbean peoples. *J Hum Hypertens.* 1996;10:31-5.
6. Zhu X, Bouzekri N, Southam L, Cooper RS, Adeyemo A, McKenzie CA,

Luke A, Chen G, Elston RC, Ward R. Linkage and association analysis of angiotensin I-converting enzyme (ACE)-gene polymorphisms with ACE concentration and blood pressure. *Am J Hum Genet.* 2001;68:1139-48.

7. Matsubara M, Suzuki M, Fujiwara T, Kikuya M, Metoki H, Michimata M, Araki T, Kazama I, Satoh T, Hashimoto J, Hozawa A, Ohkubo T, Tsuji I, Katsuya T, Higaki J, Ogihara T, Satoh H, Imai Y. Angiotensin-converting enzyme I/D polymorphism and hypertension: the Ohasama study. *J Hypertens.* 2002;20:1121-6.
8. Turner ST, Boerwinkle E, Sing CF. Context-dependent associations of the ACE I/D polymorphism with blood pressure. *Hypertension.* 1999;34:773-8.
9. Kario K, Matsuo T, Kobayashi H, Kanai N, Hoshide S, Mitsuhashi T, Ikeda U, Nishiuma S, Matsuo M, Shimada K. Endothelial cell damage and angiotensin-converting enzyme insertion/deletion genotype in elderly hypertensive patients. *J Am Coll Cardiol.* 1998;32:444-50.
10. Butler R, Morris AD, Burchell B, Struthers AD. DD angiotensin-converting enzyme gene polymorphism is associated with endothelial dysfunction in normal humans. *Hypertension.* 1999;33:1164-8.
11. Perticone F, Ceravolo R, Maio R, Ventura G, Zingone A, Perrotti N, Mattioli PL. Angiotensin-converting enzyme gene polymorphism is associated with endothelium-dependent vasodilation in never treated hypertensive patients. *Hypertension.* 1998;31:900-5.
12. Drexler H, Hornig B. Endothelial dysfunction in human disease. *J Mol Cell Cardiol.* 1999;31:51-60.
13. Hofman A, Grobbee DE, de Jong PT, van den Ouweland FA. Determinants of disease and disability in the elderly: the Rotterdam Elderly Study. *Eur J Epidemiol.* 1991;7:403-22.
14. 1999 World Health Organization-International Society of Hypertension Guidelines for the Management of Hypertension. Guidelines Subcommittee. *J Hypertens.* 1999;17:151-83.
15. van Gent CM, van der Voort HA, de Bruyn AM, Klein F. Cholesterol determinations. A comparative study of methods with special reference to enzymatic procedures. *Clin Chim Acta.* 1977;75:243-51.
16. Lindpaintner K, Pfeffer MA, Kreutz R, Stampfer MJ, Grodstein F, LaMotte F, Buring J, Hennekens CH. A prospective evaluation of an angiotensin-converting-enzyme gene polymorphism and the risk of ischemic heart disease. *N Engl J Med.* 1995;332:706-11.

17. Fornage M, Amos CI, Kardia S, Sing CF, Turner ST, Boerwinkle E. Variation in the region of the angiotensin-converting enzyme gene influences interindividual differences in blood pressure levels in young white males. *Circulation.* 1998;97:1773-9.
18. O'Donnell CJ, Lindpaintner K, Larson MG, Rao VS, Ordovas JM, Schaefer EJ, Myers RH, Levy D. Evidence for association and genetic linkage of the angiotensin- converting enzyme locus with hypertension and blood pressure in men but not women in the Framingham Heart Study. *Circulation.* 1998;97:1766-72.
19. Higaki J, Baba S, Katsuya T, Sato N, Ishikawa K, Mannami T, Ogata J, Ogihara T. Deletion allele of angiotensin-converting enzyme gene increases risk of essential hypertension in Japanese men : the Suita Study. *Circulation.* 2000;101:2060-5.
20. Harrap SB, Davidson HR, Connor JM, Soubrier F, Corvol P, Fraser R, Foy CJ, Watt GC. The angiotensin I converting enzyme gene and pre disposition to high blood pressure. *Hypertension.* 1993;21:455-60.
21. Fuentes RM, Perola M, Nissinen A, Tuomilehto J. ACE gene and physical activity, blood pressure, and hypertension: a population study in Finland. *J Appl Physiol.* 2002;92:2508-12.
22. Cambien F, Poirier O, Lecerf L, Evans A, Cambou JP, Arveiler D, Luc G, Bard JM, Bara L, Ricard S, et al. Deletion polymorphism in the gene for angiotensin-converting enzyme is a potent risk factor for myocardial infarction. *Nature.* 1992;359:641-4.
23. Hibi K, Ishigami T, Kimura K, Nakao M, Iwamoto T, Tamura K, Nemoto T, Shimizu T, Mochida Y, Ochiai H, Umemura S, Ishii M. Angiotensin-converting enzyme gene polymorphism adds risk for the severity of coronary atherosclerosis in smokers. *Hypertension.* 1997;30:574-9.
24. Green MS, Jucha E, Luz Y. Blood pressure in smokers and nonsmokers: epidemiologic findings. *Am Heart J.* 1986;111:932-40.
25. Primatesta P, Falaschetti E, Gupta S, Marmot MG, Poulter NR. Association between smoking and blood pressure: evidence from the health survey for England. *Hypertension.* 2001;37:187-93.
26. Halimi JM, Giraudeau B, Vol S, Caces E, Nivet H, Tichet J. The risk of hypertension in men: direct and indirect effects of chronic smoking. *J Hypertens.* 2002;20:187-93.
27. Mahmud A, Feely J. Effect of smoking on arterial stiffness and pulse pressure amplification. *Hypertension.* 2003;41:183-7.
28. Davis D, Liyou N, Johnson A. The ACE gene I/D polymorphism, but not

the angiotensin II type I receptor gene A1166C polymorphism is associated with isolated systolic hypertension.
J Hum Hypertens. 2001;15:653-4.

29. Johnson AG, Simons LA, Friedlander Y, Simons J, Davis DR, MaCallum J. I/D polymorphism of the angiotensin-converting enzyme gene does not predict isolated systolic or systolic-diastolic hypertension in the elderly. *J Hum Hypertens.* 1996;10:167-9.
30. Rathaus M, Bernheim J. Oxygen species in the microvascular envi ronment: regulation of vascular tone and the development of hypertension. *Nephrol Dial Transplant.* 2002;17:216-21.
31. Zhang S, Day I, Ye S. Nicotine induced changes in gene expression by human coronary artery endothelial cells.
Atherosclerosis. 2001;154:277-83.

2.2. A STUDY OF GENE-ENVIRONMENT INTERACTION ON THE ANGIOTENSIN CONVERTING ENZYME GENE: A COMBINED FUNCTIONAL AND POPULATION-BASED APPROACH

Introduction: Studies on the role of the insertion/deletion (I/D) polymorphism of the angiotensin-converting-enzyme (ACE) gene in atherosclerosis have been inconsistent. In a meta-analysis, we recently showed that this relationship is stronger in high-risk populations. In this paper we used a combined functional and population-based approach to investigate a gene-environment interaction of the ACE I/D polymorphism in relation to carotid artery wall thickness.

Methods: The study was embedded in the Rotterdam Study, a prospective population-based cohort study. In 5321 subjects, IMT was measured in the carotid arteries by ultrasonography and ACE genotype was determined by size-analysis of polymerase chain reaction products.

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

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

Introduction

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

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

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

Methods

Study population

This study is embedded in the Rotterdam Study, an ongoing population-based follow-up study. The study is designed to investigate the determinants of chronic diseases in the

elderly and has been described in more detail elsewhere.[13] In brief, baseline data were collected between March 1990 and July 1993 on 7983 subjects, aged 55 years or older, living in Rotterdam, the Netherlands.[13] The study was approved by the Medical Ethics Committee of Erasmus Medical Center Rotterdam, and written informed consent was obtained from all participants. All participants were interviewed at home by a trained research assistant using computerized questionnaires, and they subsequently visited the study center. Smoking history was assessed during the interview at home and was categorized as never, former, or current smoker. At the study center an extensive physical examination was performed, including ultrasonography of carotid arteries. Blood sample was drawn and serum and plasma were stored at -80°C.

Carotid ultrasonography

Carotid atherosclerosis was assessed by carotid duplex scan ultrasonographic investigation of the carotid arteries, by means of a 7.5 MHz linear array transducer (ATL, Ultramark IV). Measurements of intima media thickness were performed offline from the frozen images recorded on videotape. Details about this measurement have been published previously.[14] Briefly, the interfaces of the far and near wall of the distal common carotid artery are marked over a length of 10 mm. We used the average of the measurements of 3 frozen images of both the left and right arteries. Carotid IMT was determined as the mean of the maximum IMT of near and far wall measurements of both the left and right arteries. Results from a reproducibility study of IMT measurements have been published elsewhere.[15] In short, mean differences (SD) in common carotid IMT between paired measurements of sonographers, readers and visits were 0.005 mm (0.09), 0.060 mm (0.05) and 0.033 mm (0.12), respectively. We defined plaques as focal widening of the vessel wall with protrusion into the lumen, composed of calcified or non-calcified components. The protrusion was evaluated by eye, without measuring the thickness of the lesions or of the adjacent structure. The total plaque score reflected the total number of sites with plaques and ranged from 0 to 6 (left- and right-sided common carotid arteries, bifurcation, and internal carotid arteries).

Laboratory assessments

Colorimetric determination of ACE activity was performed in the stored plasma samples (-80°C). Because of cost considerations, ACE levels were assessed in a random group of 215 individuals. The measurements were carried out with a kit by Fujirebio Inc, which uses a *p*-hydroxy-Hip-His-Leu substrate.[16] Fluorimetric assay of ACE activity in plasma was performed by measuring the release of His-Leu from the substrates Hip-His-Leu and Z-Phe-His-Leu.[17,18] DNA was isolated from the blood samples using standard procedures (salting out method).[19] The II, ID and DD genotypes were detected by PCR according to the method of Lindpaintner et al[20] with some modifications. The insertion and deletion alleles of the ACE gene were identified by using a set of oligonucleotide primers flanking

the polymorphic site in intron 16. The final volume of the PCR mix was 20 ml containing 50 ng DNA as template and 1 x PCR buffer (Gibco), 1.3 mM MgCl2, 200 mmol dNTPS, 20 pmol primer mix and 0.35 units *Taq* polymerase in a PE9600 PCR machine. The thermocycling procedure was completely identical to the method of Lindpaintner et al[20]. The result of amplification were 319-bp and 597-bp amplicons for the D and I alleles respectively. Because the D allele in heterozygous samples is preferentially amplified, there is a tendency of misclassification for about 4 – 5 % of ID genotypes to DD. In order to avoid this misclassification, a second independent PCR has been performed with a primer pair that recognizes an insertion specific sequence. To optimize the second PCR 10% DMSO, 0.35 units Ampli*Taq* Gold DNA polymerase and 1 x GeneAmp PCR Gold buffer (Applied Biosystems) was added to the PCR mix with annealing temperature of 67°C. The reaction yielded a 335-bp amplicon only if the I allele was present. All reactions were performed in 96-well plates and handled by a robot (Beckman Biomek® 2000). In the post-PCR analyses, 10 ml of PCR product was loaded on 3% agarose gel. Fragments were visualized using ethidium bromide staining and UV transillumination. Two independent investigators read pictures from each gel and all ambiguous samples were analyzed a second time.

Statistical analyses

Hardy-Weinberg equilibrium was tested with a χ^2-test. We analyzed the distribution of conventional cardiovascular risk factors among three genotype groups using χ^2-statistics for dichotomous variables and ANOVA analyses for continuous variables. Multiple linear regression analysis was used to analyze the relation between vascular risk factors and ACE activity. The differences of ACE activity and IMT between the smoking and genotype groups were tested using a general linear model univariate procedure, adjusted for age and gender. In addition, subjects were divided into two groups based on the number of plaques in the carotid arteries (0-2 plaques and 3 or more plaques). Logistic regression was used to estimate the adjusted odds ratios for the different genotype groups using subjects with the II genotype as reference. No adjustments were initially made for vascular risk factors such as hypertension, since they may act as intermediate factors. Interaction of the ACE genotype with smoking was tested using a multiplicative model. All statistical analyses were conducted using SPSS for windows version 11.0.

Results

We performed our analysis on 5321 subjects for whom the complete data of ACE genotyping, IMT measurements and smoking status were available. Data for at least one of the variables was missing in the remaining subjects due to logistic reasons; mainly because the subjects were too old or disabled to visit the research center for the examination. In our sample, the frequency of the D allele was 53.3% and the distribution of the genoty-

Table 1. Demographic characteristics of subjects stratified by ACE I/D Genotype

	ACE genotype		
	II (n = 1156)	ID (n = 2657)	DD (n = 1508)
Sex - % male	41.70	40.80	39.66
Age – yrs	68.03 ± 8.53	69.08 ± 8.67†	69.25 ± 8.89†
Body mass index – kg/m^2	26.39 ± 3.82	26.22 ± 3.65	26.31 ± 3.63
Current smokers - %	22.88	23.60	20.82
Total cholesterol - mmol/l	6.62 ± 1.28	6.66 ± 1.17	6.61 ± 1.21
HDL cholesterol - mmol/l	1.34 ± 0.35	1.35 ± 0.35	1.35 ± 0.37
SBP – mmHg	137.67 ± 21.61	139.09 ± 22.66	140.01 ± 22.84†
DBP – mmHg	73.48 ± 11.64	73.53 ± 11.63	73.73 ± 11.29
Hypertension* - %	31.78	36.03†	35.05
Common carotid IMT - $mm10^{-1}$	7.88 ± 1.53	7.99 ± 1.59	8.03 ± 1.59†
No. of carotid plaques >= 3 - %	20.90	23.58	23.75

*Values are presented as percentage or mean ± standard deviation. ACE, angiotensin-converting enzyme; I/D, insertion/deletion; HDL, high density lipoprotein; BP, blood pressure; IMT, intima media thickness. *Hypertension defined as SBP >= 160 mmHg or DBP >=100 mmHg or medication use. †p<0.05 compared to II genotype.*

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

Variable	ß	SE	p-value
ACE I/D polymorphism*	2.71	0.35	<0.01
Current smoking	1.92	0.61	<0.01
Male sex	0.63	0.54	0.24
Age	-0.07	0.06	0.20
Body mass index	0.07	0.08	0.37
Total cholesterol	-0.19	0.23	0.42
HDL cholesterol	0.95	0.76	0.22
SBP	-0.01	0.02	0.84
DBP	-0.02	0.04	0.56

ß: *regression coefficient; SE: standard error of the coefficient.*
**Number of the D alleles used in the equation (II = 0, ID = 1 and DD = 2).*

pes and allele frequencies were in Hardy-Weinberg equilibrium (II, 21.7%; ID, 49.9%; and DD, 28.3%; p-value = 0.82). Among the remaining subjects for whom the genotype frequencies were available (n = 1548), no deviation from Hardy-Weinberg equilibrium was observed. Furthermore, there was no significant difference in the genotype frequencies between the two samples (p-value= 0.44).

Table 1 shows the demographic characteristics of the participants stratified by ACE genotype. No significant differences were found among the 3 genotypes with respect to classical cardiovascular risk factors. However, the carriers of the D allele were significantly older than the II carriers but the difference was only 1.22 years. Furthermore, carriers of the DD genotype had a significantly higher mean systolic blood pressure as well as a higher mean of carotid IMT compared to the II genotype. Although there was a slight increase in the percentage of subjects with three or more plaques with the number of D alleles present, the frequency did not differ significantly between carriers of the D allele and carriers of the II genotype (Table 1).

In the sample of 212 individuals for whom both plasma ACE activity and I/D genotypes were measured, the allele and genotype proportions were consistent with Hardy-Weinberg equilibrium (p-value = 0.48). In multiple linear regression analysis, ACE I/D polymorphism and smoking were the only determinants of plasma ACE activity (Table 2). These two factors together explained 28% of the variation of the enzyme levels in serum ($r = 0.53$, $p<0.01$). The activity of ACE was not significantly different between former smokers and non-smokers (14.83 $\pm$ 3.57 and 15.58 $\pm$ 3.87 U/l, respectively); therefore we combined these two groups. Overall, the ACE activity was 1.8 U/l higher in current smokers compared to non- and former smokers ($p<0.01$). Although the difference in ACE activity between smokers and non- and former smokers was largest in the ID and DD group, it was not significantly different from the difference in the II group (Figure 1).

When studying the joint effects of smoking and the ACE I/D polymorphsim on IMT, there was a significant increase in mean carotid IMT in the number of D alleles present in current smokers (p for trend = 0.04). The difference in IMT (mean $\pm$ SE) between DD and II genotypes in smokers was $0.26 \pm 0.12\ 10^{-1}$ mm, (p-value=0.03). In contrast, IMT was not significantly associated with the D allele among non- and former smokers (Figure 2). The p-value for interaction between ACE genotypes and smoking status was 0.08. The differences in IMT between the ACE genotype groups in smokers reduced but did not disappear when adjusted for systolic blood pressure. Subjects carrying only one of the risk factors (smoking or the D allele) did not show significant differences in IMT compared to the non- and former smokers group carrying the II genotype, while carriers of both risk factors had significant higher IMT ($p<0.01$).

The odds ratios of having three or more plaques in carotid arteries in current smokers were 1.14 (95% CI: 0.81-1.59) and 1.40 (95% CI: 0.96-2.04) for ID and DD genotypes res-

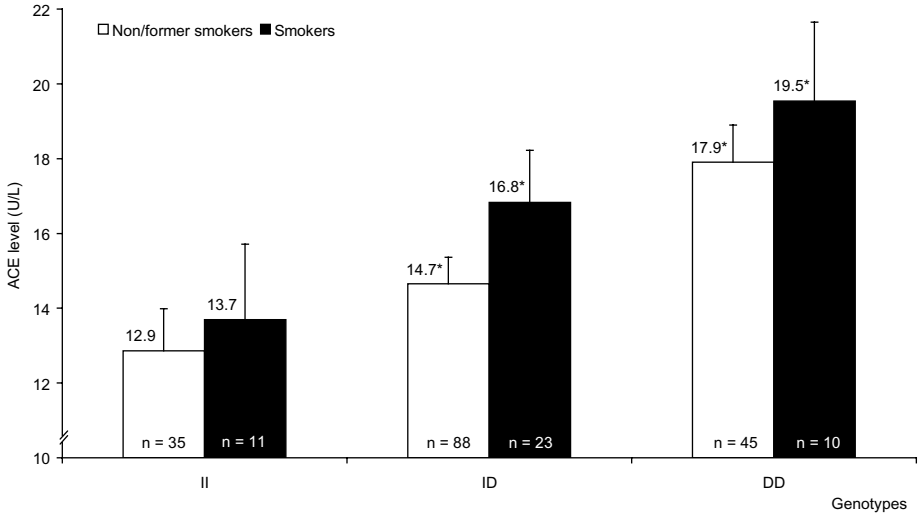


Figure 1. Plasma ACE activity in different genotypes groups, stratified by smoking status. Data are adjusted for age and gender, *Significantly different from non- and former smokers in II genotype group.

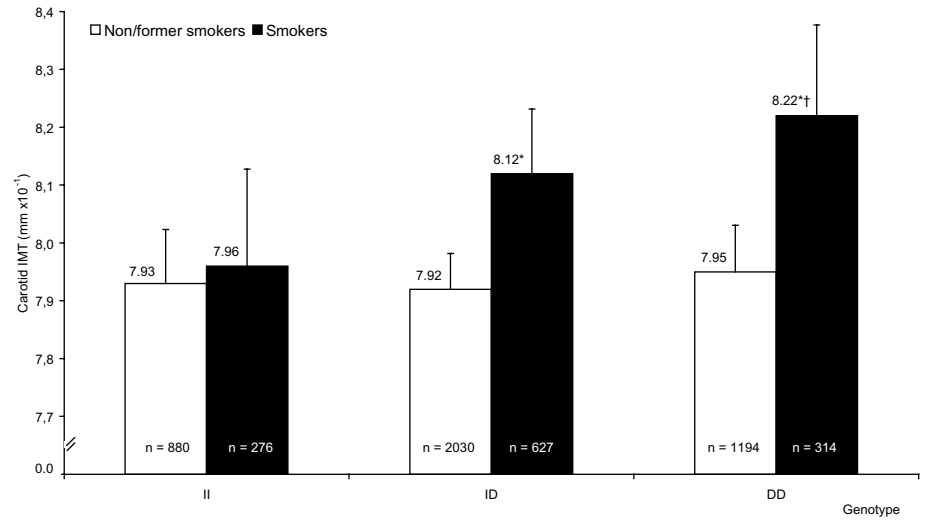


Figure 2. Carotid intima media thickness (IMT) in different genotype groups stratified by smoking status. Data are adjusted for age and gender. *Significantly different from non- and former smokers in II genotype group. †Significantly different from smokers in II genotype group.

pectively. This increase of odds ratios with the number of the D alleles was borderline significant (p=0.07) while in non- and former smokers the odds ratios remained the same between genotype groups.

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

Discussion

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

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

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

To unravel the role of interactions of the ACE I/D polymorphism with other vascular

risk factors, we focused on identifying determinants in the renin angiotensin system, more specifically those associated with serum ACE activity. In our population-based study, we found that besides the ACE I/D polymorphism, smoking is the only other factor related to plasma ACE activity, suggesting they use the same pathways. The vascular risk factors that might be related to serum ACE activity are not well known. In one report smoking and blood pressure[30], and in another male sex and history of hypertension[31] were correlated with serum ACE activity. In our sample, the I/D polymorphism and smoking together explained 28% of the variance in ACE activity. On average, current smokers had 1.8 U/L higher ACE activity in plasma. The effect of smoking on ACE activity was larger in the ID and DD genotype groups than in the II genotype group, although the differences were not significant. A possible effect of smoking on cleavage secretion of ACE from the endothelial cells may explain this finding.[32] Additionally, there are indications that nicotine increases expression of a number of genes in endothelium, including ACE.[33]

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

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

In summary, we found a positive association between the D allele of the ACE I/D polymorphism and carotid IMT in the presence of smoking. This association provides further evidence that genetic and environmental factors interact in the formation of arterial lesions. There may be various pathways underlying the observation of an effect of the

ACE gene on IMT in smokers only, but on the basis of the present results it is not possible to fully explain the underlying mechanism. Our findings remain to be confirmed in future studies.

References

1. Erdos EG, Skidgel RA. The angiotensin I-converting enzyme. *Lab Invest.* 1987;56:345-8.
2. Rigat B, Hubert C, Alhenc-Gelas F, Cambien F, Corvol P, Soubrier F. An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. *J Clin Invest.* 1990;86:1343-6.
3. Tiret L, Rigat B, Visvikis S, Breda C, Corvol P, Cambien F, Soubrier F. Evidence, from combined segregation and linkage analysis, that a variant of the angiotensin I-converting enzyme (ACE) gene controls plasma ACE levels. *Am J Hum Genet.* 1992;51:197-205.
4. Danser AH, Schalekamp MA, Bax WA, van den Brink AM, Saxena PR, Riegger GA, Schunkert H. Angiotensin-converting enzyme in the human heart. Effect of the deletion/insertion polymorphism. *Circulation.* 1995;92:1387-8.
5. Castellano M, Muiesan ML, Rizzoni D, Beschi M, Pasini G, Cinelli A, Salvetti M, Porteri E, Bettoni G, Kreutz R, Lindpaintner K, Rosei EA. Angiotensin-converting enzyme I/D polymorphism and arterial wall thickness in a general population. The Vobarno Study. *Circulation.* 1995;91:2721-4.
6. Nergizoglu G, Keven K, Gurses MA, Aras O, Erturk S, Duman N, Ates K, Akar H, Akar N, Karatan O, Erbay B, Ertug AE. Carotid intima-media thickness and ACE-gene polymorphism in hemodialysis patients. *J Nephrol.* 1999;12:261-5.
7. Pujia A, Motti C, Irace C, Cortese C, Biagiotti L, Mattioli PL, Federici G, Gnasso A. Deletion polymorphism in angiotensin converting enzyme gene associated with carotid wall thickening in a healthy male population. *Coron Artery Dis.* 1996;7:51-5.
8. Hosoi M, Nishizawa Y, Kogawa K, Kawagishi T, Konishi T, Maekawa K, Emoto M, Fukumoto S, Shioi A, Shoji T, Inaba M, Okuno Y, Morii H. Angiotensin-converting enzyme gene polymorphism is associated with carotid arterial wall thickness in non-insulin-dependent diabetic patients. *Circulation.* 1996;94:704-7.
9. Diamantopoulos EJ, Andreadis E, Kakou M, Vlachonikolis I,

Vassilopoulos C, Giannakopoulos N, Tarassi K, Papasteriades C, Nicolaides A, Raptis S. Atherosclerosis of carotid arteries and the ace insertion/deletion polymorphism in subjects with diabetes mellitus type 2. *Int Angiol.* 2002;21:63-9.

10. Dessi-Fulgheri P, Catalini R, Sarzani R, Sturbini S, Siragusa N, Guazzarotti F, Offidani M, Tamburrini P, Zingaretti O, Rappelli A. Angiotensin converting enzyme gene polymorphism and carotid atherosclerosis in a low-risk population. *J Hypertens.* 1995;13:1593-6.
11. Lohmueller KE, Pearce CL, Pike M, Lander ES, Hirschhorn JN. Meta-analysis of genetic association studies supports a contribution of common variants to susceptibility to common disease. *Nat Genet.* 2003;33:177-82.
12. Sayed-Tabatabaei FA, Houwing-Duistermaat JJ, van Duijn CM, Witteman JC. Angiotensin-converting enzyme gene polymorphism and carotid artery wall thickness: a meta-analysis. *Stroke.* 2003;34:1634-9.
13. Hofman A, Grobbee DE, de Jong PT, van den Ouweland FA. Determinants of disease and disability in the elderly: the Rotterdam Elderly Study. *Eur J Epidemiol.* 1991;7:403-22.
14. Bots ML, Hofman A, De Jong PT, Grobbee DE. Common carotid intima-media thickness as an indicator of atherosclerosis at other sites of the carotid artery. The Rotterdam Study. *Ann Epidemiol.* 1996;6:147-53.
15. Bots ML, Mulder PG, Hofman A, van Es GA, Grobbee DE. Reproducibility of carotid vessel wall thickness measurements. The Rotterdam Study. *J Clin Epidemiol.* 1994;47:921-30.
16. Kasahara Y, Ashihara Y. Colorimetry of angiotensin-I converting enzyme activity in serum. *Clin Chem.* 1981;27:1922-5.
17. Friedland J, Silverstein E. A sensitive fluorimetric assay for serum angiotensin-converting enzyme. *Am J Clin Pathol.* 1976;66:416-24.
18. Piquilloud Y, Reinharz A, Roth M. Studies on the angiotensin conver ting enzyme with different substrates. *Biochim Biophys Acta.* 1970;206:136-42.
19. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* 1988;16:1215.
20. Lindpaintner K, Pfeffer MA, Kreutz R, Stampfer MJ, Grodstein F,

LaMotte F, Buring J, Hennekens CH. A prospective evaluation of an angiotensin-converting-enzyme gene polymorphism and the risk of ischemic heart disease [see comments].
N Engl J Med. 1995;332:706-11.

21. Mannami T, Katsuya T, Baba S, Inamoto N, Ishikawa K, Higaki J, Ogihara T, Ogata J. Low potentiality of angiotensin-converting enzyme gene insertion/deletion polymorphism as a useful predictive marker for carotid atherogenesis in a large general population of a Japanese city: the Suita study. *Stroke.* 2001;32:1250-6.
22. Hibi K, Ishigami T, Kimura K, Nakao M, Iwamoto T, Tamura K, Nemoto T, Shimizu T, Mochida Y, Ochiai H, Umemura S, Ishii M. Angiotensin-converting enzyme gene polymorphism adds risk for the severity of coronary atherosclerosis in smokers. *Hypertension.* 1997;30:574-9.
23. Kauma H, Paivansalo M, Savolainen MJ, Rantala AO, Kiema TR, Lilja M, Reunanen A, Kesaniemi YA. Association between angiotensin converting enzyme gene polymorphism and carotid atherosclerosis.
J Hypertens. 1996;14:1183-7.
24. Sass C, Zannad F, Herbeth B, Salah D, Chapet O, Siest G, Visvikis S. Apolipoprotein E4, lipoprotein lipase C447 and angiotensin-I converting enzyme deletion alleles were not associated with increased wall thickness of carotid and femoral arteries in healthy subjects from the Stanislas cohort. *Atherosclerosis.* 1998;140:89-95.
25. Zhu X, McKenzie CA, Forrester T, Nickerson DA, Broeckel U, Schunkert H, Doering A, Jacob HJ, Cooper RS, Rieder MJ. Localization of a small genomic region associated with elevated ACE.
Am J Hum Genet. 2000;67:1144-53.
26. Soubrier F, Martin S, Alonso A, Visvikis S, Tiret L, Matsuda F, Lathrop GM, Farrall M. High-resolution genetic mapping of the ACE-linked QTL influencing circulating ACE activity.
Eur J Hum Genet. 2002;10:553-61.
27. Rieder MJ, Taylor SL, Clark AG, Nickerson DA. Sequence variation in the human angiotensin converting enzyme.
Nat Genet. 1999;22:59-62.
28. Keavney B, McKenzie CA, Connell JM, Julier C, Ratcliffe PJ, Sobel E, Lathrop M, Farrall M. Measured haplotype analysis of the angiotensin-I converting enzyme gene. *Hum Mol Genet.* 1998;7:1745-51.
29. Watanabe Y, Ishigami T, Kawano Y, Umahara T, Nakamori A, Mizushima S, Hibi K, Kobayashi I, Tamura K, Ochiai H, Umemura S,

Ishii M. Angiotensin-converting enzyme gene I/D polymorphism and carotid plaques in Japanese. *Hypertension.* 1997;30:569-73.

30. Ucar G, Yildirim Z, Ataol E, Erdogan Y, Biber C. Serum angiotensin converting enzyme activity in pulmonary diseases: correlation with lung function parameters. *Life Sci.* 1997;61:1075-82.
31. Hung J, McQuillan BM, Nidorf M, Thompson PL, Beilby JP. Angiotensin-converting enzyme gene polymorphism and carotid wall thickening in a community population. *Arterioscler Thromb Vasc Biol.* 1999;19:1969-74.
32. Ramchandran R, Sen GC, Misono K, Sen I. Regulated cleavage-secretion of the membrane-bound angiotensin-converting enzyme. *J Biol Chem.* 1994;269:2125-30.
33. Zhang S, Day I, Ye S. Nicotine induced changes in gene expression by human coronary artery endothelial cells. *Atherosclerosis.* 2001;154:277-83.
34. Tabara Y, Kohara K, Nakura J, Miki T. Risk factor-gene interaction in carotid atherosclerosis: effect of gene polymorphisms of renin-angiotensin system. *J Hum Genet.* 2001;46:278-84.
35. Butler R, Morris AD, Burchell B, Struthers AD. DD angiotensin-converting enzyme gene polymorphism is associated with endothelial dysfunction in normal humans. *Hypertension.* 1999;33:1164-8.
36. Jeerooburkhan N, Jones LC, Bujac S, Cooper JA, Miller GJ, Vallance P, Humphries SE, Hingorani AD. Genetic and environmental determinants of plasma nitrogen oxides and risk of ischemic heart disease. *Hypertension.* 2001;38:1054-61.
37. Celermajer DS, Sorensen KE, Georgakopoulos D, Bull C, Thomas O, Robinson J, Deanfield JE. Cigarette smoking is associated with dose-related and potentially reversible impairment of endothelium-dependent dilation in healthy young adults. *Circulation.* 1993;88:2149-55.
38. Barua RS, Ambrose JA, Eales-Reynolds LJ, DeVoe MC, Zervas JG, Saha DC. Dysfunctional endothelial nitric oxide biosynthesis in healthy smokers with impaired endothelium-dependent vasodilatation. *Circulation.* 2001;104:1905-10.
39. Tsuchiya M, Asada A, Kasahara E, Sato EF, Shindo M, Inoue M. Smoking a single cigarette rapidly reduces combined concentrations of nitrate and nitrite and concentrations of antioxidants in plasma. *Circulation.* 2002;105:1155-7.

40. Clara JG, Coelho C, Breitenfeld L, Siqueira C, Bicho M, de Padua F. [Acute effects of tobacco and vascular risk modulated by genetic factors]. *Rev Port Cardiol.* 2000;19:1279-83.

2.3. ANGIOTENSIN CONVERTING ENZYME INSERTION/DELETION POLYMORPHISM AND RISK OF HEART FAILURE IN HYPERTENSIVE SUBJECTS

Aims: Cardiac angiotensin-I converting enzyme (ACE) activity is influenced by the ACE I/D polymorphism. Evidence suggests that the DD-genotype may be a risk factor for cardiac hypertrophy and heart failure, especially in hypertensive subjects. We assessed the relation between the ACE I/D polymorphism and the risk of incident heart failure in normotensive and hypertensive subjects.

Methods and Results: We investigated 4264 normotensive and 2174 hypertensive participants of the Rotterdam Study; a population based prospective cohort study. All subjects were available for follow-up from 1990 until 2000. Incidence rates (IR) of heart failure in normotensive subjects were the same over all genotype strata (10 per 1000 personyears). In hypertensive subjects, the IR increased with the number of D-alleles present (II: IR=13, ID: IR=18 and DD: IR=20 per 1000 personyears). Hypertensive subjects carrying the II-genotype did not have an increased risk of heart failure compared to normotensive II subjects. However, hypertensive subjects carrying one or two copies of the D-allele did have a significantly increased risk of heart failure (ID: RR: 1.4 (1.1-1.9) and DD: RR: 1.5 (1.2-2.1)).

Conclusion: Our findings suggest that the ACE I/D polymorphism may play a modifying role in the development of heart failure in hypertensive subjects.

Introduction

Heart failure is a complex clinical syndrome that can result from any structural or functional cardiac disorder that impairs adequate ventricular filling or ejecting of blood. Coronary artery disease and hypertension are among the most common risk factors. Regardless of the initial cause of cardiac stress, the heart will respond with a set of adaptive mechanisms in order to maintain its pumping function. Both clinical and experimental data suggest that activation of local renin angiotensin system (RAS) in the heart plays an important role in this compensatory mechanism to maintain adequate haemodynamic function.[1] Recent studies have shown that cardiac expression of angiotensin-I converting enzyme (ACE) and angiotensinogen is increased in experimental heart failure.[2,3] In patients with chronic heart failure, cardiac expression of ACE was found to be increased up to threefold compared to the hearts of subjects without heart failure.[4]

An Insertion/Deletion (I/D) polymorphism, characterized by the presence or absence of a 287-base pair alu repeat sequence in intron 16 of the ACE gene, has been reported to be responsible for about 50% of the interindividual variability in serum ACE levels.[5,6] Both serum ACE levels and cardiac ACE activity were highest in subjects carrying two copies of the D-allele.[5-7] The DD-genotype has been put forward as a risk factor for left ventricular remodeling in hypertensive subjects.[8,9] Raynolds et al. observed an increased frequency of the DD-genotype in patients with both ischaemic and idiopathic dilated cardiomyopathy.[10]

We examined the role of the ACE I/D polymorphism in the development of heart failure in a population-based cohort study. Since several studies reported an effect of the D-allele on cardiac disease in hypertensive subjects only, we analyzed normotensive and hypertensive subjects separately.

Methods

Study Population

The study was conducted within the Rotterdam Study, a single-center prospective follow-up study in which all residents aged 55 years and over of the Rotterdam suburb of Ommoord were invited to take part. The baseline examination of the Rotterdam Study was conducted between 1990 and 1993. The Medical Ethics Committee of Erasmus Medical Center Rotterdam approved the study. Written informed consent was obtained from all participants. The design of the study has been described previously.[11] 7983 participants were examined (response 78%). In 6869 subjects, the ACE I/D polymorphism was genotyped successfully (86%). In the remaining 1114 subjects, no genotypes were available. We excluded 211 subjects because no information on blood pressure levels was available.

At baseline, information concerning medical history, medication use and smoking behavior was obtained with a computerized questionnaire.[11] Blood pressure was measured twice, after a minimum of 5 minutes rest, in the sitting position at the right upper arm using a random zero sphygmomanometer. Participants were asked to abstain from smoking and drinking alcoholic or caffeine-containing beverages at least two hours before blood pressure measurements were taken. The average of two measurements was used for analysis. Hypertension was defined as a diastolic blood pressure (DBP) of 100 mmHg or higher and/or a systolic blood pressure (SBP) of 160 mmHg or higher and/or use of anti-hypertensive medication indicated for treatment of hypertension (grade 2 and 3 of the 1999 WHO/ISH criteria and 2003 ESH/ESC criteria).[12,13]

Heart failure assessment

Assessment of prevalent heart failure at the baseline examination in the Rotterdam Study has been described in detail earlier.[14] We excluded subjects with prevalent heart failure from our study (n=220). All participants of the Rotterdam Study were continuously monitored for the occurrence of heart failure during follow-up from 1990 until 2000, using automated linkage with files from general practitioners. All available medical data, such as hospital discharge letters and notes from general practitioners, were obtained from the medical records in case of possible heart failure. Apart from this systematic follow-up procedure, we used verified hospital discharge diagnoses for case finding, gathered from all hospitals in the Rotterdam area as described above.

The diagnosis of heart failure was classified as definite, probable, possible or unlikely. Definite heart failure was defined as a combination of heart failure diagnosed by a medical specialist and the presence of typical symptoms of heart failure, such as breathlessness at rest or during exertion, ankle edema and pulmonary crepitations, confirmed by objective evidence of cardiac dysfunction (chest X-ray, echocardiography). This definition is in accordance with the criteria of the European Society of Cardiology.[15] Probable heart failure was defined as heart failure diagnosed by a general practitioner, with at least two typical symptoms suggestive of heart failure, and at least 1 of the following: history of cardiovascular disease (e.g. myocardial infarction, hypertension), response to treatment for heart failure, or objective evidence of cardiac dysfunction, while symptoms could not be attributed to another underlying disease.

Two research physicians independently classified all information on potential heart failure events. If there was disagreement, a consensus was reached in a separate session. Finally, a cardiologist verified all probable and possible cases, and all cases in which the two physicians could not reach consensus. If the cardiologist disagreed with the research physicians, the cardiologist's judgment was considered decisive. The research physicians and the cardiologist based their decisions on the same data. Only definite and probable cases were included in the analyses.

After heart failure cases were diagnosed as definite or probable, the date of incident heart failure was defined as the day of the first occurrence of symptoms suggestive of heart failure or the date of the first prescription of a loop diuretic or an ACE-inhibitor.

Genotyping

The II, ID and DD genotypes were detected using the polymerase chain reaction technique (PCR) according to the method of Lindpaintner et al with some modifications.[16] In order to avoid misclassification of ID genotypes into DD genotypes, a second PCR was performed using an I-specific primer.

Statistical Analysis

Overall general characteristics of normotensive and hypertensive subjects and those stratified by ACE genotype, were compared using univariate analysis of variance for continuous variables and chi-square statistics for dichotomous variables. Differences in median follow-up between the normotensive and hypertensive group, and between genotypes within the normotensive and hypertensive group were tested using a Mann-Whitney non-parametric test for independent samples. Incidence rates (IR) were expressed as number of cases per 1000 personyears and presented with 95% confidence intervals (CI), based on the assumed Poisson distribution for the observed number of cases. We constructed a numerical variable for the ACE-genotype groups (1-2-3; II-ID-DD) and performed a linear regression in order to test for trend of IR in the normotensive and hypertensive groups. Relative risks of incident heart failure were assessed using Cox proportional hazard regression analysis. Proportionality of hazards was assessed and satisfied by means of a log-minus-log plot. All risk estimates are presented with 95% confidence intervals (CI). We adjusted for age and sex in all analyses. To assess the effect of the ACE I/D polymorphism independent of possible confounding or mediating factors, analyses were repeated adding body mass index (BMI), diabetes mellitus, smoking, myocardial infarction, total and HDL-cholesterol to the model. We tested for statistical interaction between the ACE I/D polymorphism and hypertension by adding an interaction term to the regression model: hypertension (dichotomous) x ACE-genotype (categorical). All presented p-values are two-sided. We performed all analyses with SPSS version 11.0.

Results

A total of 6438 subjects were available for follow-up until January 1, 2000. Baseline descriptives of the total study population are presented in table 1. We included 4264 normotensive subjects and 2174 hypertensive subjects in our study. Both groups followed Hardy-Weinberg Equilibrium proportions for the ACE I/D polymorphism. Median follow-up was 7.2 (6.7;8.1) years for normotensive subjects and 7.0 (5.3;8.0) years for hypertensive subjects. In hypertensive subjects, median follow-up was significantly shorter for subjects carrying two copies of the D-allele than for subjects carrying two copies of the

Table 1. Baseline descriptives normotensive and hypertensive subjects: overall and stratified by ACE genotype

	Normotensive subjects				Hypertensive subjects			
ACE genotype	Overall	II	ID	DD	Overall	II	ID	DD
Number - n (%)	4264 (66.0)	962 (22.6)	2106 (49.4)	1196 (28.0)	2174 (34.0)	441(20.3)	1116 (51.3)	617 (28.4)
Median follow-up (yrs)	7.2 (6.7;8.1)	7.2 (6.7;8.1)	7.2 (6.7;8.2)	7.2 (6.7;8.1)	7.0 (5.3;8.0)*	7.1 (5.7;8.2)†	7.1 (5.6;8.0)	7.0 (4.8;7.9)
Age - yrs	68.0 ± 8.9	67.6 ± 8.8	68.1 ± 8.8	68.4 ± 9.2	71.0 ± 8.8*	70.6 ± 9.0	71.1 ± 8.6	71.2 ± 9.1
Sex - % male	42.9	43.2	43.0	42.4	35.3*	34.9	36.1	34.0
SBP - mmHg	130.7 ± 16.1	130.6 ± 16.3	130.2 ± 16.4	131.4 ± 15.8	156.8 ± 22.4*	156.3 ± 22.7	156.5 ± 22.2	157.6 ± 22.5
DBP - mmHg	70.9 ± 10.0	70.8 ± 10.1	70.6 ± 9.9	71.4 ± 9.9**	80.0 ± 12.0*	80.4 ± 12.5	80.0 ± 12.0	79.4 ± 11.7
Diabetes mellitus - %	7.1	6.5	8.0	6.0	15.0*	15.9	15.8	13.0
Myocardial Infarction-%	11.3	10.2	11.8	11.3	15.6*	14.6	16.0	15.5
BMI - kg/m^2	25.8 ± 3.5	25.8 ± 3.7	25.7 ± 3.5	25.9 ± 3.5	27.2 ± 3.9*	27.5 ± 3.8†	27.2 ± 4.0	27.1 ± 3.7
Total cholesterol - mmol	6.6 ± 1.2	6.5 ± 1.3	6.6 ± 1.2	6.6 ± 1.2	6.7 ± 1.2*	6.8 ± 1.3	6.7 ± 1.3	6.7 ± 1.2
HDL-cholesterol - mmol	1.4 ± 0.4	1.4 ± 0.4	1.4 ± 0.4	1.4 ± 0.4	1.3 ± 0.4*	1.3 ± 0.3	1.3 ± 0.4	1.3 ± 0.4
Smoking (current) - %	24.7	25.9	25.5	22.4	18.4*	16.6	19.3	17.9

*Values are presented as percentage or mean ± standard deviation, except for follow up, which is presented as median (interquartile range). *Significantly different from normotensive subjects, p<0.005. **Significantly different from normotensive ID-subjects, p<0.05. †Significantly different from hypertensive DD-subjects, p<0.05.*

Table 2. Number of cases and incidence rates of heart failure stratified by ACE genotype in normotensive and hypertensive subjects.

	Normotension			Hypertension		
ACE genotype	Number of cases	Person years	IR (95%CI)	Number of cases	Person years	IR (95% CI)
II	67	6823.4	10 (8-12)	39	3020.2	13 (9-17)
ID	131	14970.7	9 (7-10)	138	7616.4	18 (15-21)
DD	88	8377.2	11 (8-13)	80	4075.2	20 (16-24)

Incidence rate (IR) presented as number of cases per 1000 personyears with 95% confidence interval.

Table 3. Risk of heart failure in normotensive and hypertensive subjects: overall and stratified by ACE genotype

	Model 1		Model 2	
	Normotension	Hypertension	Normotension	Hypertension
Risk overall:	1.0 (ref)	1.6 (1.4-1.9)†	1.0 (ref)	1.4 (1.2-1.7)†
Risk stratified by ACE genotype:				
II	1.0 (ref)	1.1 (0.8-1.7)	1.0 (ref)	1.2 (0.8-1.8)
ID	0.9 (0.6-1.2)	1.5 (1.1-2.0)**	1.0 (0.7-1.3)	1.4 (1.1-1.9)*
DD	1.0 (0.7-1.4)	1.6 (1.2-2.2)**	1.1 (0.8-1.6)	1.5 (1.2-2.1)*

*Model 1: adjusted for age and sex. Model 2: adjusted for age, sex, BMI, smoking, diabetes mellitus, myocardial infarction, total and HDL-cholesterol. Significantly different from overall normotensive group, †p<0.001. Significantly different from II normotensive group, *p<0.05, **p<0.01.*

I-allele. Hypertensive subjects were significantly older and less often male than normotensive subjects. This difference was the same over all genotype strata. Within the normotensive group, DBP was significantly higher in subjects carrying the DD-genotype compared to subjects carrying the ID-genotype. Prevalence of diabetes mellitus and myocardial infarction, mean BMI and total cholesterol levels were significantly higher in hypertensive subjects compared to normotensive subjects. In the hypertensive group, BMI was significantly higher in subjects carrying the II-genotype compared to subjects carrying the DD-genotype. HDL-cholesterol and percentage current smokers were significantly lower in hypertensive subjects than in normotensive subjects. This difference was the same over all genotype strata.

Table 2 shows number of cases, personyears and incidence rates (IR) of heart failure observed in normotensive and hypertensive subjects stratified by ACE genotype. During 44,883.1 personyears of follow-up 543 participants developed heart failure. In normotensive subjects, the IR of heart failure was about 10 per 1000 personyears, independent of genotype status. In hypertensive subjects, the IR of heart failure significantly

increased with the number of D-alleles present (p for trend<0.05). In subjects carrying the II-genotype the IR of heart failure was 13 per 1000 personyears (95%CI: 9;17). In subjects carrying one or two copies of the D-allele the IR of heart failure increased up to 18 (15;21) and 20 (16;24) per 1000 personyears, respectively.

In table 3, the relative risks (RR) of heart failure for hypertensive and normotensive subjects, overall and stratified by ACE genotype, are presented. Overall, hypertensive subjects had a significantly increased risk of 1.4 (1.2;1.7) of heart failure compared to normotensive subjects. In the normotensive group, the risk of heart failure did not differ between the different ACE genotype groups. Hypertensive subjects carrying two copies of the I-allele did not have an increased risk of heart failure compared to normotensive subjects carrying two copies of the I-allele (RR: 1.2 (0.8;1.8) (model 2). However, hypertensive subjects carrying one or two copies of the D-allele had a significantly increased risk of heart failure compared to normotensive II subjects (ID: RR: 1.4 (1.1;1.9) and DD: RR: 1.5 (1.2;2.1)) (model 2). Additional analyses including also mild hypertensive subjects in the hypertensive group (cut-of value for diagnosis hypertension: SBP ≥ 140 mmHg or DBP ≥ 90 mmHg) did not show a significant effect of the ACE-genotype on the risk of heart failure. Although the direction of the risk estimates was the same (RR: ID: 1.2 (0.8;1.8) and DD: 1.4 (0.8;2.5) (results not shown in table).

The statistical interaction term between the D-allele of the ACE-genotype and hypertension was borderline significant, (p=0.059).

Discussion

We observed an increased risk of heart failure in hypertensive subjects compared to normotensive subjects that was dependent on the presence of the D-allele of the ACE I/D polymorphism. Hypertensive subjects did not have a significantly increased risk of heart failure compared to normotensive subjects, unless they carried one or two copies of the D-allele. The incidence rate of heart failure in hypertensive subjects increased with the number of D-alleles present. As the incidence of heart failure marks the end of the follow-up period, this may also explain the shorter follow-up period observed in hypertensive DD subjects compared to II subjects.

Hypertension is the most common condition antedating heart failure in the general population.[17,18] Especially in the elderly, heart failure is often preceded by long standing high blood pressure and LVH.[19,20] However, the extent of cardiac remodeling does not always seem to correlate with the extent of cardiac damage. In fact, hypertension may lead to severe heart failure in one patient whereas hypertension may be without any perceivable effects on cardiac function in another patient. As a consequence, it has been hypothesized that genetic factors may modulate the manifestation or progression of cardiac remodelling.[21]

The ACE I/D polymorphism is by far the most frequently studied candidate gene in the development of left ventricular hypertrophy and heart failure. Homozygosity for the D-allele has been associated with higher prevalence of LVH and increased heart weight in (untreated) hypertensive subjects.[8, 9,22-24] Raynolds et al. were the first to report an association between the ACE I/D polymorphism and heart failure. They observed an increased frequency of the DD-genotype in subjects with ischaemic and dilated cardiomyopathy.[10] Since local formation of angiotensin II (ANG II) within the myocardium is thought to be involved in the cardiac remodeling process, elevated cardiac ANG II levels in subjects carrying the D-allele, may partly explain the association between the DD-genotype and various cardiac disorders.[7]

Although cardiac chymase is also an important ANG II generating enzyme in the heart, two studies observed that in failing hearts ACE enzyme gene expression was increased, whereas cardiac chymase enzyme gene expression was not.[4,33] This may suggest that in failing hearts ANGII formation is more dependent on ACE than on cardiac chymase and therefore can potentially be modified by the ACE I/D polymorphism.

Nevertheless, findings remain controversial and so far positive and negative results seem to outweigh eachother.[25-27] Many of the conflicting findings on the ACE I/D polymorphism and cardiac disease are most likely due to small sample sizes and large heterogeneity of the populations that were studied. Another reason for the inconsistent findings may be that the ACE I/D polymorphism by itself does not have enough biological significance to exert an effect on cardiac tissue, especially since the RAS is normally under strict negative feedback inhibition. This has led to the hypothesis that an effect of the ACE I/D polymorphism on cardiac function may only become clinically relevant under specific conditions in which the cardiac growth machinery is already activated.[28] In line with this hypothesis, Montgomery et al. observed increased left ventricular mass after rigorous exercise only in those participants who carried a copy of the D-allele.[29] Another study observed increased adverse cardiac remodeling in subjects with the ACE ID- and DD-genotype after they had experienced a myocardial infarction.[30]

We believe our findings provide additional evidence for a modifying effect of the ACE I/D polymorphism in the development of cardiac disease. In our study, the D-allele was associated with an increased risk of heart failure in hypertensive subjects only, which may suggest that the D-allele has an effect on the heart merely when local RAS is already activated because of increased haemodynamic load. Since asymptomatic cardiac remodeling usually precedes the development of clinically overt heart failure in hypertensive subjects, we believe our findings are in accordance with the observation that, especially in subjects with hypertension, the D-allele of the ACE I/D polymorphism is associated with increased levels of various echocardiography measures of cardiac hypertrophy.[8,9,31,32]

Until now, our study is the largest population based study that assessed the role of the ACE I/D polymorphism in heart failure in a relatively homogenous population, as 98 % of the participants in our study are Caucasians and they all live in the same area of Rotterdam. In contrast to case-control studies on heart failure that have been conducted so far, the prospective nature of our study makes our results less prone to survival bias. Still several issues need to be addressed. We observed a significantly increased risk of heart failure in moderate and severe hypertensive subjects only. Additional analyses including also mild hypertensive subjects did not show a significantly increased risk of heart failure for ID and DD-carriers, although the risk estimates were in the same direction as those for moderate to severe hypertensive subjects. We believe this implies a "threshold" effect of the ACE I/D polymorphism, as its detrimental effects on cardiac function only become present when the heart already is under severe cardiac stress due to substantially elevated blood pressure levels. Second, we did not account for lifestyle or dietary factors that may have influenced our genotype-phenotype relationship. Kuznetsova et al. recently observed that the relationship between left ventricular mass index and the ACE I/D polymorphism might be modulated by sodium intake.[34] Finally, we were not able to discern the different etiologies of heart failure (idiopathic, ischemic or other) in our study. However, we think that the ACE I/D polymorphism may be more important as a modulator in the way the myocardium responds to cardiac damage ("remodeling") than in the events leading to cardiac damage.

The ACE I/D polymorphism may increase the risk of heart failure through an effect on blood pressure or an increased risk of myocardial infarction; however, our results do not support this. On the contrary, our findings suggest that hypertension by itself is not a real strong predictor of heart failure, unless one or two copies of the D-allele are present. Furthermore, correction for baseline and incident MI in our analyses did not change the association between heart failure and the ACE I/D polymorphism. In addition, the prevalence of MI did not differ significantly between the genotype groups in hypertensive subjects.

In conclusion, our findings suggest that the ACE I/D polymorphism may play a modifying role in the development of heart failure in hypertensive subjects, regardless of the initial cause of cardiac damage. We believe these findings may provide an additional genetic clue as to whether some hypertensive subjects do develop cardiac hypertrophy resulting in heart failure, whereas others do not.

References

1. Dostal DE, Baker KM. The cardiac renin-angiotensin system: coceptual, or a regulator of cardiac function? *Circ Res.* 1999;85:643-50.
2. Finckh M, Hellmann W, Ganten D, Furtwangler A, Allgeier J, Boltz M,

Holtz J. Enhanced cardiac angiotensinogen gene expression and angiotensin converting enzyme activity in tachypacing-induced heart failure in rats. *Basic Res Cardiol.* 1991;86:303-16.

3. Hirsch AT, Talsness CE, Schunkert H, Paul M, Dzau VJ. Tissue-specific activation of cardiac angiotensin converting enzyme in experimental heart failure. *Circ Res.* 1991;69:475-82.
4. Studer R, Reinecke H, Muller B, Holtz J, Just H, Drexler H. Increased angiotensin-I converting enzyme gene expression in the failing human heart. Quantification by competitive RNA polymerase chain reaction. *J Clin Invest.* 1994;94:301-10.
5. Rigat B, Hubert C, Alhenc-Gelas F, Cambien F, Corvol P, Soubrier F. An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. *J Clin Invest.* 1990;86:1343-6.
6. Tiret L, Rigat B, Visvikis S, Breda C, Corvol P, Cambien F, Soubrier F. Evidence, from combined segregation and linkage analysis, that a variant of the angotensin I-converting enzyme (ACE) gene controls plasma ACE levels. *Am J Hum Genet.* 1992;51:197-205.
7. Danser AH, Schalekamp MA, Bax WA, van den Brink AM, Saxena PR, Riegger GA, Schunkert H. Angiotensin-converting enzyme in the human heart. Effect of the deletion/insertion polymorphism. *Circulation.* 1995;92:1387-8.
8. Gharavi AG, Lipkowitz MS, Diamond JA, Jhang JS, Phillips RA. Deletion polymorphism of the angiotensin-converting enzyme gene is independently associated with left ventricular mass and geometric remodeling in systemic hypertension. *Am J Cardiol.* 1996;77:1315-9.
9. Perticone F, Maio R, Cosco C, Ceravolo R, Iacopino S, Chello M, Mastroroberto P, Tramontano D, Mattioli PL. Hypertensive left ventricular remodeling and ACE-gene polymorphism. *Cardiovasc Res.* 1999;43:192-9.
10. Raynolds MV, Bristow MR, Bush EW, Abraham WT, Lowes BD, Zisman LS, Taft CS, Perryman MB. Angiotensin-converting enzyme DD genotype in patients with ischaemic or idiopathic dilated cardiomyopathy. *Lancet.* 1993;342:1073-5.
11. Hofman A, Grobbee DE, de Jong PT, van den Ouweland FA. Determinants of disease and disability in the elderly: the Rotterdam Elderly Study. *Eur J Epidemiol.* 1991;7:403-22.
12. 2003 European Society of Hypertension-European Society of

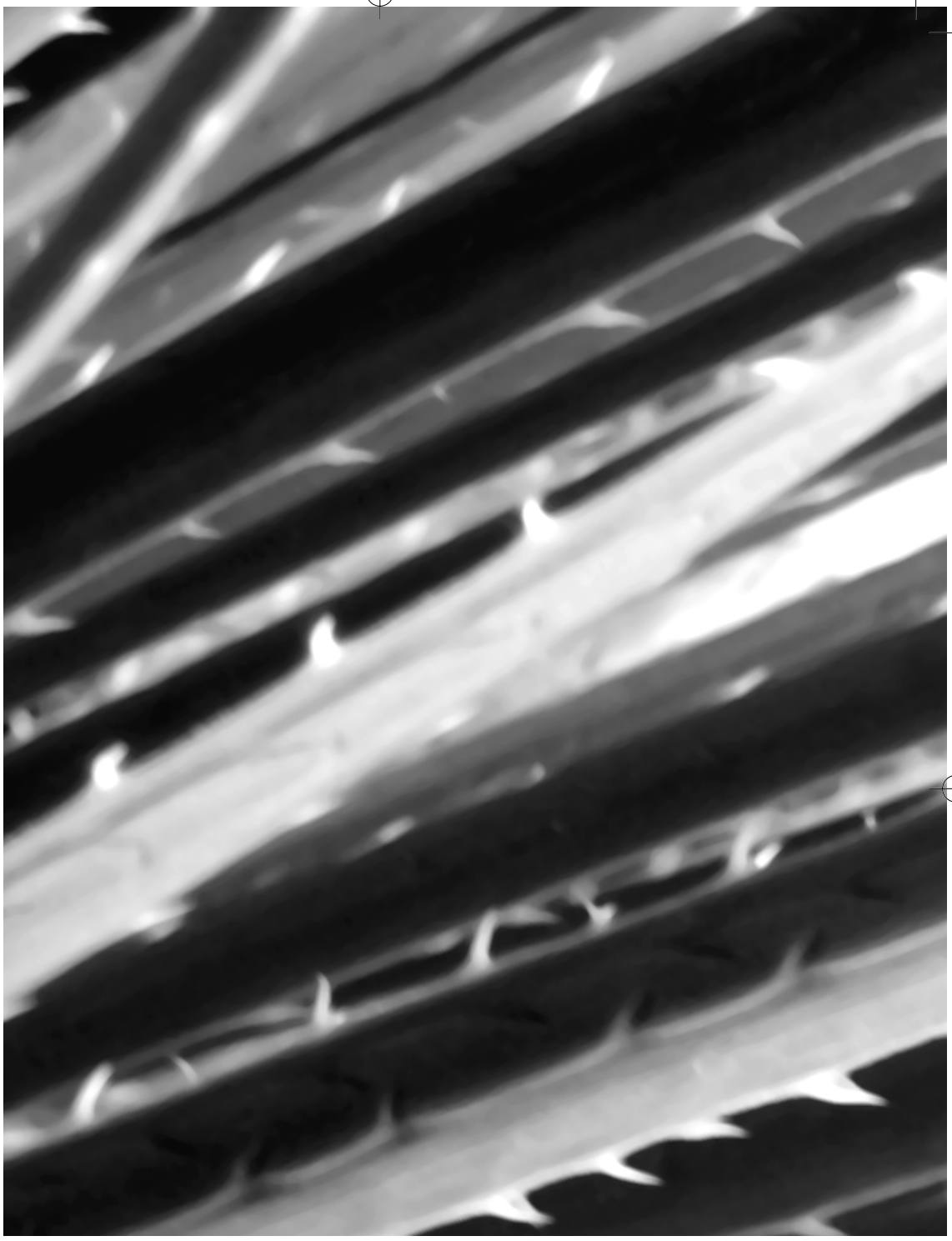
Cardiology guidelines for the management of arterial hypertension. *J Hypertens.* 2003;21:1011-53.
13. 1999 World Health Organization-International Society of Hypertension Guidelines for the Management of Hypertension. Guidelines Subcommittee. *J Hypertens.* 1999;17:151-83.
14. Mosterd A, Hoes AW, de Bruyne MC, Deckers JW, Linker DT, Hofman A, Grobbee DE. Prevalence of heart failure and left ventricular dysfunction in the general population; The Rotterdam Study. *Eur Heart J.* 1999;20:447-55.
15. Remme WJ, Swedberg K. Guidelines for the diagnosis and treatment of chronic heart failure. *Eur Heart J.* 2001;22:1527-60.
16. Lindpaintner K, Pfeffer MA, Kreutz R, Stampfer MJ, Grodstein F, LaMotte F, Buring J, Hennekens CH. A prospective evaluation of an angiotensin-converting-enzyme gene polymorphism and the risk of ischemic heart disease. *N Engl J Med.* 1995;332:706-11.
17. Levy D, Larson MG, Vasan RS, Kannel WB, Ho KK. The progression from hypertension to congestive heart failure. *JAMA.* 1996;275:1557-62.
18. Vasan RS, Levy D. The role of hypertension in the pathogenesis of heart failure. A clinical mechanistic overview. *Arch Intern Med.* 1996;156:1789-96.
19. Ho KK, Pinsky JL, Kannel WB, Levy D. The epidemiology of heart failure: the Framingham Study. *J Am Coll Cardiol.* 1993;22:6A-13A.
20. Francis GS, Tang WH. Pathophysiology of congestive heart failure. *Rev Cardiovasc Med.* 2003;4 Suppl 2:S14-20.
21. Schunkert H. Molecular genetics of congestive heart failure. *Scand Cardiovasc J Suppl.* 1998;47:37-43.
22. Kuznetsova T, Staessen JA, Wang JG, Gasowski J, Nikitin Y, Ryabikov A, Fagard R. Antihypertensive treatment modulates the association between the D/I ACE gene polymorphism and left ventricular hypertrophy: a meta-analysis. *J Hum Hypertens.* 2000;14:447-54.
23. Celentano A, Mancini FP, Crivaro M, Palmieri V, Ferrara LA, De Stefano V, Di Minno G, de Simone G. Cardiovascular risk factors, angiotensin-converting enzyme gene I/D polymorphism, and left ventricular mass in systemic hypertension. *Am J Cardiol.* 1999;83:1196-200.
24. Nakahara K, Matsushita S, Matsuoka H, Inamatsu T, Nishinaga M, Yonawa M, Aono T, Arai T, Ezaki Y, Orimo H. Insertion/deletion poly-

morphism in the angiotensin-converting enzyme gene affects heart weight. *Circulation.* 2000;101:148-51.

25. Montgomery HE, Keeling PJ, Goldman JH, Humphries SE, Talmud PJ, McKenna WJ. Lack of association between the insertion/deletion polymorphism of the angiotensin-converting enzyme gene and idiopathic dilated cardiomyopathy. *J Am Coll Cardiol.* 1995;25:1627-31.
26. Sanderson JE, Yu CM, Young RP, Shum IO, Wei S, Arumanayagam M, Woo KS. Influence of gene polymorphisms of the renin-angiotensin system on clinical outcome in heart failure among the Chinese. *Am Heart J.* 1999;137:653-7.
27. Sanderson JE, Young RP, Yu CM, Chan S, Critchley JA, Woo KS. Lack of association between insertion/deletion polymorphism of the angiotensin-converting enzyme gene and end-stage heart failure due to ischemic or idiopathic dilate cardiomyopathy in the Chinese. *Am J Cardiol.* 1996;77:1008-10.
28. Schunkert H. Controversial association of left ventricular hypertrophy and the ACE I/D polymorphism--is the mist clearing up? *Nephrol Dial Transplant.* 1998;13:1109-12.
29. Montgomery HE, Clarkson P, Dollery CM, Prasad K, Losi MA, Hemingway H, Statters D, Jubb M, Girvain M, Varnava A, World M, Deanfield J, Talmud P, McEwan JR, McKenna WJ, Humphries S. Association of angiotensin-converting enzyme gene I/D polymorphism with change in left ventricular mass in response to physical training. *Circulation.* 1997;96:741-7.
30. Ohmichi N, Iwai N, Maeda K, Shimoike H, Nakamura Y, Izumi M, Sugimoto Y, Kinoshita M. Genetic basis of left ventricular remodeling after myocardial infarction. *Int J Cardiol.* 1996;53:265-72.
31. West MJ, Summers KM, Burstow DJ, Wong KK, Huggard PR. Renin and angiotensin-converting enzyme genotypes in patients with essential hypertension and left ventricular hypertrophy. *Clin Exp Pharmacol Physiol.* 1994;21:207-10.
32. Pontremoli R, Sofia A, Tirotta A, Ravera M, Nicolella C, Viazzi F, Bezante GP, Borgia L, Bobola N, Ravazzolo R, Sacchi G, Deferrari G. The deletion polymorphism of the angiotensin I-converting enzyme gene is associated with target organ damage in essential hypertension. *J Am Soc Nephrol.* 1996;7:2550-8.
33. Serneri GGN, Boddi M, Cecioni I, Vanni S, Coppo M, Papa ML, Bandinelli B, Bertolozzi I, Polidori I, Toscano T, Maccherini M, Modesti

PA. Cardiac angiotensin II formation in the Clinical Course of heart failure and its relationship with left ventricular function. *Circ Res.* 2001;88:961-8.

34. Kuznetsova T, Staessen JA, Stolarz K, Ryabikov A, Tikhonoff V, Olszanecka A, Bianchi G, Brand E, Casiglia E, Dominiczak A, Fagard R, Malyutina S, Nikitin Y, Kawecka-Jaszcz K. Relationship between left ventricular mass and the ACE D/I polymorphism varies according to sodium intake. *J Hypertens* 2004;22:287-295.



3. CANDIDATE GENE STUDIES

INSULIN-LIKE GROWTH FACTOR-I PROMOTER POLYMORPHISM

3.1. POLYMORPHISM IN THE PROMOTER REGION OF THE INSULIN-LIKE GROWTH FACTOR I GENE IS RELATED TO CAROTID INTIMA MEDIA THICKNESS AND AORTIC PULSE WAVE VELOCITY IN SUBJECTS WITH HYPERTENSION

Background and Purpose: Low circulating levels of Insulin-like Growth factor I (IGF-I) have been associated with an increased risk for atherosclerosis. Absence of the 192-bp (wildtype) allele in the promoter region of the IGF-I gene has been associated with low circulating IGF-I levels. We examined the role of this polymorphism in relation to blood pressure and two early markers of atherosclerosis: carotid intima media thickness (IMT) and aortic pulse wave velocity (PWV).

Methods: 5132 subjects of the Rotterdam study, aged 55-75 years, were included in this study. In 3769 subjects, who did not use blood pressure lowering medication, the association between the IGF-I polymorphism and blood pressure was examined. In the total population, 3484 normotensive subjects, 1648 hypertensive and 462 non-treated hypertensive subjects, the association between this polymorphism and IMT and PWV was examined.

Results: Mean systolic and diastolic blood pressure did not differ between genotypes. In hypertensive subjects IMT was significantly increased in non carriers of the 192-bp allele (0.83 mm) compared to heterozygous or homozygous carriers (0.80 mm) (p=0.04). PWV was also significantly higher in hypertensive subjects who were non carriers of the 192-bp allele (14.3 m/s) compared to heterozygous (14.1 m/s) or homozygous carriers (13.7 m/s) (p=0.02). Findings were more pronounced in hypertensive subjects without medication use. In normotensive subjects no association between this polymorphism, IMT and PWV was observed.

Conclusion: Our study suggests that hypertensive subjects who have low IGF-I levels because of a genetic polymorphism in the IGF-I gene are at increased risk of developing atherosclerosis.

Introduction

Insulin-like Growth Factor I (IGF-I) may play an important role in the development of cardiovascular disease.[1,2] Its contribution to the development of atherosclerosis is a topic of increasing interest in both human and animal studies.[3-5] Low circulating IGF-I levels have been associated with the early development of cardiovascular disease.[6-8] Because of its growth mediating and vasodilator properties, IGF-I is assumed to be an important mediator in the pathophysiological response to increased blood pressure in the vessel wall. Animal studies have shown that an increase in haemodynamic load is accompanied by increased IGF-I expression in both cardiac and vascular tissues.[9-11]

We have recently demonstrated that a polymorphism in the promoter region of the IGF-I gene is associated with serum IGF-I levels. In our studies, absence of the 192-bp (wildtype) allele was associated with 20% lower circulating IGF-I serum levels at middle age, lower body height and a reduction in birth weight.[12,13] This polymorphism can be used to study subjects with a genetic predisposition towards chronic low exposure of IGF-I in all tissues of the body, including those of cardiovascular origin.

In this population-based study we examined the effect of this polymorphism on blood pressure and the development of atherosclerosis. We used two early markers of atherosclerosis in this study: intima media thickness of the carotid arteries (IMT) and aortic pulse wave velocity (PWV).

Materials and Methods

Study Population

The study was performed within the Rotterdam Study, a single-center prospective follow-up study, in which all residents aged 55 years and over of the Rotterdam suburb Ommoord were invited to take part. The baseline examination of the Rotterdam Study was conducted between 1990 and 1993. The study was approved by the Medical Ethics Committee of Erasmus Medical Center Rotterdam. Written informed consent was obtained from all participants. The design of the study has been described previously.[14]

We examined 7983 participants (response 78%). Because no DNA was available for 948 subjects and in 23 subjects genotyping failed, 7012 subjects were successfully genotyped for the IGF-I gene. The relation between the IGF-I polymorphism and serum IGF-I levels was assessed in a subgroup of 150 subjects, consisting of 50 subjects randomly drawn from each genotype group. Since serum IGF-I levels show an age-dependent decline and are related to the development of cardiovascular disease, we excluded all subjects aged over 75 years in order to avoid any bias in our results because of selective mortality. Therefore only subjects who were between 55-75 years of age at baseline examination and successfully genotyped were included in this study (n=5132).

In a subgroup of 3769 subjects, who did not use blood pressure lowering medication, the association between the IGF-I polymorphism and systolic and diastolic blood pressure was examined. In the total study population (n=5132), the IGF-I polymorphism was examined in relation to intima media thickness and pulse wave velocity. This relation was also examined separately in a subgroup of 1648 hypertensive subjects and in a subgroup of 3484 normotensive subjects. Since lowering of blood pressure is known to influence the development of atherosclerosis, the relation between the IGF-I polymorphism, IMT and PWV was also assessed in 462 hypertensive subjects who did not use any blood pressure lowering medication.

Measurements

At the baseline examination, information concerning medical history, medication use and smoking behavior was obtained with a computerized questionnaire.[14] Height and weight were measured and body mass index (BMI in kg/m2) was calculated. Blood pressure was measured in sitting position at the right upper arm using a random zero sphygmomanometer. The average of two measurements was used for analysis. Hypertension was defined as a diastolic blood pressure of 100 mmHg or higher and/or a systolic blood pressure of 160 mmHg or higher and/or use of anti-hypertensive medication indicated to treat high blood pressure (grade 2 and 3 of the 1999 WHO criteria).[15] Diabetes mellitus was defined as the use of blood glucose-lowering medication and/or random serum glucose level ≥ 11.1 mmol/l. Total serum cholesterol and HDL-cholesterol were determined with an automated enzymatic procedure.[16] Total IGF-I levels were determined in non-fasting serum by a commercially available radioimmunoassay (Medgenix Diagnostics, with an intra- and interassay variation of 6.1% and 9.9%).

Intima media thickness of the left and right carotid artery was assessed by ultrasound.[17,18] The beginning of the dilatation of the distal common carotid artery served as a reference point for the start of the measurement and IMT was measured over an average distance of 10 mm. The lumen-intima interface and the media-adventitia interface of the near and far walls of the distal common carotid artery were measured offline. In each subject mean IMT of far and near wall ((left + right)/2) was taken as measure for wall thickness of the distal common carotid artery. The intraclass correlation coefficient for assessment of common carotid IMT was 0.74.[19] Carotid -femoral Pulse Wave Velocity was assessed using an automatic device (Complior, Colson, Garges-les-Gonesse Cx, France) that recorded the time delay between the rapid upstroke of the feet of simultaneously recorded pulse waves in the carotid artery and femoral artery.[20] The distance traveled by the pulse, between the carotid and the femoral artery, was measured over the surface of the body using a tape measure. PWV was calculated as the ratio between the distance traveled by the pulse wave and the foot-to-foot delay and expressed in meters per second. We used the average of at least 10 successive measurements, to cover a com-

plete respiratory cycle, in the analysis. The intraclass correlation coefficient for carotid-femoral PWV was 0.80.[19]

The IGF-I gene promoter polymorphism was genotyped as described earlier.[12] Based on our previous studies, three genotype groups were distinguished: homozygous carriers of the 192-bp allele, heterozygous carriers and non carriers of this allele.

Data Analysis

Hardy-Weinberg equilibrium of the IGF-I promoter polymorphism genotypes was tested using the GENEPOP-package (Raymond M. & Rousset F, 1995. GENEPOP version). General characteristics of the total study population, stratified by genotype, were compared using the univariate analysis of variance for continuous variables and χ^2- statistics for dichotomous variables. To examine the effect of the IGF-I genotype on intima media thickness and pulse wave velocity in hypertensive and normotensive subjects separately, we stratified all subjects based on their genotype and the presence or absence of hypertension. Subjects homozygous for the 192-bp allele and normotensive were used as the reference category in these analyses. All analyses on systolic and diastolic blood pressure, intima media thickness and pulse wave velocity were adjusted for possible confounders: age, sex, BMI, total cholesterol, HDL-cholesterol, smoking and diabetes mellitus. Additional analyses, adjusting for myocardial infarction and stroke, were performed to correct for possible confounding by the presence of prevalent cardiovascular disease. SPSS for Windows software package, version 10.0, was used to perform all analyses.

Results

Genotype frequencies in the total study population and in the normotensive and hypertensive subjects were in Hardy Weinberg Equilibrium (p=0.4).

In table 1, the general characteristics of the total study population for each genotype group are presented. Serum total IGF-I levels were significantly lower in non carriers of the 192-bp allele (16.7 nmol/l) compared to homozygous carriers (20.5 nmol/l) (p=0.003). Levels of cardiovascular risk factors are in the high normal range, as can be expected in a population of elderly subjects. No significant differences between homozygous, heterozygous and non carriers of the 192-bp allele were observed.

In table 2, systolic and diastolic blood pressures, prevalence of hypertension, mean IMT of the carotid arteries and aortic PWV are presented for each genotype group. Crude results (not shown) did not differ significantly from those after adjustment for possible confounders. Systolic and diastolic blood pressure and the prevalence of hypertension did not differ between the genotype groups. IMT was significantly increased in non carriers of the 192-bp allele compared to heterozygous and homozygous carriers (p for trend=0.02). PWV did not differ between genotype groups in the overall analysis.

In figure 1, mean IMT by genotype for all normotensive, all hypertensive subjects and

Table 1. General characteristics of total study population stratified by IGF-I genotype

	Homozygous carriers 192-bp allele	Heterozygous carriers 192-bp allele	Non carriers 192-bp allele
Number of subjects	2209	2244	602
Serum total IGF-I levels* - nmol/l	20.5 ± 6.2 (n=50)	19.6 ± 6.4 (n=50)	16.7 ± 5.0 (n=50)†
Men -%	43.3	44.2	40.2
Age – yrs	65.0 ± 5.5	64.9 ± 5.5	64.9 ± 5.5
Body mass index - kg/m^2	26.3 ± 3.7	26.2 ± 3.5	26.4 ± 3.6
Current smoking - %	25.7	26.8	21.4
Total cholesterol - mmol/l	6.7 ± 1.2	6.7 ± 1.2	6.7 ± 1.1
HDL-cholesterol - mmol/l	1.4 ± 0.4	1.3 ± 0.4	1.4 ± 0.4
Diabetes mellitus - %	8.2	8.4	11.9

*All values are presented as mean ± standard deviation or percentage. *Measured in a subset of the total study population (n=150). †Significantly different from homozygous carriers (p=0.003).*

Table 2. Systolic and diastolic blood pressure, prevalence of hypertension, intima media thickness and pulse wave velocity in total study population stratified by IGF-I genotype

	Homozygous carriers 192-bp allele	Heterozygous carriers 192-bp allele	Non carriers 192-bp allele
Systolic blood pressure* - mmHg	135.2 ± 21.2	135.1 ± 21.0	134.0 ± 21.2
Diastolic blood pressure* - mmHg	73.5 ± 11.0	74.0 ± 11.2	72.8 ± 10.7
Hypertension - %	31.1	31.5	30.3
Intima media thickness - mm	0.76 ± 0.14	0.77 ± 0.15	0.78 ± 0.14†
Pulse wave velocity - m/s	13.3 ± 2.9	13.3 ± 3.0	13.3 ± 2.9

*All values are presented as mean ± standard deviation or percentage and adjusted for age, sex, BMI, total cholesterol, HDL-cholesterol, diabetes and smoking. *Only subjects without use of any blood pressure lowering medication (n=3769). †Significantly different from homozygous carriers; p<0.05.*

non-treated hypertensive subjects is presented. IMT was significantly higher in hypertensive subjects compared to normotensive subjects in all genotype groups (p<0.005). In all hypertensive subjects IMT was significantly higher in non carriers of the 192-bp allele (0.83 mm) compared to heterozygous and homozygous carriers (0.80 mm) (p=0.04). In non-treated hypertensive subjects IMT was also higher in non carriers (0.85 mm) compared to heterozygous (0.80 mm) and homozygous carriers (0.80 mm) (p=0.01). In normotensive subjects, IMT did not differ significantly between the genotype groups.

In figure 2, mean PWV by genotype for all normotensive, all hypertensive subjects and non-treated hypertensive subjects is presented. PWV was significantly higher in hypertensive subjects than in normotensive subjects in all genotype groups (p<0.005). In

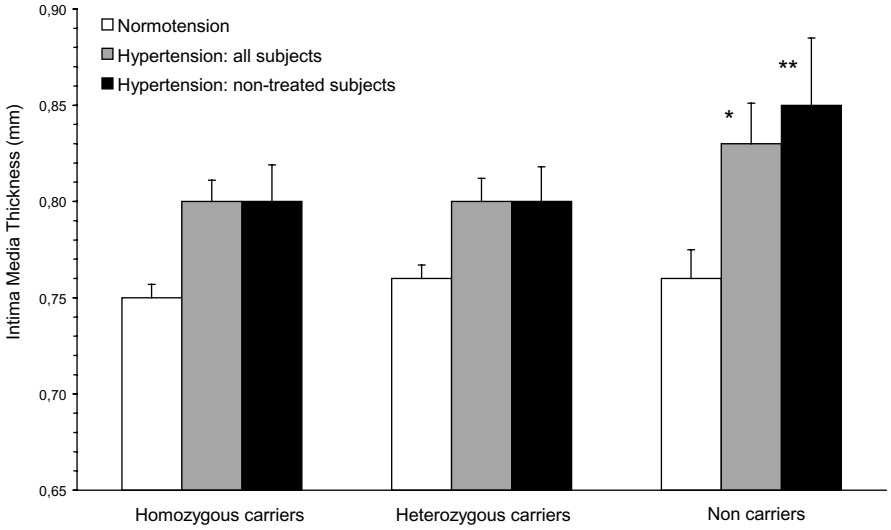


Figure 1. Intima media thickness (mm) by IGF-I genotype in normotensive subjects (white bars), all hypertensive subjects (grey bars) and non-treated hypertensive subjects (black bars).
*Significantly different from homozygous carriers in all hypertensive subjects, p<0.05.
**Significantly different from homozygous carriers in non-treated hypertensive subjects, p<0.01.
Adjusted for age, sex, BMI, total cholesterol, HDL-cholesterol, diabetes mellitus and smoking.

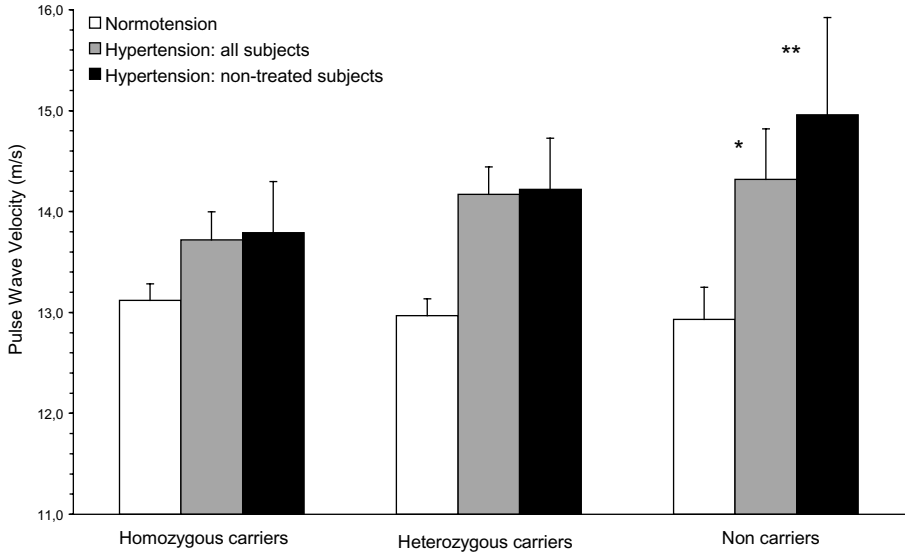


Figure 2. Pulse wave velocity (m/s) by IGF-I genotype in normotensive subjects (white bars), all hypertensive subjects (grey bars) and non-treated hypertensive subjects (black bars).
*Significantly different from homozygous carriers in all hypertensive subjects, p<0.05.
**Significantly different from homozygous carriers in non-treated hypertensive subjects, p<0.05.
Adjusted for age, sex, BMI, total cholesterol, HDL-cholesterol, diabetes mellitus and smoking.

hypertensive subjects, PWV was significantly higher in non carriers of the 192-bp allele (14.3 m/s) compared to heterozygous carriers (14.1 m/s) and homozygous carriers (13.7 m/s) (p=0.02). In non-treated hypertensive subjects, the difference in PWV between non carriers and carriers of the 192-bp allele increased. Non carriers had significantly higher PWV (15.0 m/s) than heterozygous (14.2 m/s) and homozygous carriers (13.8 m/s) (p=0.03). In normotensive subjects PWV did not differ significantly between the genotype groups. Additional analyses adjusting for myocardial infarction and stroke did not significantly change the results presented in table 2 and the figures.

Discussion

In this population based study we found an association between a genetic polymorphism in the promoter region of the IGF-I gene and IMT of the carotid arteries in the general population. Further analysis revealed that the association between this polymorphism and atherosclerosis was most pronounced in hypertensive subjects. Hypertensive subjects who did not carry a copy of the 192-bp allele had an increased carotid IMT and higher aortic PWV compared to heterozygous and homozygous carriers of the 192-bp allele. The effect of this polymorphism was even stronger in subjects with untreated hypertension. In normotensive subjects no association was found between this polymorphism and IMT or PWV.

Since IMT and PWV are considered reliable indicators of the structural and functional changes in the vasculature, we used both as early markers of atherosclerosis.21 Recent findings in our study population have shown a strong positive association between aortic stiffness (as measured by PWV) and common carotid IMT.[19] PWV and IMT have been reported to be strongly associated with vascular risk factors and the prevalence of cerebrovascular and cardiovascular disease.[18,22-24]

We have previously observed that non carriers of the 192-bp allele have significantly lower circulating IGF-I levels than heterozygous or homozygous carriers of this allele.[12] Findings by Janssen et al., who observed an inverse relation between circulating IGF-I levels and atherosclerosis in another population-based study, suggested that low IGF-I levels may play a role in the development of atherosclerosis in the general population.[7] Studies in GH-deficient patients have already indicated that low circulating IGF-I levels may play an important role in the development of cardiovascular disease.[25] GH-deficient patients have significantly increased intima media thickness, decreased systemic nitric oxide (NO) generation and a tendency towards impaired flow-mediated vasodilatation.[26-28] They also have increased levels of circulating inflammatory cardiovascular risk markers, such as CRP, IL-6 and TNF-α.[29,30] In addition, an increase in circulating IGF-I levels, observed during GH-replacement therapy, is accompanied by a reduction in intima media thickness and a decrease in circulating inflammatory

markers.[29,31-33] Although the exact mechanism by which IGF-I influences the development of atherosclerosis is still unknown, the effects of IGF-I on the vascular endothelium are thought to be partly mediated by NO, which is not only known to induce vasorelaxation but also inhibits platelet aggregation, leukocyte adhesion and smooth muscle cell growth.[28,34,35]

Our findings support the observation that low levels of serum IGF-I may affect the development of atherosclerosis. Of interest is that the effect of this polymorphism on IMT and PWV was observed in hypertensive subjects only. One explanation for this finding may be that a relatively lower expression of the IGF-I gene, in non carriers of the 192-bp allele, only becomes clinically relevant in subjects who have an increased demand for IGF-I. We hypothesize that in hypertensive subjects, because of increased haemodynamic load, more IGF-I is needed to protect the vessel wall than in normotensive subjects. So, hypertensive subjects who do not carry a copy of the 192-bp allele may not have enough (reserve) capacity to adequately fulfill the increased demand for IGF-I. As a consequence, the anti-atherogenic effects of IGF-I in these subjects may no longer be sufficient to prevent the development of atherosclerosis.

In our study we found an even stronger effect of this polymorphism in subjects with untreated hypertension. This observation supports our hypothesis that especially in subjects at high risk for atherosclerosis, e.g. due to increased haemodynamic load, the need for IGF-I is increased. In line with this is the observation in animal studies that IGF-I expression increases during periods of high haemodynamic load.[9-11]

Since IGF-I is also known to induce vasorelaxation, it seems surprising that we did not observe an effect of this polymorphism in the IGF-I gene on blood pressure or the prevalence of hypertension. This suggests that IGF-I is not so much a determinant in the chronic blood pressure lowering response, but rather is a mediator in limiting the damaging effects of high blood pressure on the vasculature. Another important issue that needs to be addressed is possible confounding by other cardiovascular risk factors such as diabetes mellitus, impaired glucose tolerance, increased BMI, hyperlipidemia and preexisting cardiovascular disease. These risk factors have also been associated with low IGF-I levels.[30,29] However, our results did not change significantly after adjustment for these risk factors, suggesting that the effects of the IGF-I polymorphism on the vascular endothelium are independent of other cardiovascular risk factors.

In conclusion, our findings support the opinion that IGF-I plays a role in the pathogenesis of atherosclerosis. The polymorphism we studied in the IGF-I gene most likely is a modifier of the risk for atherosclerosis in subjects with hypertension. Ongoing studies on the role of IGF-I and the interaction with its receptor and binding proteins will help to further elucidate the exact mechanism by which IGF-I exerts its effects on the development of atherosclerosis in subjects with high blood pressure.

References

1. Sowers JR. Insulin and insulin-like growth factor in normal and pathological cardiovascular physiology. *Hypertension.* 1997;29:691-699.
2. Delafontaine P. Insulin-like growth factor I and its binding proteins in the cardiovascular system. *Cardiovasc Res.* 1995;30:825-834.
3. Bayes-Genis A, Conover CA, Schwartz RS. The insulin-like growth factor axis: A review of atherosclerosis and restenosis. *Circ Res.* 2000;86:125-130.
4. Ferns GA, Motani AS, Anggard EE. The insulin-like growth factors: their putative role in atherogenesis. *Artery.* 1991;18:197-225.
5. Delafontaine P, Lou H, Alexander RW. Regulation of insulin-like growth factor I messenger RNA levels in vascular smooth muscle cells. *Hypertension.* 1991;18:742-747.
6. Juul A, Scheike T, Davidsen M, Gyllenborg J, Jorgensen T. Low serum insulin-like growth factor I is associated with increased risk of ischemic heart disease: a population-based case-control study. *Circulation.* 2002;106:939-944.
7. Janssen JA, Stolk RP, Pols HA, Grobbee DE, Lamberts SW. Serum total IGF-I, free IGF-I, and IGFB-1 levels in an elderly population: relation to cardiovascular risk factors and disease. *Arterioscler Thromb Vasc Biol.* 1998;18:277-282.
8. Spallarossa P, Brunelli C, Minuto F, Caruso D, Battistini M, Caponnetto S, Cordera R. Insulin-like growth factor-I and angiographically documented coronary artery disease. *Am J Cardiol.* 1996;77:200-202.
9. Du J, Delafontaine P. Inhibition of vascular smooth muscle cell growth through antisense transcription of a rat insulin-like growth factor I receptor cDNA. *Circ Res.* 1995;76:963-972.
10. Fath KA, Alexander RW, Delafontaine P. Abdominal coarctation increases insulin-like growth factor I mRNA levels in rat aorta. *Circ Res.* 1993;72:271-277.
11. Wickman A, Friberg P, Adams MA, Matejka GL, Brantsing C, Guron G, Isgaard J. Induction of growth hormone receptor and insulin-like growth factor-I mRNA in aorta and caval vein during hemodynamic challenge. *Hypertension.* 1997;29:123-130.
12. Vaessen N, Heutink P, Janssen JA, Witteman JC, Testers L, Hofman A, Lamberts SW, Oostra BA, Pols HA, van Duijn CM. A polymorphism in the gene for IGF-I: functional properties and risk for type 2 diabetes

and myocardial infarction. *Diabetes.* 2001;50:637-642.
13. Vaessen N, Janssen JA, Heutink P, Hofman A, Lamberts SW, Oostra BA, Pols HA, van Duijn CM. Association between genetic variation in the gene for insulin-like growth factor-I and low birthweight. *Lancet.* 2002;359:1036-1037.
14. Hofman A, Grobbee DE, de Jong PT, van den Ouweland FA. Determinants of disease and disability in the elderly: the Rotterdam Elderly Study. *Eur J Epidemiol.* 1991;7:403-422.
15. 1999 World Health Organization-International Society of Hypertension Guidelines for the Management of Hypertension. Guidelines Subcommittee. *J Hypertens.* 1999;17:151-183.
16. van Gent CM, van der Voort HA, de Bruyn AM, Klein F. Cholesterol determinations. A comparative study of methods with special reference to enzymatic procedures. *Clin Chim Acta.* 1977;75:243-251.
17. Bots ML, de Jong PT, Hofman A, Grobbee DE. Left, right, near or far wall common carotid intima-media thickness measurements: associations with cardiovascular disease and lower extremity arterial atherosclerosis. *J Clin Epidemiol.* 1997;50:801-807.
18. Bots ML, Hoes AW, Koudstaal PJ, Hofman A, Grobbee DE. Common carotid intima-media thickness and risk of stroke and myocardial infarction: the Rotterdam Study. *Circulation.* 1997;96:1432-1437.
19. van Popele NM, Grobbee DE, Bots ML, Asmar R, Topouchian J, Reneman RS, Hoeks AP, van der Kuip DA, Hofman A, Witteman JC. Association between arterial stiffness and atherosclerosis: the Rotterdam Study. *Stroke.* 2001;32:454-460.
20. Asmar R, Benetos A, Topouchian J, Laurent P, Pannier B, Brisac AM, Target R, Levy BI. Assessment of arterial distensibility by automatic pulse wave velocity measurement. Validation and clinical application studies. *Hypertension.* 1995;26:485-490.
21. Wada T, Kodaira K, Fujishiro K, Maie K, Tsukiyama E, Fukumoto T, Uchida T, Yamazaki S. Correlation of ultrasound-measured common carotid artery stiffness with pathological findings. *Arterioscler Thromb.* 1994;14:479-482.
22. Salonen R, Tervahauta M, Salonen JT, Pekkanen J, Nissinen A, Karvonen MJ. Ultrasonographic manifestations of common carotid atherosclerosis in elderly eastern Finnish men. Prevalence and associations with cardiovascular diseases and risk factors. *Arterioscler Thromb.* 1994;14:1631-1640.

23. Blacher J, Asmar R, Djane S, London GM, Safar ME. Aortic pulse wave velocity as a marker of cardiovascular risk in hypertensive patients. *Hypertension.* 1999;33:1111-1117.
24. Blacher J, Pannier B, Guerin AP, Marchais SJ, Safar ME, London GM. Carotid arterial stiffness as a predictor of cardiovascular and all-cause mortality in end-stage renal disease. *Hypertension.* 1998;32:570-574.
25. McCallum RW, Petrie JR, Dominiczak AF, Connell JM. Growth hormone deficiency and vascular risk. *Clin Endocrinol* (Oxf). 2002;57:11-24.
26. Markussis V, Beshyah SA, Fisher C, Sharp P, Nicolaides AN, Johnston DG. Detection of premature atherosclerosis by high-resolution ultrasonography in symptom-free hypopituitary adults. *Lancet.* 1992;340:1188-1192.
27. Markussis V, Beshyah SA, Fisher C, Parker KH, Nicolaides AN, Johnston DG. Abnormal carotid arterial wall dynamics in symptom-free hypopituitary adults. *Eur J Endocrinol.* 1997;136:157-164.
28. Boger RH, Skamira C, Bode-Boger SM, Brabant G, von zur Muhlen A, Frolich JC. Nitric oxide may mediate the hemodynamic effects of recombinant growth hormone in patients with acquired growth hormone deficiency. A double- blind, placebo-controlled study. *J Clin Invest.* 1996;98:2706-2713.
29. Sesmilo G, Biller BM, Llevadot J, Hayden D, Hanson G, Rifai N, Klibanski A. Effects of growth hormone administration on inflammatory and other cardiovascular risk markers in men with growth hormone deficiency. A randomized, controlled clinical trial. *Ann Intern Med.* 2000;133:111-122.
30. Sesmilo G, Miller KK, Hayden D, Klibanski A. Inflammatory cardiovascular risk markers in women with hypopituitarism. *J Clin Endocrinol Metab.* 2001;86:5774-5781.
31. Kvasnicka J, Marek J, Kvasnicka T, Weiss V, Markova M, Stepan J, Umlaufova A. Increase of adhesion molecules, fibrinogen, type-1 plasminogen activator inhibitor and orosomucoid in growth hormone (GH) deficient adults and their modulation by recombinant human GH replacement. *Clin Endocrinol (Oxf).* 2000;52:543-548.
32. Pfeifer M, Verhovec R, Zizek B, Prezelj J, Poredos P, Clayton RN. Growth hormone (GH) treatment reverses early atherosclerotic changes in GH-deficient adults. *J Clin Endocrinol Metab.* 1999;84:453-457.

33. Christ ER, Chowienczyk PJ, Sonksen PH, Russel-Jones DL. Growth hormone replacement therapy in adults with growth hormone deficiency improves vascular reactivity.
Clin Endocrinol (Oxf). 1999;51:21-25.
34. Radomski MW, Salas E. Nitric oxide--biological mediator, modulator and factor of injury: its role in the pathogenesis of atherosclerosis.
Atherosclerosis. 1995;118 Suppl:S69-80.
35. Tsukahara H, Gordienko DV, Tonshoff B, Gelato MC, Goligorsky MS. Direct demonstration of insulin-like growth factor-I-induced nitric oxide production by endothelial cells. *Kidney Int.* 1994;45:598-604.

3.2. ABSENCE OF THE 192-BASE PAIR ALLELE IN A PROMOTER POLYMORPHISM OF THE INSULIN-LIKE GROWTH FACTOR-I GENE IS ASSOCIATED WITH AN INCREASED RISK OF LEFT VENTRICULAR HYPERTROPHY

Objective: Altered serum levels of insulin-like growth factor I (IGF-I) have been associated with adverse cardiac remodeling. A promoter polymorphism in the IGF-I gene may alter serum IGF-I levels. We investigated the association between this polymorphism and left ventricular hypertrophy.

Methods and results: This study was performed in the Rotterdam Study, a population-based prospective cohort study among elderly. Analyses were performed with baseline measurements in subjects aged between 55 and 75 years, without a history of myocardial infarction (n=1,678). Left ventricular hypertrophy was defined as a left ventricular mass index ≥ 104 g/m^2 in women and ≥ 116 g/m^2 in men. Frequencies of left ventricular geometry patterns by genotype were also studied. Non-carriers of the 192-base pair allele of a cytosine-adenosine repeat in the IGF-I gene had a 1.5 fold increased risk of left ventricular hypertrophy as compared to subjects homozygous for this wild type allele. Although we found no clearly increased association in heterozygotes, there was a significant allele-effect relationship (p-trend<0.05). The small difference in distribution of left ventricular geometry patterns between genotypes was not significant.

Conclusion: Non-carriers of a 192-base pair polymorphism in the IGF-I gene are more susceptible to the development of left ventricular hypertrophy than individuals homozygous for this allele.

Introduction

Left ventricular hypertrophy is a strong predictor for cardiovascular morbidity and mortality.[1] The structure of the left ventricle is influenced by several factors such as blood pressure, age, gender, body mass index, diabetes mellitus, and pre-existing cardiovascular disease.[2,3] These factors, however, do not completely explain the variability in left ventricular mass. Of the other factors involved, insulin-like growth factor I (IGF-I) may explain part of this variability.[4] IGF-I is a polypeptide growth factor that is expressed in many organs. It is the product of the IGF-I gene, which has been mapped to chromosome 12.[5] Evidence has accumulated that, in addition to its growth-promoting and metabolic effects, IGF-I has cardioprotective effects by reducing myocardial apoptosis and injury in response to ischemia.[6] Moreover, findings suggest that lowered free IGF-I levels are associated with a higher prevalence of cardiovascular disease.[7]

There are several limitations of studies conducted on serum IGF-I levels in relation to cardiovascular disease. Most of these studies have been performed cross-sectional, making it difficult to distinguish whether altered serum IGF-I levels are a cause or a consequence of the underlying disease. In addition, serum levels of IGF-I are profoundly influenced by various factors such as growth hormone, insulin, age, diet, physical activity and genetic factors.[8,9] A genetic polymorphism in the IGF-I promoter region has been identified which influences IGF-I production.[10,11] Recently, we observed lower circulating total IGF-I levels in non-carriers of the wild type allele of this polymorphism than in homozygous carriers.[12,13] Studying the effect of this polymorphism in relation to pathology may better reflect the effects of long-term IGF-I exposure than studies on circulating serum IGF-I levels, which may fluctuate considerably. Furthermore, studies of genetic determinants of IGF-I levels may suffer less from the confounding influence of other factors.

This study aims to investigate the association between a promoter polymorphism of the IGF-I gene and the occurrence of left ventricular hypertrophy on the echocardiogram.

Methods

Setting and study population

The Rotterdam Study is a population-based prospective cohort study of cardiovascular-, locomotor-, neurologic- and ophthalmologic diseases in the elderly.[14] All inhabitants of Ommoord, a suburb of Rotterdam in the Netherlands, who were 55 years of age or older were invited to participate. Of the 10,275 eligible subjects, 7,983 agreed to participate (78%). The baseline examination was conducted between 1990 and 1993. Participants were visited at home for a standardized questionnaire and were subsequently examined at the research center, where echocardiography was performed. Due to costs and logistic

problems, cardiac ultrasound was carried out in a random subpopulation of the Rotterdam Study consisting of 2,823 subjects, who were not living in nursing homes. In 19.7% (n=556), echocardiographic registrations were considered inadequate for reliable measurement of left ventricular dimensions. This percentage is comparable to other population-based studies.[15]

The present study was performed using baseline measurements in subjects between 55 and 75 years of age. Participants with a history of myocardial infarction were excluded. The analyses were restricted to persons for whom blood specimens were available for IGF-I typing, and for whom all measurements were available to determine left ventricular mass index (n=1,678).

Left ventricular hypertrophy

For assessment of the presence of left ventricular hypertrophy, echocardiography was carried out with the participant in the partial left decubitus position using a 2.75-MHz transducer (Toshiba SSH-60A Nasuworks, Otawara, Japan). Measurements were made by experienced staff, trained at the echo lab of the Thorax Center in the Erasmus MC in Rotterdam, according to a protocol based on the recommendations of the American Society of Echocardiography (ASE).[16] Two-dimensional imaging using parasternal long-axis views were performed to aid M-mode studies. Measurements of the left ventricle were performed at end-diastole, as defined by the onset of the QRS complex, according to ASE recommendations. Left ventricular mass was determined using the Devereux-modified ASE cube formula.[17] left ventricular mass (grams) = $0.8x(1.04x[(LVED+IVS+LVPW)^3-(LVED)^3])+0.6$, where LVED = left ventricular end diastolic diameter, IVS = interventricular septum thickness, and LVPW = left ventricular posterior wall thickness. Left ventricular mass was indexed to body surface area.

Cases with left ventricular hypertrophy were defined as having a left ventricular mass index equal to or greater than 104 g/m^2 in women and 116 g/m^2 in men.[18,19] Since there is no consensus concerning left ventricular hypertrophy thresholds in the medical literature, left ventricular mass index was also divided into gender specific quintiles, which has been demonstrated to provide a good prediction of cardiovascular risk estimates.[20] For this second analysis, cases were defined as being in the highest gender specific quintile, and controls as being in the remaining quintiles.

To study differences in left ventricular remodeling patterns according to IGF-I genotype, relative wall thickness (RWT) was also determined and calculated as RWT = 2 x LVPW / LVED.[21] Increased relative wall thickness was considered present when this ratio exceeded 0.43, according to previously published criteria.[22] Three mutually exclusive patterns were identified: normal left ventricular geometry (normal relative wall thickness and normal left ventricular mass index), concentric remodeling (increased relative wall thickness but normal left ventricular mass index), and left ventricular hypertrophy (as defined above).

IGF-I genotype

The polymorphism under study was a cytosine-adenosine repeat in the promoter region, 1 kilo base upstream from the transcription site of the IGF-I gene. Genotyping for the IGF-I polymorphism was performed as described elsewhere.[12] Earlier, we identified ten different alleles in the promoter region of the IGF-I gene in a sample of 900 subjects of the Rotterdam Study.[12] Of these participants, 88.4% carried at least one 192-base pair (bp) allele, which suggests that this is the wild type allele from which all other alleles originated. The frequency of the other 9 alleles was low. Based on this observation, using the wild type allele, our study population was divided into three genotypes: individuals homozygous for the 192-bp allele (43.6%), individuals heterozygous for the 192-bp allele (45.6%), and non-carriers of the 192-bp allele polymorphism (10.7%).

Other variables

Information on several risk factors, such as age, gender, history of myocardial infarction (confirmed by a general practitioner, cardiologist, or the electrocardiogram), smoking (classified as never/former/current), hypertension, diabetes mellitus, and body mass index (kg/m^2) was obtained at baseline. Information on the use of medication and type of medication were assessed during the home interview. Participants subsequently showed all currently used medication at the research center, where a physician determined the indication for each drug. Systolic and diastolic blood pressures from the right upper arm were measured with a random-zero sphygmomanometer twice with the patient in a sitting position. The mean of the two readings was used to determine blood pressure levels. Hypertension was defined as use of antihypertensive medication for the indication high blood pressure, or as a systolic blood pressure of 140 mmHg or over, or a diastolic blood pressure of 90 mmHg or over.[23] Diabetes mellitus was defined as use of anti-diabetic medication, or a random or post-load serum glucose level higher than 11.0 mmol/l.

Analysis

Univariate comparisons were evaluated with a logistic regression model. Agreement of the genotype frequencies with the Hardy-Weinberg equilibrium expectations was tested using a χ^2-test. To investigate the association between left ventricular hypertrophy and IGF-I genotypes, we used multivariate logistic regression to calculate odds ratios plus 95% confidence intervals (CI). An allele-effect model was assumed to explain differences between genotype groups. Participants homozygous for the 192-bp allele served as the reference category for all analyses. Age (years), gender, hypertension, diabetes mellitus, and body mass index (kg/m^2) were added to the model to adjust for potential confounding. The decision to keep potential confounders in the final model was based on biological plausibility. None of these factors changed the point estimate of the association between IGF-I genotype and left ventricular hypertrophy by more than 5% when added to the univariate model. On each potential confounder more than 97% of data was available. For categorical covariates with missing values we incorporated missing

indicator variables in the model. We performed age- and gender adjusted logistic regression analysis to test for an allele-effect relationship (trend test). To evaluate the distribution of left ventricular geometry patterns according to IGF-I genotype, percentages per genotype were calculated. To test differences between genotypes, we used an ordinal regression model adjusted for age, with the different patterns of left ventricular geometry as outcome variables.

Table 1. General characteristics of the study population.

	Cases* (n=358) [LVH +]	**Controls (n=1320) [LVH -]**	**OR (95% CI)**
Sex – n (% female)	192 (54)	754 (57)	0.87 (0.69-1.10)
Age - yrs	65 ± 5.3	63 ± 5.3	1.07 (1.05-1.09)
Diabetes mellitus – n (%)	30 (8)	62 (5)	1.85 (1.18-2.92)
Smoking – n (%)			
- Never	101 (28)	435 (33)	1.00 (ref)
- Former	171 (48)	584 (44)	1.26 (0.96-1.66)
- Current	84 (24)	296 (23)	1.22 (0.88-1.69)
Hypertension† - n (%)	223 (64)	578 (45)	2.16 (1.69-2.75)
BMI - kg/m^2	27 ± 3.1	26 ± 3.3	1.09 (1.05-1.13)
SBP - mmHg	144 ± 21.7	134 ± 21.1	1.02 (1.02-1.03)
DBP - mmHg	76 ± 12.0	74 ± 11.0	1.02 (1.01-1.03)

*Values for characteristics of cases and controls are presented as numbers (percentage) or mean ± SD. *Case definition: left ventricular hypertrophy (LVH) was considered present when left ventricular mass index was equal or greater than 104 g/m^2 in women or 116 g/m^2 in men. †Defined as systolic blood pressure of 140 mmHg or higher or diastolic blood pressure of 90 mmHg or higher or use of antihypertensive drugs with the indication hypertension. SBP, systolic blood pressure; DBP, diastolic blood pressure.*

Table 2. Association between echocardiographically determined left ventricular hypertrophy and IGF-I genotypes

192-bp allele	**Cases*** (n=358)	**Controls** (n=1320)	**OR model 1†**	**OR model 2‡**
Homozygous carriers	145 (41%)	587 (44%)	1.00 (ref.)	1.00 (ref.)
Heterozygous carriers	166 (46%)	600 (46%)	1.13 (0.88-1.46)	1.11 (0.86-1.43)
Non carriers	47 (13%)	133 (10%)	1.50 (1.02-2.21)*	1.49 (1.01-2.20)*

*Number (% among cases or controls); odds ratio (OR) with 95% confidence interval. *Case definition: left ventricular hypertrophy was considered present when left ventricular mass index was equal or greater than 104 g/m^2 in women and 116 g/m^2 in men. †Adjusted for age (years) and gender. ‡Adjusted for age (years), gender, body mass index (kg/m^2), hypertension, and diabetes mellitus. *Allele-effect relationship: p-trend <0.05.*

Results

In total, 358 cases were identified with left ventricular hypertrophy. Genotype and allele distributions were in Hardy-Weinberg equilibrium (total population: p-value = 0.33).

Table 1 presents the baseline characteristics of the study population. Participants with left ventricular hypertrophy were on average two years older than patients with normal left ventricular mass index (mean age 65 and 63 years respectively, t-test p<0.001). In addition, cases had a higher mean body mass index, systolic- and diastolic blood pressure, and had more co-morbidity such as diabetes mellitus and hypertension. The number of smokers did not differ significantly between participants with or without left ventricular hypertrophy. The difference in gender distribution was not statistically significant.

In table 2, point estimates are presented for the association between IGF-I genotypes and left ventricular hypertrophy on the echocardiogram. Non-carriers of the 192-bp allele had a 1.50 fold increased odds of echocardiographically determined left ventricular hypertrophy as compared to individuals homozygous for the 192-bp allele. This association remained significant after adjustment for potential confounding factors (OR 1.49; 95%CI 1.01-2.20). The frequency of left ventricular hypertrophy was slightly but non-significantly increased in participants heterozygous for the 192-bp allele. However, there was a significant allele-effect relationship (p-trend <0.05). According to the model,

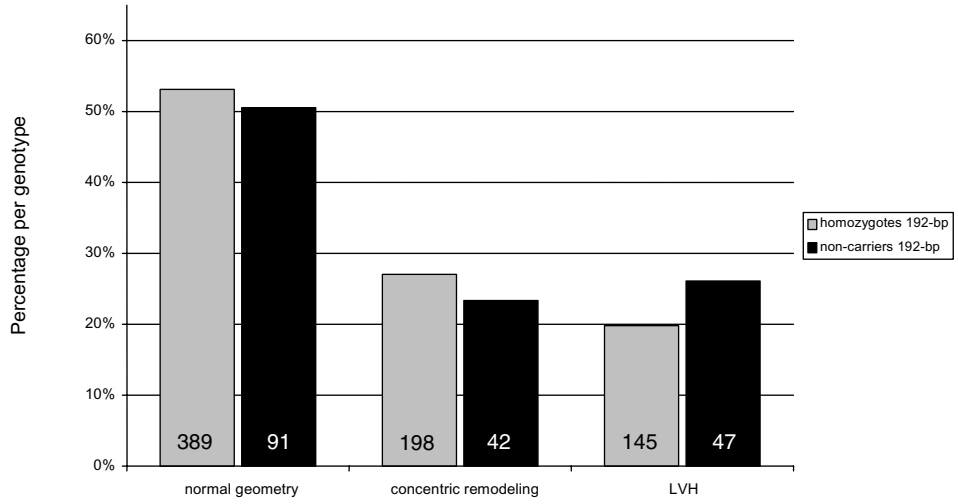


Figure 1. Left ventricular remodeling and IGF-I genotypes. LVH, left ventricular hypertrophy. Bars add up to 100 % per genotype. Numbers in the bars are the absolute number per genotype. Ordinal regression, p=0.17. OR LVH versus concentric remodeling: 1.6 (1.0-2.6), OR LVH versus normal geometry 1.5 (1.0-2.2) (age and sex adjusted).

the age- and gender adjusted odds of left ventricular hypertrophy is multiplied by 120% per risk allele present (non-wild type). Findings were similar for the association between IGF-I genotypes and the highest versus other gender specific quintiles of left ventricular mass index (age-adjusted OR non-carriers 192-bp allele 1.48, 95%CI 1.001-2.18). However, after additional adjustment for body mass index, hypertension, and diabetes mellitus this association was not statistically significant (OR 1.46, 95%CI 0.99-2.17).

Figure 1 shows the percentages per IGF-I genotype of geometric patterns of the left ventricle for non-carriers and participants homozygous for the 192-bp allele. Although ordinal regression analysis estimates were not statistically significant (p=0.17), this graph shows a tendency of percentages for non-carriers to be higher in the most unfavorable pattern of left ventricular geometry, while concentric remodeling was more frequent in individuals homozygous for the wild type allele.

Discussion

In this study, non-carriers of the 192-bp allele of a cytosine-adenosine repeat in the promoter region of the IGF-I gene were more susceptible to the development of left ventricular hypertrophy than participants homozygous for the wild type allele. To our knowledge, this is the first population-based study on the association between this IGF-I polymorphism and left ventricular hypertrophy. The risk of elevated left ventricular mass index detected by echocardiography in these subjects was significantly increased by 50%. Although there was no clearly increased difference in risk for participants heterozygous for the wild type allele, there was a significant allele-effect relationship in this population. In addition, there was a tendency for non-carriers to have a higher frequency of adverse cardiac remodeling with cardiac enlargement than participants homozygous for the 192-bp allele. In contrast, concentric remodeling, which is a more appropriate response, was more frequent in individuals homozygous for the wild type allele. Cardiovascular morbidity and mortality are known to increase as the geometric pattern of the left ventricle changes from normal to concentric remodeling and finally to left ventricular hypertrophy.[24] In vitro and in vivo studies suggest that IGF-I may help maintain appropriate myocardial remodeling in reaction to an injury to the heart.[25] This may provide an explanation for our findings.

Most studies on the association between IGF-I serum levels and left ventricular mass have been performed cross-sectional, making it difficult to distinguish whether altered serum IGF-I levels are a cause or a consequence of left ventricular hypertrophy. In addition, serum levels of IGF-I are influenced by several factors, which vary over time, including growth hormone, insulin, nutrition and physical activity. Therefore, residual confounding can be an issue in these studies. Studying the association with the IGF-I polymorphism may circumvent these difficulties, since this approach will probably

better proxy long-term exposure to circulating IGF-I. Moreover, disturbances by factors that regulate IGF-I serum levels will not influence the genetic background. Accordingly, potential confounding factors did not have an effect on the point estimates in this study.

It cannot be not excluded that the IGF-I polymorphism itself is not functional but just serves as a marker for a nearby genetic variant functionally involved in IGF-I expression. However, the polymorphism investigated in the present study has been associated with serum IGF-I concentrations in several studies, albeit with opposite directions.[11,12] Recently, we found in a sample of 900 participants of the Rotterdam Study that non-carriers of the 192-bp allele had lower circulating total IGF-I levels and lower body height than carriers of this polymorphism. The main finding of this study was that the absence of this allele was also significantly associated with an increased risk of type-2 diabetes mellitus and myocardial infarction.[12] Moreover, we observed that the normal gradual decline in circulating serum IGF-I levels during aging was highly influenced by the presence of two 192-bp alleles in the IGF-I gene.[13]

In recent years, several studies provided support to the hypothesis that low serum IGF-I is a risk factor for ischaemic heart disease and atherosclerosis.[7,26-28] In addition to its favorable effects on glycaemic control and the lipid profile[29], IGF-I has beneficial effects on cardiac remodeling by reducing myocyte apoptosis in response to ischemia[25,30], and by improving myocardial contractility and stroke volume[31]. Furthermore, IGF-I promotes cardiac hypertrophy of a physiologic phenotype[32,33] and causes systemic vascular vasodilatation[34]. Moreover, basal IGF-I levels are reduced in patients with dilated cardiomyopathy[35], and a recent study demonstrated an inverse association between the severity of heart failure, by both clinical assessment and left ventricular performance, and IGF-I levels.[36] This may implicate that progression of disease from compensated to decompensated heart failure may be partly influenced by the ability to generate IGF-I for cardiac remodeling. Hence, evidence suggests that IGF-I deficiency may lead to diminished cardiac performance and adverse remodeling in reaction to an injury to the heart. On the other hand, IGF-I directly stimulates growth of cardiac myocytes through induction of cardiac protein synthesis. In patients with acromegaly, IGF-I hypersecretion leads to ventricular hypertrophy with interstitial fibrosis.[37] Also, in a cross-sectional study of patients with untreated essential hypertension and normal glucose tolerance, IGF-I was a powerful independent determinant of left ventricular mass.[4] Therefore, both low and high levels of IGF-I seem to have adverse effects on cardiac function and structure.

This observational study is potentially limited by selection- and information bias and confounding. Old and diseased individuals were less likely to participate, resulting in a healthier study population. Random misclassification of the outcome may have occurred due to measurement error, but this will only lead to conservative risk estimates. Potential

confounding factors were dealt with in the analyses. None of these factors had a major influence on the association studied.

In conclusion, non-carriers of a 192-bp allele polymorphism in the promoter region of the IGF-I gene are more susceptible to the development of left ventricular hypertrophy than participants homozygous for the wild type allele. This may be the consequence of a relative IGF-I deficiency, leading to a faulty remodeling in response to myocardial injury.

References

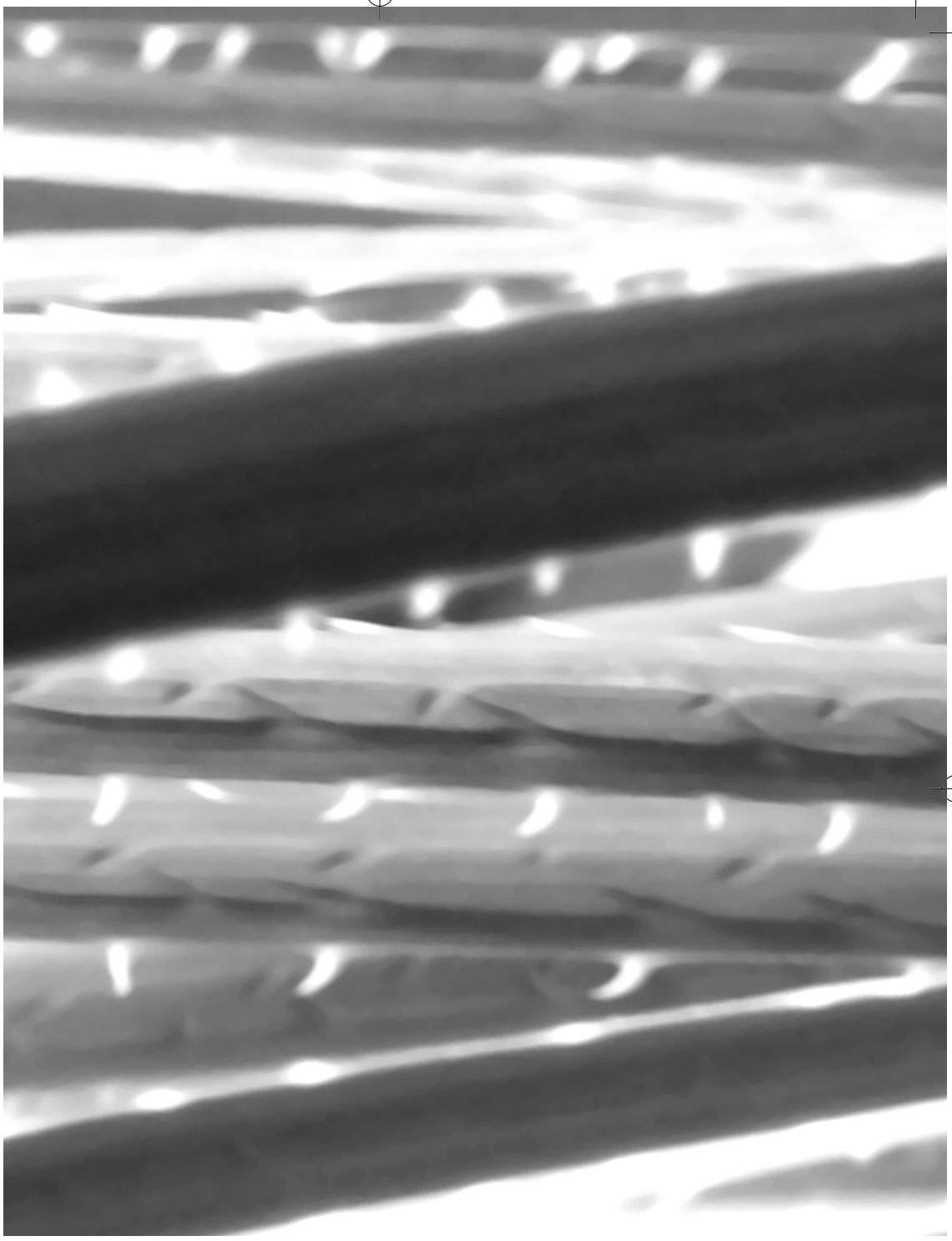
1. Levy D, Garrison RJ, Savage DD, Kannel WB, Castelli WP. Prognosticimplications of echocardiographically determined left ventricular mass in the Framingham Heart Study. *N Engl J Med.* 1990;322:1561-6.
2. Devereux RB, Roman MJ, Paranicas M, O'Grady MJ, Lee ET, Welty TK, Fabsitz RR, Robbins D, Rhoades ER, Howard BV. Impact of diabetes on cardiac structure and function: the strong heart study. *Circulation.* 2000;101:2271-6.
3. Gardin JM, Siscovick D, Anton-Culver H, Lynch JC, Smith VE, Klopfenstein HS, Bommer WJ, Fried L, O'Leary D, Manolio TA. Sex, age, and disease affect echocardiographic left ventricular mass and systolic function in the free-living elderly. The Cardiovascular Health Study. *Circulation.* 1995;91:1739-48.
4. Verdecchia P, Reboldi G, Schillaci G, Borgioni C, Ciucci A, Telera MP, Santeusanio F, Porcellati C, Brunetti P. Circulating insulin and insulin growth factor-1 are independent determinants of left ventricular mass and geometry in essential hypertension. *Circulation.* 1999;100:1802-7.
5. Delafontaine P. Insulin-like growth factor I and its binding proteins in the cardiovascular system. *Cardiovasc Res.* 1995;30:825-34.
6. Janssen JA, Lamberts SW. The role of IGF-I in the development of cardiovascular disease in type 2 diabetes mellitus: is prevention possible? *Eur J Endocrinol.* 2002;146:467-77.
7. Janssen JA, Stolk RP, Pols HA, Grobbee DE, Lamberts SW. Serum total IGF-I, free IGF-I, and IGFB-1 levels in an elderly population: relation to cardiovascular risk factors and disease. *Arterioscler Thromb Vasc Biol.* 1998;18:277-82.
8. Froesch ER, Hussain MA, Schmid C, Zapf J. Insulin-like growth factor I: physiology, metabolic effects and clinical uses.

Diabetes Metab Rev. 1996;12:195-215.

9. Ruotolo G, Bavenholm P, Brismar K, Efendic S, Ericsson CG, de Faire U, Nilsson J, Hamsten A. Serum insulin-like growth factor-I level is independently associated with coronary artery disease progression in young male survivors of myocardial infarction: beneficial effects of bezafibrate treatment. *J Am Coll Cardiol.* 2000;35:647-54.
10. Weber JL, May PE. Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *Am J Hum Genet.* 1989;44:388-96.
11. Rosen CJ, Kurland ES, Vereault D, Adler RA, Rackoff PJ, Craig WY, Witte S, Rogers J, Bilezikian JP. Association between serum insulin growth factor-I (IGF-I) and a simple sequence repeat in IGF-I gene: implications for genetic studies of bone mineral density. *J Clin Endocrinol Metab.* 1998;83:2286-90.
12. Vaessen N, Heutink P, Janssen JA, Witteman JC, Testers L, Hofman A, Lamberts SW, Oostra BA, Pols HA, van Duijn CM. A polymorphism in the gene for IGF-I: functional properties and risk for type 2 diabetes and myocardial infarction. *Diabetes.* 2001;50:637-42.
13. Rietveld I, Janssen JA, Hofman A, Pols HA, van Duijn CM, Lamberts SW. A polymorphism in the IGF-I gene influences the age-related decline in circulating total IGF-I levels. *Eur J Endocrinol.* 2003;148:171-5.
14. Hofman A, Grobbee DE, de Jong PT, van den Ouweland FA. Determinants of disease and disability in the elderly: the Rotterdam Elderly Study. *Eur J Epidemiol.* 1991;7:403-22.
15. Savage DD, Garrison RJ, Kannel WB, Anderson SJ, Feinleib M, Castelli WP. Considerations in the use of echocardiography in epidemiology. The Framingham Study. *Hypertension.* 1987;9:II40-4.
16. Sahn DJ, DeMaria A, Kisslo J, Weyman A. Recommendations regarding quantitation in M-mode echocardiography: results of a survey of echocardiographic measurements. *Circulation.* 1978;58:1072-83.
17. Devereux RB, Alonso DR, Lutas EM, Gottlieb GJ, Campo E, Sachs I, Reichek N. Echocardiographic assessment of left ventricular hypertrophy: comparison to necropsy findings. *Am J Cardiol.* 1986;57:450-8.
18. Okin PM, Devereux RB, Jern S, Julius S, Kjeldsen SE, Dahlof B. Relation of echocardiographic left ventricular mass and hypertrophy to per sistent electrocardiographic left ventricular hypertrophy in hyper-

tensive patients: the LIFE Study. *Am J Hypertens.* 2001;14:775-82.
19. Palmieri V, Dahlof B, DeQuattro V, Sharpe N, Bella JN, de Simone G, Paranicas M, Fishman D, Devereux RB. Reliability of echocardiographic assessment of left ventricular structure and function: the PRESERVE study. Prospective Randomized Study Evaluating Regression of Ventricular Enlargement. *J Am Coll Cardiol.* 1999;34:1625-32.
20. Schillaci G, Verdecchia P, Porcellati C, Cuccurullo O, Cosco C, Perticone F. Continuous relation between left ventricular mass and cardiovascular risk in essential hypertension. *Hypertension. 2000;35:580-6.*
21. Reichek N, Devereux RB. Reliable estimation of peak left ventricular systolic pressure by M-mode echographic-determined end-diastolic relative wall thickness: identification of severe valvular aortic stenosis in adult patients. *Am Heart J.* 1982;103:202-3.
22. Wachtell K, Bella JN, Liebson PR, Gerdts E, Dahlof B, Aalto T, Roman MJ, Papademetriou V, Ibsen H, Rokkedal J, Devereux RB. Impact of different partition values on prevalences of left ventricular hypertrophy and concentric geometry in a large hypertensive population : the LIFE study. *Hypertension.* 2000;35:6-12.
23. 1999 World Health Organization-International Society of Hypertension Guidelines for the Management of Hypertension. Guidelines Subcommittee. *J Hypertens.* 1999;17:151-83.
24. Koren MJ, Devereux RB, Casale PN, Savage DD, Laragh JH. Relation of left ventricular mass and geometry to morbidity and mortality in uncomplicated essential hypertension. *Ann Intern Med.* 1991;114:345-52.
25. Lee WL, Chen JW, Ting CT, Lin SJ, Wang PH. Changes of the insulin-like growth factor I system during acute myocardial infarction: implications on left ventricular remodeling. *J Clin Endocrinol Metab.* 1999;84:1575-81.
26. Juul A, Scheike T, Davidsen M, Gyllenborg J, Jorgensen T. Low serum insulin-like growth factor I is associated with increased risk of ischemic heart disease: a population-based case-control study. *Circulation.* 2002;106:939-44.
27. Spallarossa P, Brunelli C, Minuto F, Caruso D, Battistini M, Caponnetto S, Cordera R. Insulin-like growth factor-I and angiogra phically documented coronary artery disease. *Am J Cardiol.* 1996;77:200-2.

28. Goodman-Gruen D, Barrett-Connor E, Rosen C. IGF-1 and ischemic heart disease in older people. *J Am Geriatr Soc.* 2000;48:860-1.
29. Froesch ER, Zenobi PD, Hussain M. Metabolic and therapeutic effects of insulin-like growth factor I. *Horm Res.* 1994;42:66-71.
30. Wang L, Ma W, Markovich R, Chen JW, Wang PH. Regulation of cardiomyocyte apoptotic signaling by insulin-like growth factor I. *Circ Res.* 1998;83:516-22.
31. Ren J, Samson WK, Sowers JR. Insulin-like growth factor I as a cardiac hormone: physiological and pathophysiological implications in heart disease. *J Mol Cell Cardiol.* 1999;31:2049-61.
32. Duerr RL, Huang S, Miraliakbar HR, Clark R, Chien KR, Ross J, Jr. Insulin-like growth factor-1 enhances ventricular hypertrophy and function during the onset of experimental cardiac failure. *J Clin Invest.* 1995;95:619-27.
33. Serneri GG, Modesti PA, Boddi M, Cecioni I, Paniccia R, Coppo M, Galanti G, Simonetti I, Vanni S, Papa L, Bandinelli B, Migliorini A, Modesti A, Maccherini M, Sani G, Toscano M. Cardiac growth factors in human hypertrophy. Relations with myocardial contractility and wall stress. *Circ Res.* 1999;85:57-67.
34. Donath MY, Sutsch G, Yan XW, Piva B, Brunner HP, Glatz Y, Zapf J, Follath F, Froesch ER, Kiowski W. Acute cardiovascular effects of insulin-like growth factor I in patients with chronic heart failure. *J Clin Endocrinol Metab.* 1998;83:3177-83.
35. Broglio F, Fubini A, Morello M, Arvat E, Aimaretti G, Gianotti L, Boghen MF, Deghenghi R, Mangiardi L, Ghigo E. Activity of GH/IGF-I axis in patients with dilated cardiomyopathy. *Clin Endocrinol (Oxf).* 1999;50:417-30.
36. Al-Obaidi MK, Hon JK, Stubbs PJ, Barnes J, Amersey RA, Dahdal M, Laycock JF, Noble MI, Alaghband-Zadeh J. Plasma insulin-like growth factor-1 elevated in mild-to-moderate but not severe heart failure. *Am Heart J.* 2001;142:E10.
37. Lombardi G, Colao A, Marzullo P, Ferone D, Longobardi S, Esposito V, Merola B. Is growth hormone bad for your heart? Cardiovascular impact of GH deficiency and of acromegaly. *J Endocrinol.* 1997;155 Suppl 1:S33-7; discussion S39.



4. STUDIES IN A GENETICALLY ISOLATED POPULATION

4.1. GENETIC AND ENVIRONMENTAL CONTRIBUTIONS TO BLOOD PRESSURE VARIANCE IN AN EXTENDED PEDIGREE OF A DUTCH GENETICALLY ISOLATED POPULATION

Background: Genetic factors play an important role in the development of hypertension. Up to now, most heritability estimates for blood pressure have been based on nuclear family data and did not account for shared environment and genetic dominance effects. In addition, the proportion of shared genetic factors between different blood pressure traits has not been reported.

Aim: The present study assessed the heritability and genetic correlations of four blood pressure traits in the first 1000 participants of the Erasmus Rucphen Family (ERF) Study, a family-based cohort study.

Material and methods: All participants are members of an extended pedigree from a Dutch genetically isolated population. An extensive phenotypic assessment consisted of blood pressure and anthropometric measurements, laboratory tests and a personal interview. Heritability and genetic correlations of systolic (SBP), diastolic (DBP), mean arterial (MAP) and pulse pressure (PP) were assessed using a variance components approach (SOLAR).

Results: We were able to explain 50-70 % of the blood pressure variance in our population. Heritability estimates were significant for all four blood pressure traits, ranging between 0.25-0.37. We observed high genetic correlations between SBP, DBP and MAP (0.93-0.98). PP showed modest to low genetic correlations with the other blood pressure traits (0.05-0.70).

Conclusion: Genetic factors contribute to a substantial proportion of blood pressure variance, encouraging a search for genes involved in blood pressure variance in this population. Including PP in this search may lead to the identification of genes involved in different aspects of blood pressure regulation, such as arterial stiffness.

Introduction

The significance of genetic effects on blood pressure regulation has been acknowledged for a long time. Twin, adoption and nuclear family studies indicate that a substantial proportion of systolic and diastolic blood pressure variance is due to the effect of genes.[1-8] However, heritability estimates range widely between different study populations, depending heavily on the type of relative pairs used. Heritability estimates for blood pressure normally vary around 60 % in twin studies and around 25 % in nuclear family studies.[1,3,5,6,9] As these estimates are based on blood pressure correlations between first-degree relatives only, they are likely to be confounded by the effects of shared familial environment, which causes an overestimation of blood pressure heritability. Large family-based samples, including second and third degree relatives, who do not usually share the same household, may therefore generate more accurate heritability estimates of blood pressure.

In the present study we aim to assess to what extent genes and environment influence blood pressure variance in 1000 inhabitants of a genetically isolated community in the Southwest part of the Netherlands. They were all related to each other in one extended pedigree. The additive effect of genes (narrow-sense heritability) was estimated for four quantitative blood pressure traits: systolic (SBP), diastolic (DBP), mean arterial (MAP) and pulse pressure (PP). Together with our heritability estimates, we modeled the effects of shared environment and possible genetic dominance effects, by constructing a parameter based on sibships. We also estimated the proportion of blood pressure variance explained by environmental factors. Next, we assessed the phenotypic, genetic and environmental correlations between the four blood pressure traits.

Material and Methods

Setting

Analyses were performed on phenotypic data collected from Dutch inhabitants of an isolated community in the Southwest part of the Netherlands, who participated in the Erasmus Rucphen Family (ERF) study. The ERF study is a family-based cohort study and part of an ongoing research program called Genetic Research in Isolated Populations (GRIP). This research program aims to identify genetic risk factors in the development of complex disorders.[10] The study was approved by the Medical Ethics Committee of Erasmus Medical Center Rotterdam. Written informed consent was obtained from all participants.

Participants

Genealogical records trace back almost all inhabitants of this isolated population to about 150 individuals who founded this community around 1750. Minimal inward migration and considerable population growth have for years characterized this population. About 20.000 inhabitants are now scattered over eight adjacent villages. Genealogical

information of this population is currently available in the form of a large pedigree-database including over 63.000 individuals.

Eligibility for enrolment in this study was dependent on genealogical background of the participant. Twenty couples that had at least 6 children baptized in the community church between 1880-1900 were identified with the help of genealogical records of the church and the municipality. All third, fourth and fifth generational descendants of these couples and their spouses were invited to participate in the study. Data collection started in June 2002 and is still ongoing. In this study, we focus on the first 928 participants for whom complete phenotypic and genealogical information is available at present.

Data collection

Participants were invited for a series of clinical examinations at our research center located within the community. At the start of the clinical examinations, fasting blood samples were drawn for the storage of DNA and measurement of lipids, glucose, plasma creatinine, and plasma albumin levels according to a standardized procedure.[11,12] Serum samples were obtained from whole blood after clotting. Plasma samples were obtained from whole blood collected in disodium EDTA.

Hyperlipidemia was defined as the use of lipid lowering medication or total cholesterol levels between 6.5-9.0 mmol/l and a total cholesterol-HDL cholesterol ratio above 5.0, or total cholesterol below 6.5 mmol/l and a ratio above 8.0, or total cholesterol above 9.0 mmol/l, independent of the ratio, or triglycerides above 4.0 mmol/l. These criteria are in accordance with those of the Dutch college of general practitioners. Diabetes mellitus was defined as the use of blood glucose-lowering medication or fasting serum glucose level above 7.0 mmol/l.[13]

Blood pressure was measured twice in the sitting position at the right upper arm using an automated device (OMRON 711, automatic IS). The average of these two measurements was used for our analyses. Mean arterial pressure (1/3 SBP + 2/3 DBP) and pulse pressure (SBP-DBP) were calculated. Hypertension was defined as a diastolic blood pressure (DBP) of 90 mmHg or higher and/or a systolic blood pressure (SBP) of 140 mmHg or higher and/or use of anti-hypertensive medication indicated for treatment of hypertension.[14,15] Height and weight were measured with the participant dressed in light under clothing and body mass index was calculated (kg/m^2). Waist and hip circumference were measured on uncovered skin using a tape measure with the participant in upright position. Waist circumference was measured halfway between the rib cage and the pelvic bone. Hip circumference was measured at the maximal circumference of the hips. Waist to Hip ratio (WHR) was calculated from these data. Total body and trunk fat mass (grams) were obtained from DEXA scans performed in a Prodigy™ total body fan-beam densitometer and analyzed with the enCORE™ 2002 software V. 6.70.021 (GE Lunar

Corporation Madison, WI). Total body scans were auto analyzed by the software that employs an algorithm that divides body measurements into areas corresponding to head, trunk, arms and legs. The trunk region was limited by an upper horizontal border below the chin, vertical borders lateral to the ribs, and a lower border formed by oblique lines passing through the femoral necks without touching the pelvis. All analyzes were verified by a trained technician who performed adjustments when necessary. Daily quality assurance tests were performed with a calibration block supplied by the manufacturer. Repeated measurements on the calibration block had coefficients of variation less than 1%. Finally, a research physician obtained information on medical history, medication use, smoking and alcohol use in a personal interview.

Statistical analysis

Inbreeding coefficients were calculated using PEDIG software (Bouchard D: http://dga.jouy.inra.fr/sgqa/diffusions/pedig/pedigE.htm). The inbreeding coefficient is the probability that the two alleles at some locus in an individual are identical by descent (are copies of the same ancestral allele).

A variance component maximum likelihood approach, implemented in SOLAR software package, was used to estimate heritability and genetic correlations, respectively, for SBP, DBP, MAP and PP.[16] Heritability (h^2) was defined as the ratio of the variance of the trait explained by additive polygenic effects to the total phenotypic variance of the trait. In the heritability analyses, the relative contribution of these additive genetic factors to total blood pressure variance is estimated. In order to reduce the confounding effects of shared environment on our heritability estimates, we constructed a parameter identifying sibships (denoted as c^2). This parameter estimates the compound effects of shared environment and possible genetic dominance effects (which are predominantly present in bilineal relatives, e.g. sibships). Furthermore, we identified significant (environmental) covariates for each blood pressure trait in order to estimate the contribution of environmental factors to blood pressure variance.

We selected a broad range of (biologically plausible) covariates that might influence blood pressure, which were tested in a model in which the genetic parameter and the sibship parameter were constrained to zero and phenotypic variation was influenced by measured covariates and residual variance only. Covariates were allowed to enter the model in a stepwise procedure. Significant effects of each covariate were tested using a likelihood ratio test with 1 *df*. To make sure all covariates with possible important effects were included in the final model, all covariates that were significant at the 0.10 level were retained for the heritability model. Covariates that were tested included age, sex, age^2, age x sex interaction-term, various lipid measures (total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides and the diagnosis hyperlipidemia), diabetes status (fas-

ting serum glucose levels and diagnosis diabetes mellitus), lifestyle measures (smoking status and alcohol use), blood pressure lowering medication (β-blocker, diuretics, Ca-channel antagonists, ACE inhibitors and angiotensin receptor blockers), various anthropometrical measures (body mass index, waist to hip ratio, total fat mass and trunk fat mass) and inbreeding coefficients.

Finally, the value of each blood pressure trait was modeled as a linear combination of additive genetic effects (heritability=h^2), sibship effects (c^2), environmental effects (significant covariates) and residual effects. In order to obtain a multivariate normal distribution of the residuals of the blood pressure traits, SBP, DBP, MAP and PP were natural logarithmically transformed for the analyses. Heritability and sibship effects were first estimated including only age and sex as covariates (Model 1), then the analyses were repeated including all significant covariates (Model 2). The significance of the genetic and sibship effects was tested using the likelihood ratio test, where the likelihood of a model in which heritability and the sibship parameter are estimated is compared with the likelihood of a model in which either the heritability or the sibship parameter is constrained to zero. Twice the difference in the natural ln-likelihoods values of these models yields a test statistic that is asymptotically distributed as a χ^2-statistic with df equal to the difference in number of estimated parameters in the two models being compared.[17]

Subsequently, a bivariate analysis was performed to estimate the genetic and environmental correlations between the four blood pressure traits.[18,19] The phenotypic correlations between the blood pressure traits were then calculated by the following formula:[20,21]

$$\rho_P = \sqrt{h_1^2}\,\sqrt{h_2^2}\,\rho_G + \sqrt{(1-h_1^2)}\,\sqrt{(1-h_2^2)}\,\rho_E,$$

where h_1^2 and h_2^2 are the heritability estimates of the two blood pressure traits, for which the phenotypic correlation is calculated, and ρ_G and ρ_E are the genetic and environmental correlations between these two traits (as estimated in the bivariate analyses). Significance of the phenotypic, additive genetic and environmental correlations was determined using a likelihood ratio test. Whether a given correlation between two blood pressure traits was significantly different form zero, was tested by comparing the likelihood of a model in which this correlation was constrained to zero with a model in which the same correlation was estimated. Twice the difference in ln-likelihoods of these models yields a test statistic that is asymptotically distributed as a χ^2-statistic with df equal to the difference in number of parameters estimated in the two models. All bivariate analyses were adjusted for age and sex.

The analysis of each phenotype was restricted to those individuals for whom all covariate data were complete (n=928).

Table 1. Number and types of relative pairs in pedigree

Degree	Relationship	No of pairs
First	Parent-offspring	371
	Sibling	536
Second	Half-sibling	43
	Avuncular	875
	Grandparent-grandchild	35
Third	Half-avuncular	55
	First cousins	2262
	Half first cousins	53

Table 2. General characteristics of the study population

Number	928
Age – yrs	54.4 ± 15.2 (range: 18-92)
Sex – % male	40.0
SBP – mmHg	141.4 ± 22.0
DBP – mmHg	80.2 ± 10.1
Pulse pressure – mmHg	61.2 ± 17.6
Mean arterial pressure – mmHg	100.6 ± 12.7
Hypertension – %	46.3
Anti-hypertensive treatment – %	21.3 (42.9 % of HT-subjects)
Total-cholesterol – mmol/l	5.6 ± 1.1
HDL-cholesterol – mmol/l	1.3 ± 0.4
LDL-cholesterol – mmol/l	3.7 ± 1.0
Triglycerides – mmol/l	1.4 ± 0.8
Glucose – mmol/l	4.8 ± 1.1
Diabetes Mellitus – %	5.9
Hyperlipidemia – %	27.6
BMI – kg/m^2	27.1 ± 4.6
WHR	0.88 ± 0.1
Trunk fat mass – kg	14.0 ± 5.5
Total fat mass – kg	24.3 ± 9.3
Current smokers – %	37.0
Former smokers – %	29.9
Alcohol use – units/week	4.3 ± 9.2

Values are presented as percentage or mean ± SD. SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; WHR, waist to hip ratio.

Table 3. Total number of individuals, transformations and covariates included in heritability analyses

Phenotype	n	Transformation	Model 1	Model 2
SBP	928	ln	age and sex	age, sex, age^2, age x sex interaction term, anti-hypertensive medication, trunk fat mass, total fat mass, HDL-cholesterol, total cholesterol, alcohol intake and glucose levels
DBP				
MAP				
PP				

Results

In total 928 participants were available for analysis. As they were recruited from one extended pedigree, a large number of relative pairs were included. Information on 907 first-degree relative pairs, 659 second-degree relative pairs and 2370 third-degree relative pairs was available for this study (table 1). In 349 subjects no evidence for inbreeding was detected based on genealogy. In the remaining 579 subjects the mean inbreeding coefficient was 0.0086 (0.4.10-6 – 0.0436), slightly higher than that of a marriage of second cousins once removed.

Table 2 presents the general descriptives of the total study population. The mean age was 54 years, but as participants were ascertained within three generations, the age range was very broad (18-92 years). Mean systolic blood pressure was 141.4 ± 15.2 mmHg and mean diastolic blood pressure was 80.2 ± 10.1 mmHg. The prevalence of hypertension was 46.3 %, and 42.9 % of the hypertensive participants were currently on anti-hypertensive treatment (21.3 % of total study population). The prevalence of diabetes mellitus was 5.9 % and the prevalence of hyperlipidemia was 27.6 %. Furthermore, over one third of the population was a current smoker and about one third was a former smoker.

In table 3 total numbers of individuals, transformations and the significant covariates included in the heritability analyses are presented. Besides age and sex, anti-hypertensive medication use, trunk and total fat mass, total cholesterol and HDL-cholesterol, fasting glucose levels and alcohol intake had significant effects on SBP, DBP, MAP and PP.

Model-fit statistics for all four blood pressure traits (in model 1 and 2) are presented in table 4. A general model, including both polygenic and sibship effects, was the best model for SBP and PP. For both traits, the hypotheses of no sibship or no polygenic effects were rejected. For DBP and MAP the likelihood of the models including both sibship and polygenic effects and a model including polygenic effects only, were almost identical. Thus, the effect of the sibship parameter for these traits was negligible. Results of hypothesis testing were the same for the age and sex adjusted and multivariate adjusted analyses.

In table 5 the components of variance for SBP, DBP, MAP and PP are presented. The proportion of phenotypic variance explained by (environmental) covariates, additive genetic effects (heritability), sibship effects and residual environmental effects are presented for model 1 and model 2. Under both models, heritability estimates for SBP, DBP, MAP and PP were significant, and ranged from 0.25 for SBP to 0.37 for DBP (model 2). Sibship effects were significant for SBP and PP (0.09 and 0.10, respectively) (model 2). For DBP and MAP no significant sibship effects were observed. Covariates explained about 16 % of the variance of DBP and up to 35 % of the variance of SBP.

Table 4. Model fit statistics for SBP, DBP, MAP, PP

		SBP			DBP			MAP			PP		
	df	-2 ln L	X^2	p	-2 ln L	X^2	p	-2 ln L	X^2	p	-2 ln L	X^2	p
Model 1													
General	1	2627.4			2504.6			2384.0			3683.0		
c=0, h=0	2	2656.2	28.8	<0.001	2528.8	24.2	<0.001	2409.8	25.8	<0.001	3716.4	33.4	<0.001
c=0	1	2631.6	4.2	0.04	2504.6	0	0.5	2384.4	0.4	0.5	3687.	4.4	0.04
h=0	1	2634.8	7.4	0.003	2517.8	13.2	<0.001	2395	11.0	<0.001	3691.8	8.8	0.002
Model 2													
General	1	2462.8			2367.0			2224.0			3527.2		
c=0, h=0	2	2491.8	29.0	<0.001	2393.8	26.8	<0.001	2252.6	28.6	<0.001	3557.6	30.4	<0.001
c=0	1	2466.6	3.8	0.05	2367.5	0.5	0.40	2225.0	1.0	0.15	3531.4	4.2	0.04
h=0	1	2469.0	6.3	0.005	2379.2	12.2	<0.001	2233.8	9.8	<0.001	3535.8	8.6	0.001

General model: sibling and polygenic effects, c=0: no sibling effects, h=0: no polygenic effects.

Table 5. Heritability estimates and proportion of variance explained by sibship and covariate effects for SBP, DBP, MAP and PP

		Model 1				Model 2			
Phenotypes	n	h^2 ± se	c^2 ± se	% variance due to covariates	variance Residual	h^2 ± se	c^2 ± se	% variance due to covariates	Residual variance
SBP	928	0.24 ± 0.09**	0.11 ± 0.06*	28.7	0.36	0.25 ± 0.10**	0.10 ± 0.06*	34.9	0.30
DBP	928	0.35 ± 0.09†	0	9.8	0.55	0.37 ± 0.09**	0	15.5	0.48
MAP	928	0.30 ± 0.09**	0	19.4	0.51	0.32 ± 0.09†	0	26.5	0.41
PP	928	0.27 ± 0.09*	0.10 ± 0.06*	30.3	0.33	0.28 ± 0.10**	0.09 ± 0.06*	34.0	0.29

h^2: additive genetic effects (heritability), c^2: sibship effects. Model 1: Age and sex adjusted.

*Model 2: Multivariate adjusted. Significance levels: *0.01<p<0.05, **0.001<p<0.01, †p<0.001.*

Table 6. Phenotypic, genetic and environmental correlations between SBP, DBP, MAP and PP

	ρ_P	ρ_G	ρ_E
SBP-DBP	0.68 ± 0.03	0.93 ± 0.13	0.60 ± 0.05
SBP-MAP	0.90 ± 0.01	0.98 ± 0.04	0.89 ± 0.02
SBP-PP	0.80 ± 0.02	0.70 ± 0.11	0.84 ± 0.03
DBP-MAP	0.90 ±0.01	0.93 ± 0.03	0.88 ± 0.02
DBP-PP	0.15 ± 0.05	0.05 ± 0.22*	0.19 ± 0.09
MAP-PP	0.52 ± 0.03	0.64 ± 0.21	0.50 ± 0.06

*ρ_P is the phenotypic correlation, ρ_G is the genetic correlation, ρ_E is the environmental correlation. *All correlations are significant with p<0.001, except for ρ_P and ρ_G between DBP and PP.*

Table 6 shows the results of the bivariate analyses. All correlations were positive and significant, except for the genetic correlation between DBP and PP. The phenotypic correlations ranged between 0.15 for DBP-PP (not significant) and 0.90 for SBP-MAP and DBP-MAP. Genetic correlations between SBP, DBP and MAP were very high (0.93-0.98), whereas genetic correlations between PP and the other blood pressure traits were considerably lower (0.05-0.70). Environmental correlations ranged widely between 0.19 for DBP-PP and 0.89 for SBP-MAP.

Discussion

In this study we investigated to what extent genetic and environmental factors contribute to blood pressure variance in 928 members of an extended pedigree living in a genetically isolated Dutch community. Furthermore, we estimated the phenotypic, genetic and environmental correlations between SBP, DBP, MAP and PP. General descriptives of our study population indicate that this community is characterized by a relative adverse cardiovascular risk profile, including obesity, hyperlipidemia, and a high prevalence of hypertension. Inbreeding effects may partly explain the increased prevalence of hypertension, due to an increased frequency of recessive deleterious alleles as a result of increased homozygosity in our population. In support of this finding, several studies of small inbred communities world wide also reported an increased prevalence of hypertension.[22-26]

We have simultaneously estimated the proportion of blood pressure variance explained by the additive effects of genes (heritability), sibship effects (including shared environment and genetic dominance effects) and various environmental covariates. Additive genetic, sibship and covariate effects together accounted for 50 to 70 % of the total blood pressure variance observed in our population. All our heritability estimates were significant and ranged between 0.25 for SBP and 0.37 for DBP. Although differences in study design, exposure to environmental risk factors, and adjustments for

covariates make a direct comparison of our results with that of other studies difficult, our heritability estimates are within the high range of those reported in other family-based studies, which range from 0.15 to 0.40.[5,7,8,27-29].

Covariates accounted for about 16 % of DBP variance and up to one third of SBP and PP variance. Besides age and sex, total body fat and trunk body fat mass, fasting glucose levels and alcohol intake were the most important covariates influencing blood pressure in this population.

As our heritability estimates were based on first, second and third degree relatives we diminished the effects of shared familial environment on these estimates. In order to further reduce confounding by shared environmental factors, we modeled a parameter based on sibships. However, siblings do not only share a large part of their (childhood) environment but also share a large part of their genes. In this respect, sibships may contribute to the dominance genetic variance of a trait, because they can potentially share two alleles identical by descent (IBD) if they inherited the same two alleles from the same two parents at a specific locus. Therefore, our sibship parameter does not only reflect the effects of shared environment but also includes possible genetic dominance effects on blood pressure variance in our population. This sibship parameter explained a small proportion of SBP and PP variance ($\pm$10 %), and no sibship effects were observed for DBP and MAP. Significant genetic dominance effects for SBP in an isolated population have been observed by Abney et al.[28,30] Tambs et al. have also reported evidence for sibship effects on SBP variance in a large Norwegian population-based survey.[31] In addition, several studies have reported evidence for major (recessive) genes influencing SBP.[30,32,33] However, by modeling this sibship parameter, we were not able to determine the relative contribution of genetic dominance effects, if any, to that of shared environmental effects in siblings. Future complex segregation or linkage analysis will be needed in order to generate a more precise estimate of the magnitude of genetic dominance effects influencing blood pressure variance in our study population.

Although were able to explain up to 70 % of the blood pressure variance in our study population, a large proportion (30-50 %) of all blood pressure variance was ascribed to residual environmental variance. This may not only reflect random environmental variation but also the effects of unexplained gene-environment and gene-gene interactions. Furthermore, we were not able to assess the effects of all possible environmental risk factors influencing blood pressure. For example, we did not have information on dietary habits or level of physical activity, which are both associated with blood pressure levels. Although we believe that a large part of the effects of the above-mentioned parameters are reflected in our body mass indices (trunk fat mass and total body fat mass), dietary habits and physical activity may have influenced blood pressure variance in our population independent of their effects on body composition.

We observed high genetic correlations between SBP, DBP and MAP, indicating that these traits may share a common genetic background. In other words, the genes that influence SBP variance also influence DBP and MAP variance or are in strong linkage disequilibrium with each other. In contrast, the genetic correlations between PP and the other blood pressure traits were lower or even absent (PP and DBP). This suggests the existence of an independent set of genes influencing PP variance. Pulse pressure is an important measure of arterial stiffness and a strong predictor of cardiovascular morbidity and mortality, independent of blood pressure.[34] Hence, significant heritability estimates for PP and the results of our bivariate analyses, make this trait a suitable candidate for future genetic analyses aimed at identifying genes involved in arterial stiffness.

We feel the strength of our study lies within its population-based nature, embedded in a family-based study design. The extended pedigree structure of our population enabled us to include a broad range of different types of relatives in our heritability analyses. We believe this will have reduced the confounding effects of shared environmental factors on our heritability estimates. In addition, we introduced a parameter based on shipships, which prevented wrongful inflation of our heritability estimates due to shared environmental effects. Furthermore, the availability of information on various environmental covariates of all members in the extended pedigree enabled us to estimate the relative contribution of environmental factors on blood pressure variance in this population. Finally, the members of our extended pedigree represent a random sample of our study population and were not ascertained through persons with extreme blood pressure values. This has permitted us to make inferences about the relative importance of genetic and environmental factors on blood pressure at the population level.

In conclusion, the presence of considerable genetic effects on various blood pressure phenotypes in 928 participants of the ERF study provides a rationale and impetus to further explore the genetic background of blood pressure regulation, and hopefully identify new susceptibility genes influencing blood pressure variance in this population. Moreover, significant heritability and evidence of a distinct genetic background for pulse pressure, may offer the opportunity to uncover susceptibility genes related to arterial stiffness in future linkage and segregation analyses.

References

1. Snieder H, Harshfield GA, Treiber FA. Heritability of blood pressure and hemodynamics in African- and European-American youth. *Hypertension.* 2003;41:1196-201.
2. Rotimi CN, Cooper RS, Cao G, Ogunbiyi O, Ladipo M, Owoaje E, Ward R. Maximum-likelihood generalized heritability estimate for blood pressure in Nigerian families. *Hypertension.* 1999;33:874-8.

3. An P, Rice T, Gagnon J, Borecki IB, Perusse L, Leon AS, Skinner JS, Wilmore JH, Bouchard C, Rao DC. Familial aggregation of resting blood pressure and heart rate in a sedentary population: the HERITAGE Family Study. Health, Risk Factors, Exercise Training, and Genetics. *Am J Hypertens.* 1999;12:264-70.
4. Livshits G, Gerber LM. Familial factors of blood pressure and adiposity covariation. *Hypertension.* 2001;37:928-35.
5. Knuiman MW, Divitini ML, Welborn TA, Bartholomew HC. Familial correlations, cohabitation effects, and heritability for cardiovascular risk factors. *Ann Epidemiol.* 1996;6:188-94.
6. Vinck WJ, Fagard RH, Loos R, Vlietinck R. The impact of genetic and environmental influences on blood pressure variance across age-groups. *J Hypertens.* 2001;19:1007-13.
7. Mitchell BD, Kammerer CM, Blangero J, Mahaney MC, Rainwater DL, Dyke B, Hixson JE, Henkel RD, Sharp RM, Comuzzie AG, VandeBerg JL, Stern MP, MacCluer JW. Genetic and environmental contributions to cardiovascular risk factors in Mexican Americans. The San Antonio Family Heart Study. *Circulation.* 1996;94:2159-70.
8. North KE, Howard BV, Welty TK, Best LG, Lee ET, Yeh JL, Fabsitz RR, Roman MJ, MacCluer JW. Genetic and environmental contributions to cardiovascular disease risk in American Indians: the strong heart family study. *Am J Epidemiol.* 2003;157:303-14.
9. Perusse L, Rice T, Bouchard C, Vogler GP, Rao DC. Cardiovascular risk factors in a French-Canadian population: resolution of genetic and familial environmental effects on blood pressure by using extensive information on environmental correlates.
   Am J Hum Genet. 1989;45:240-51.
10. Vaessen N, Heutink P, Houwing-Duistermaat JJ, Snijders PJ, Rademaker T, Testers L, Batstra MR, Sandkuijl LA, van Duijn CM, Oostra BA. A genome-wide search for linkage-disequilibrium with type 1 diabetes in a recent genetically isolated population from the Netherlands. *Diabetes.* 2002;51:856-9.
11. van Gent CM, van der Voort HA, de Bruyn AM, Klein F. Cholesterol determinations. A comparative study of methods with special reference to enzymatic procedures. *Clin Chim Acta.* 1977;75:243-51.
12. Neeley WE. Simple automated determination of serum or plasma glucose by a hexokinase-glucose-6 -phosphate dehydrogenase method. *Clin Chem.* 1972;18:509-15.

13. Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care.* 2003;26 Suppl 1:S5-20.
14. 1999 World Health Organization-International Society of Hypertension Guidelines for the Management of Hypertension. Guidelines Subcommittee. *J Hypertens.* 1999;17:151-83.
15. 2003 European Society of Hypertension-European Society of Cardiology guidelines for the management of arterial hypertension. *J Hypertens.* 2003;21:1011-53.
16. Almasy L, Blangero J. Multipoint quantitative-trait linkage analysis in general pedigrees. *Am J Hum Genet.* 1998;62:1198-211.
17. Self SG LK. Asymptotic properties of maximum likelihood ratio tests under nonstandard conditions. *J Am Stat Assoc.* 1987;82:605-610.
18. Almasy L, Dyer TD, Blangero J. Bivariate quantitative trait linkage analysis: pleiotropy versus co-incident linkages. *Genet Epidemiol.* 1997;14:953-8.
19. Williams JT, Van Eerdewegh P, Almasy L, Blangero J. Joint multipoint linkage analysis of multivariate qualitative and quantitative traits. I. Likelihood formulation and simulation results. *Am J Hum Genet.* 1999;65:1134-47.
20. DS F. *Introduction to quantitative genetics.* London; 1989.
21. Luynch M WB. *Genetics and data analysis of quantitative traits.* Sunderland, MA: Sinauer; 1998.
22. Rudan I, Smolej-Narancic N, Campbell H, Carothers A, Wright A, Janicijevic B, Rudan P. Inbreeding and the genetic complexity of human hypertension. *Genetics.* 2003;163:1011-21.
23. Thomas JD, Doucette MM, Thomas DC, Stoeckle JD. Disease, lifestyle, and consanguinity in 58 American Gypsies. *Lancet.* 1987;2:377-9.
24. Martin AO, Kurczynski TW, Steinberg AG. Familial studies of medical and anthropometric variables in a human isolate. *Am J Hum Genet.* 1973;25:581-93.
25. Hurwich BJ, Rosner B, Nubani N, Kass EH, Lewitter FI. Familial aggregation of blood pressure in a highly inbred community, Abu Ghosh, Israel. *Am J Epidemiol.* 1982;115:646-56.
26. Wahid Saeed AA, al Shammary FJ, Khoja TA, Hashim TJ, Anokute CC, Khan SB. Prevalence of hypertension and sociodemographic characteristics of adult hypertensives in Riyadh City, Saudi Arabia. *J Hum Hypertens.* 1996;10:583-7.
27. Hsueh WC, Mitchell BD, Aburomia R, Pollin T, Sakul H, Gelder Ehm M,

Michelsen BK, Wagner MJ, St Jean PL, Knowler WC, Burns DK, Bell CJ, Shuldiner AR. Diabetes in the Old Order Amish: characterization and heritability analysis of the Amish Family Diabetes Study. *Diabetes Care.* 2000;23:595-601.

28. Ober C, Abney M, McPeek MS. The genetic dissection of complex traits in a founder population. *Am J Hum Genet.* 2001;69:1068-79.
29. Adeyemo AA, Omotade OO, Rotimi CN, Luke AH, Tayo BO, Cooper RS. Heritability of blood pressure in Nigerian families. *J Hypertens.* 2002;20:859-63.
30. Abney M, McPeek MS, Ober C. Broad and narrow heritabilities of quantitative traits in a founder population. *Am J Hum Genet.* 2001;68:1302-7.
31. Tambs K, Moum T, Holmen J, Eaves LJ, Neale MC, Lund-Larsen G, Naess S. Genetic and environmental effects on blood pressure in a Norwegian sample. *Genet Epidemiol.* 1992;9:11-26.
32. Cheng LS, Livshits G, Carmelli D, Wahrendorf J, Brunner D. Segregation analysis reveals a major gene effect controlling systolic blood pressure and BMI in an Israeli population. *Hum Biol.* 1998;70:59-75.
33. Chien KL, Yang CY, Lee YT. Major gene effects in systolic and diastolic blood pressure in families receiving a health examination in Taiwan. *J Hypertens.* 2003;21:73-9.
34. Franklin SS, Khan SA, Wong ND, Larson MG, Levy D. Is pulse pressure useful in predicting risk for coronary heart Disease? The Framingham heart study. *Circulation.* 1999;100:354-60.

4.2. HYPERTENSION IN A DUTCH GENETICALLY ISOLATED POPULATION

Background: Identifying genes involved in blood pressure regulation has proven difficult. Recently, genetically isolated populations have come to the attention of genetic researchers, as they may have valuable properties increasing the chance of identifying susceptibility genes for complex diseases such as hypertension.

Methods: We studied the familial aggregation and general descriptives of 366 hypertensive participants in a Dutch genetically isolated population. Blood pressure, anthropometric and laboratory parameters were measured, and medical and family history was assessed in a personal interview with a research physician. The genealogical background of all participants was traced back to 1600 with the help of church and municipal records. Inbreeding and kinship coefficients were calculated and compared to that of a normotensive control group from the same population (PEDIG software).

Results: About half of the study population was on anti-hypertensive treatment, and median age-at-diagnosis of hypertension was 50 years. Metabolically linked risk factors were present in about 80 % of the study population, the most prevalent ones being obesity and overweight. The hypertensive study population was more closely related and significantly more inbred than a normotensive control group. Sixty percent of the hypertensive participants could be linked to a common ancestor within 10 generations.

Conclusion: This genetically isolated population may offer opportunities to identify new susceptibility genes for hypertension as its population structure permits to use promising statistical tools to map genes in complex diseases. In addition, inbreeding effects may have increased the chance to discover recessive mutations involved in blood pressure regulation in this population.

Introduction

Studying the genetics of hypertension has proven difficult. Although 30 to 40 % of blood pressure variation in the population is thought to have a genetic basis, studies aimed to identify new genes involved in blood pressure regulation have not been very successful.[1] Several Mendelian forms of hypertension have been identified, but these forms are rare and not likely to explain blood pressure variation at the population level. In the majority of participants, high blood pressure is probably the outcome of the additive effects of multiple genes with complex gene-gene and gene environment interactions.

Various approaches to identify new genes for hypertension have been explored, including studies of candidate genes and whole genome searches (genome scans). Although candidate gene studies have yielded some promising results, they are restricted to variants in genes influencing physiological pathways known to be involved in blood pressure regulation. Appreciation of these limitations has led to the interest in conducting genome scans in the hope to identify new genes involved in blood pressure regulation.[2] Especially population isolates are thought to offer a promising setting for identifying new genes involved in complex diseases.[3-5] The simple population history of these isolates, characterized by a small number of founders and considerable population growth with limited inward and outward migration, is assumed to have reduced the genetic variability underlying complex traits. Availability of well-documented genealogical records allows for the construction of extended and multigenerational pedigrees, including many affected individuals. This allows for the application of specific statistical tools when analyzing the genetic data, such as identity by descent (IBD) mapping and haplotype-sharing methods.[6-8] Furthermore, inbreeding effects may increase the opportunity to map recessive genes.

In this paper we describe the population structure and give the general descriptives of 366 hypertensive patients identified in a recently genetically isolated population in the Southwest part of the Netherlands.

Methods

Setting

This study was conducted in a genetically isolated community in the Southwest part of the Netherlands. Around 1750 this population consisted of a mere 150 individuals. Demographically, this population is characterized by minimal inward migration and rapid population growth over the last two centuries. Descendants of this population, about 20.000 individuals, are now scattered over eight adjacent villages.

Enrollment of participants

Participants were ascertained as part of a research program called Genetic Research in Isolated Populations (GRIP), which aims to identify genetic risk factors in the

development of complex disorders.[9] The medical ethical committee of the Erasmus Medical Center Rotterdam approved the scientific protocol of GRIP. Participants were selected either through general practitioners or ascertained when visiting our research center as a participant of the Erasmus Rucphen Family study (ERF), a family-based study cohort study that is embedded in the GRIP research program.[10] The first 97 participants that were ascertained through their GP were collected between December 2001 and June 2002, before the start of the ERF study. The remaining 269 participants were all participants of the ERF study and included in this study between June 2002 and December 2003.

In all participants the diagnosis hypertension was based on their medical history and/or anti-hypertensive medication use, which was verified by a research physician. Hypertension was defined as a systolic blood pressure above 140 mmHg or a diastolic blood pressure above 90 mmHg, and or use of anti-hypertensive medication. These inclusion criteria are in accordance with the 1999 WHO criteria and 2003 ESH/ESC criteria.[11,12] Age-at-diagnosis was based on the medical history of the patient, either defined as date of first prescription of blood pressure lowering medication or the first recorded diagnosis of hypertension made by the GP. Participants with identifiable causes of hypertension (e.g. kidney disease) were excluded from this study. In order to increase the homogeneity of our study population and to reduce the chance of including secondary forms of hypertension, only participants born after 1940 were included.

Data collection.

A research physician assessed all participants in a personal interview, either at home or at the research center. During this visit, information concerning smoking behavior, alcohol use, medication use and medical history was recorded. In addition, all participants were asked to fill out an extensive questionnaire on their medical and family history (including genealogical information). Blood pressure was measured twice, separated by a five-minute interval, in the sitting position at the right upper arm, using an automatic device (OMRON 711, automatic IS). The mean of these two measurements was used for analyses. Body height and weight were measured and body mass-index was calculated (kg/m^2). Fasting blood samples were drawn for the storage of DNA and the measurement of lipids, glucose levels, creatinine, sodium, potassium and plasma albumin levels according to a standardized procedure.[13,14] Serum samples were obtained from whole blood after clotting, and plasma samples were obtained from whole blood collected in disodium EDTA. Hyperlipidemia was defined as the use of lipid lowering medication or total cholesterol levels between 6.5-9.0 mmol/l and a total cholesterol-HDL cholesterol ratio above 5.0, or total cholesterol below 6.5 mmol/l and a ratio above 8.0, or total cholesterol above 9.0 mmol/l, independent of the ratio, or triglycerides above 4.0 mmol/l. These criteria are in accordance with those of the Dutch college of general practitioners.

Diabetes mellitus was defined as the use of blood glucose-lowering medication or fasting serum glucose level above 7.0 mmol/l, and glucose-intolerance was defined as a fasting serum glucose level above 6.1 mmol/l and below 7.0 mmol/l.[15] We classified participants as overweight when their BMI was above 25.0 kg/m^2 and below 30.0 kg/m^2, and as obese when their BMI was above 30.0 kg/m^2. These definitions are in accordance with internationally accepted criteria.[16,17]

Genealogical data collection

Genealogical information, including name, date and place of birth of parents and grandparents, was collected for all participants. Church and municipal registers for this population are readily available and date back to 1800. Furthermore, a large computerized genealogical database including about 63.000 individuals living in our research area was available. This database consists of genealogical information dating back to 16th century. By means of these local municipal, church and computerized registers, information on the genealogical background of the participants could be extended up to 16 generations.

Statistical analysis

General descriptives of the study population, stratified by gender, were compared using χ^2- statistics for categorical and dichotomous variables and unpaired T-test and Mann-Whitney test for continuous variables.

Inbreeding coefficients and kinship coefficients were calculated using PEDIG software (Bouchard D: http://dga.jouy.inra.fr/sgqa/diffusions/pedig/pedigE.htm). The inbreeding coefficient is the probability that the two alleles at some locus in an individual are identical by descent, meaning that the identical alleles are inherited from a common ancestor. We constructed three inbreeding categories: no inbreeding, moderate inbreeding and high inbreeding. Moderate inbreeding was defined as an inbreeding coefficient higher than zero and lower than 0.5^7 (equal to a marriage of second cousins once removed) and high inbreeding was defined as an inbreeding coefficient above 0.5^7. Percentages of hypertensive participants in each inbreeding category were compared to that of a normotensive control group from the same population (spouses of participants of the ERF study, n=193). A χ^2-statistic was used to compare the number of hypertensive participants and control subjects in each inbreeding category. Kinship coefficients are a measure of the degree of relationship between two persons. We calculated kinship coefficients based on a pedigree of the total population for both the hypertensive participants and the normotensive control group. Distributions of kinship coefficients between these two groups were compared using a χ^2-statistic.

Results

In total, 366 hypertensive patients participated in the study. General descriptives of the participants, stratified by gender, are presented in table 1. Men and women were about

equally represented in our study population (48.5 % and 51.5 % respectively). The median age was 54 years for women and 55 years for men. Mean SBP was the same for both sexes (± 155.0 mmHg). Diastolic blood pressure was significantly lower in women compared to men ($p<0.001$). Mean BMI was similar for men and women (± 28.5 kg/m^2). Fasting glucose levels were significantly higher in men than women ($p<0.01$). HDL-cholesterol levels were significantly higher in women than men (1.3 mmol/l and 1.2 mmol/l, respectively) and triglyceride-levels were significantly lower in women than men (1.5 mmol/l vs. 1.8 mmol/l). Creatinine levels were higher in men (86.3 µmol/l) than women (69.4 µmol/l). Total cholesterol, sodium and potassium levels did not differ significantly between sexes. The prevalence of myocardial infarction and stroke was significantly higher in men than women. Noteworthy, about 70 % of the total study population was either a current smoker or a former smoker. Men used more alcohol than women, as about 15 % of the men drank more than three alcoholic beverages a day, while in women this was 4 % ($p<0.01$).

Table 1. General descriptives study population

	Men	Women
Number – (%)	177 (48.5)	188 (51.5)
Age – yrs	55 (47-58)	54 (49-59)
SBP – mmHg	155.6 ± 19.2	154.8 ± 19.1
DBP – mmHg	89.4 ± 11.0	84.1 ± 12.0*
BMI – kg/m^2	28.6 ± 4.4	28.4 ± 5.3
Glucose – mmol/l	5.7 ± 1.6	5.2 ± 1.3**
Total cholesterol – mmol/l	5.6 ± 1.2	5.6 ± 1.0
HDL-cholesterol – mmol/l	1.2 ± 0.6	1.3 ± 0.4**
Triglycerides – mmol/l	1.8 ± 1.1	1.5 ± 0.6**
Sodium – mmol/l	140.2 ± 2.1	139.7 ± 3.0
Potassium – mmol/l	4.3 ± 0.4	4.1 ± 0.4
Creatinine – µmol/l	86.3 ± 15.0	69.4 ± 14.6**
Myocardial infarction – (%)	7.2	0.6**
CVA – (%)	5.2	1.6
Current smokers – (%)	35.6	45.7
Former smokers – (%)	35.0	25.5
Alcohol use – > 3 U/d	14.7	3.7**

*All values are presented as mean ± standard deviation or percentage, except for age, which is presented as median (interquartile range). *p<0.001 and **p<0.01, significantly different from men.*

In table 2, clinical characteristics regarding age-at-diagnosis, medication use and family history of hypertension are presented. The median age-at-diagnosis of hypertension was about 50 years for men and women. About 53 % of the male participants and 57 % of the female participants received anti-hypertensive treatment. Within this group, 75-80 % was treated with one or two forms of blood pressure lowering medication and 20-25 % received three or more forms of anti-hypertensive medication. β-blockers and ACE-inhibitors were prescribed most in men, while in women, β-blockers and diuretics were most frequently used. ACE-inhibitor use was significantly higher among men than women (38.8 % and 23.0 % respectively). Angiotensin receptor blocker use was higher in women and men used Ca-blockers more frequently. 67 % of the hypertensive patients in our study had at least one first-degree family member with hypertension.

Table 3 presents the prevalence and number of metabolically linked risk factors in our study population. In hypertensive men, 42.1 % was overweight and 36.5 % was obese, in hypertensive women these percentages were 33.5 and 35.1 respectively. Glucose intolerance was higher among men (8.9 %) than women (5.9 %). The prevalence of diabetes mellitus was about 10 % for both sexes. Hyperlipidemia was present in about one third of the men and about one fourth of the women. In about 20 % of the participants, no metabolically linked risk factors were present. Half of the participants had one additional risk factor. The presence of two other risk factors was significantly more frequent in

Table 2. Age at diagnosis, medication use and family history of hypertension

	Men	Women
Age-at-diagnosis hypertension - yrs	49 (40-55)	50 (42-56)
Anti-hypertensive treatment – (%)	53.1	57.0
Number of anti-hypertensive medication – (%)		
1-2	75.3	80.1
3 or more	24.7	19.9
Type of medication – (%)		
-ß-blocker	48.8	48.3
-Diuretic	36.6	43.7
-ACE-inhibitor	38.8	23.0*
-Angiotensin receptor blocker	22.5	29.9
-Ca-blocker	37.5	26.4
First-degree relatives with hypertension – (%)	67.0	67.6

*All values presented as mean ± standard deviation or percentage, except age-at-diagnosis hypertension, which is presented as median (interquartile range). *Significantly different from men, p<0.05.*

Table 3. Frequency of metabolically linked risk factors

	Men	Women
Overweight – (%)	42.1	33.5*
Obesitas – (%)	36.5	35.1
Glucose intolerance – (%)	8.9	5.9
Diabetes Mellitus – (%)	12.1	11.8
Hyperlipidemia – (%)	29.9	25.5
Number of risk factors present – (%)		
0	16.9	21.3
1	47.5	53.7
2	27.1	18.6*
3	8.5	6.4

*All values are presented as percentage. *Significantly different from men, p<0.01.*

Table 4. Number of pairs in each kinship coefficient category in hypertensive patients and normotensive controls

	Hypertensive patients	Normotensive controls
0	9775 (17.6)	8407 (45.4)
$0\text{-}0.5^{15}$	3125 (5.6)	749 (4.0)
$0.5^{15}\text{-}0.5^{12}$	9773 (17.6)	1300 (7.0)
$0.5^{12}\text{-}0.5^{9}$	10248 (18.4)	1704 (9.2)
$0.5^{9}\text{-}0.5^{6}$	19577 (35.2)	5686 (30.7)
$0.5^{6}\text{-}0.5^{3}$	2874 (5.2)	661 (3.6)
$0.5^{3}\text{-}1.0$	239 (0.4)	21 (0.1)

Values are absolute number of pairs (percentage).

men than women (27. 1 % and 18.6 %, respectively). Three other risk factors were present in 8.5 % of the men and 6.4 % of the women.

As median inbreeding levels did not differ significantly between men and women, they were pooled in the analyses presented in figure 1. In this figure, percentages of the total study population in each inbreeding category are presented and compared with that of a normotensive control group of the same population. In 23.3 % of the hypertensive population no inbreeding was observed compared to 43.5 % of the control group ($p<0.001$). The number of subjects with moderate inbreeding was also higher in the hypertensive group, although not statistically significant (hypertensive group: 41.0 %

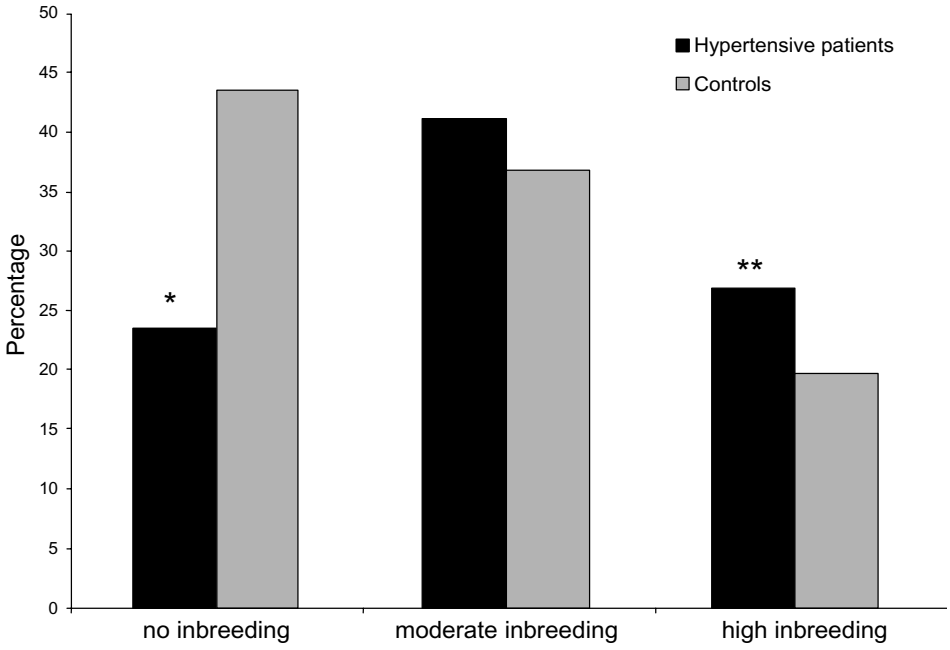


Figure1. Inbreeding levels in hypertensive patients and control group.
*Significantly lower compared to control group, p<0.001. **Significantly higher than control group, p=0.01.

and control group: 36.8 %, p=0.07). The hypertensive group consisted of significantly more participants with high inbreeding coefficients than the control group (26.8 % vs. 19.7 %, p=0.01).

Genealogical work-up of all participants revealed that 229 hypertensive participants (62.5 %) were related to a common ancestor within 10 generations. Eighty-one hypertensive participants out of these 229 participants (22.1 % of total group) were even closer related and could be traced back to a common ancestor within 7 generations. Figure 2 shows the extended pedigree for these 81 participants. Although in this pedigree multiple links be-tween participants exist, only the shortest connection with a common ancestor is presented here for ease of interpretation.

We calculated pairwise kinship coefficients for pairs of patients and for pairs of subjects in the normotensive control group. The results are presented in table 4. In the control group 45.4 % of the pairs did not have any relation with each other, whereas only 17.6 % of the hypertensive pairs were not related to each other (p<0.0001). The percentage of pairs with a kinship coefficient between 0.5^{12}-1.0 was significantly higher for hypertensive patients (59.2 %) than for normotensive controls (43.6 %) (p<0.001).

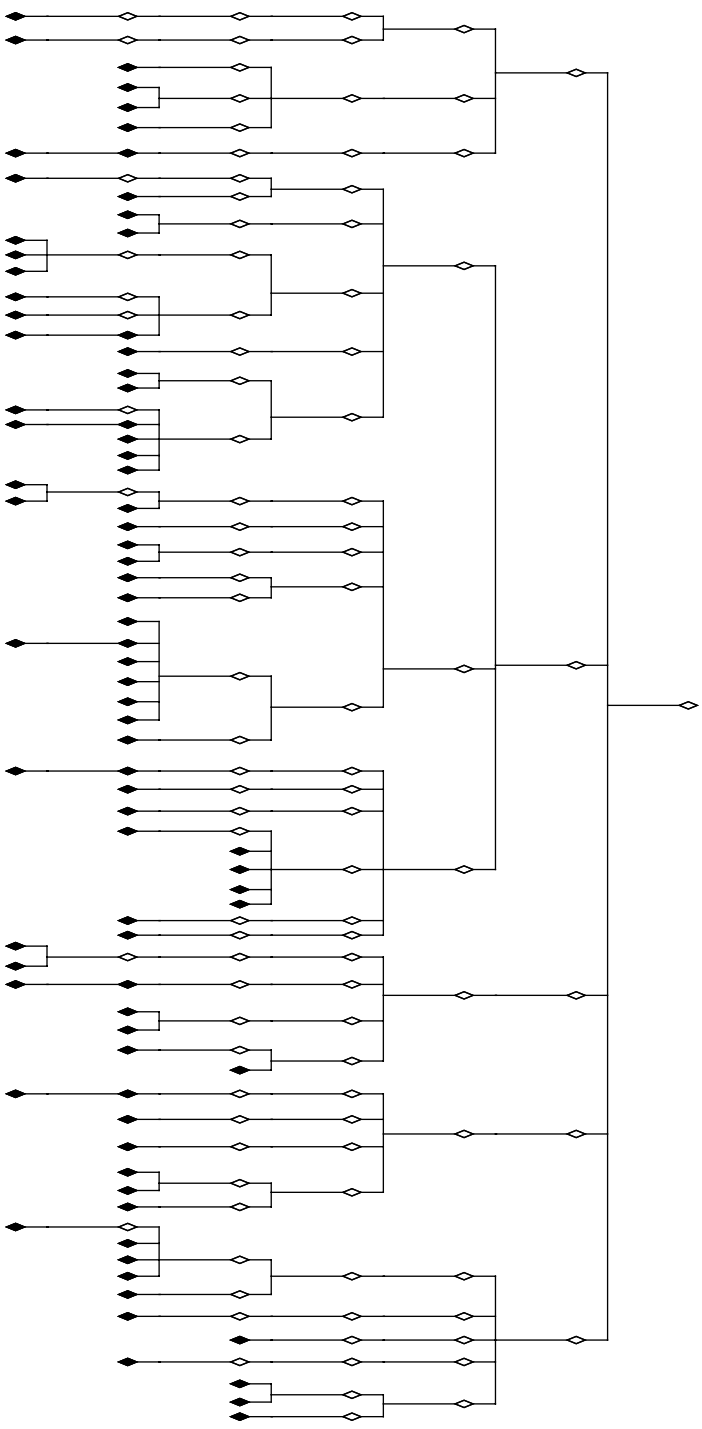


Figure 2. Pedigree of 81 hypertensive patients connected to a common ancestor within 7 generations. Filled symbols represent the hypertensive patients (phenotype of ancestors is not known).

Discussion

We identified 366 hypertensive patients in a recent genetically isolated population. Over 60 % of these patients could be linked to a common ancestor within 10 generations. Hypertensive patients were more closely related than a normotensive control group from the same population. A positive family history of hypertension (first-degree relatives) was present in almost 70 % of the participants. About half of the study population was currently receiving anti-hypertensive treatment. This is higher than recently reported by Schelleman et al., who found in a large Dutch population based survey that, in the same age group as our study population, only 18-30 % of hypertensive patients were treated.[18] This difference is probably due to a relative over sampling of treated participants in our study, as the first 97 participants were selected based on the diagnosis hypertension made by their general practitioner, whereas the other participants were ascertained in a population-based study (ERF study).[10]

The prevalence of metabolically linked risk factors in our study population was considerable, but in line with previous reports from the Framingham Heart Study.[19,20] In fact, only 20 % of the female hypertensives and even less of the male hypertensives did not have any other risk factors. Overweight and obesity were the most frequently observed metabolically linked risk factors in this study population, as 70 % of the hypertensive women were overweight or obese and almost 80 % of the hypertensive men. The second most frequent metabolically linked risk factor was hyperlipidemia, present in 25 % of the men and 30 % of the women, followed by diabetes mellitus and glucose intolerance (21 % and 18 % of men and women, respectively).

In our study, the proportion of hypertensive patients with moderate to high inbreeding was significantly larger than that of a normotensive control group of the same population. In line with this, Rudan et al. observed significant inbreeding effects on SBP and DBP in several villages of Croatian island isolates.[21] They found that persons with higher inbreeding levels also had higher blood pressure levels, and estimated that inbreeding effects may account for about 38 % of all hypertension in their study population. In addition, they observed the highest proportion of hypertensives in villages with the highest inbreeding levels.[22] We recently observed an increased prevalence of hypertension in our population.[10] Higher inbreeding levels in our hypertensive patients compared to a group of normotensive controls could point towards recessive and partially recessive deleterious alleles contributing to blood pressure variance in our hypertensive population. However, the strong familial clustering and the high number of families with patients in multiple generations indicate that most likely besides recessive mutations also dominant mutations play a role in the etiology of hypertension in this population.

As our study was performed within an isolated community, this may offer substantial advantages for a future genome screen aimed to identify new susceptibility genes for

hypertension. First, our participants are likely to share a more common environment and cultural background, which may avoid some of the environmental "noise" influencing complex diseases that are determined by a combination of nurture and nature. Second, this population originated from a limited number of founders and has known considerable population expansion with minimal inward and outward migration. This is likely to have reduced the genetic variability within this population.[4] Finally, the availability of an extended pedigree with multiple affected members, which could all be linked to a common ancestor within 10 generations, offers new statistical tools to map susceptibility genes. Participants within this pedigree are more liable to have hypertension due to the same mutations than unrelated participants in an outbred population. These mutations are likely to be introduced into the pedigree by a common ancestor and may have segregated over successive generations. In addition, participants in this pedigree may not only share a mutation but also considerable parts of DNA surrounding the mutation (genomic haplotype). This assumption forms the basis of the haplotype sharing method or linkage disequilibrium mapping.[6,8,23] The basic principle of this method is that markers located in a shared haplotype will flag the mutation in a genome screen. A big advantage of this method is the need for affecteds only. However, although this method has proven successful for some rare diseases, it remains to be demonstrated for complex diseases such as hypertension.[24,25]

Even though several genome-wide scans for hypertension or blood pressure have been performed, only few studies report significant genome wide linkage.[2] Of interest is that two of these reports come from studies performed in isolated populations.[26,27] Although these results seem promising, the use of population isolates may have its limitations. Causal mutations or susceptibility genes identified in such a specific population may be less important predictors of common disorders in larger more outbred population.

In conclusion, although our hypertensive study population phenotypically does not differ from hypertensive subjects in more outbred populations, we believe this population may offer nice prospects to identify new susceptibility genes for hypertension because of its isolated structure. Furthermore, increased inbreeding levels in this population may offer the opportunity to identify recessive mutations involved in the etiology of hypertension.

References

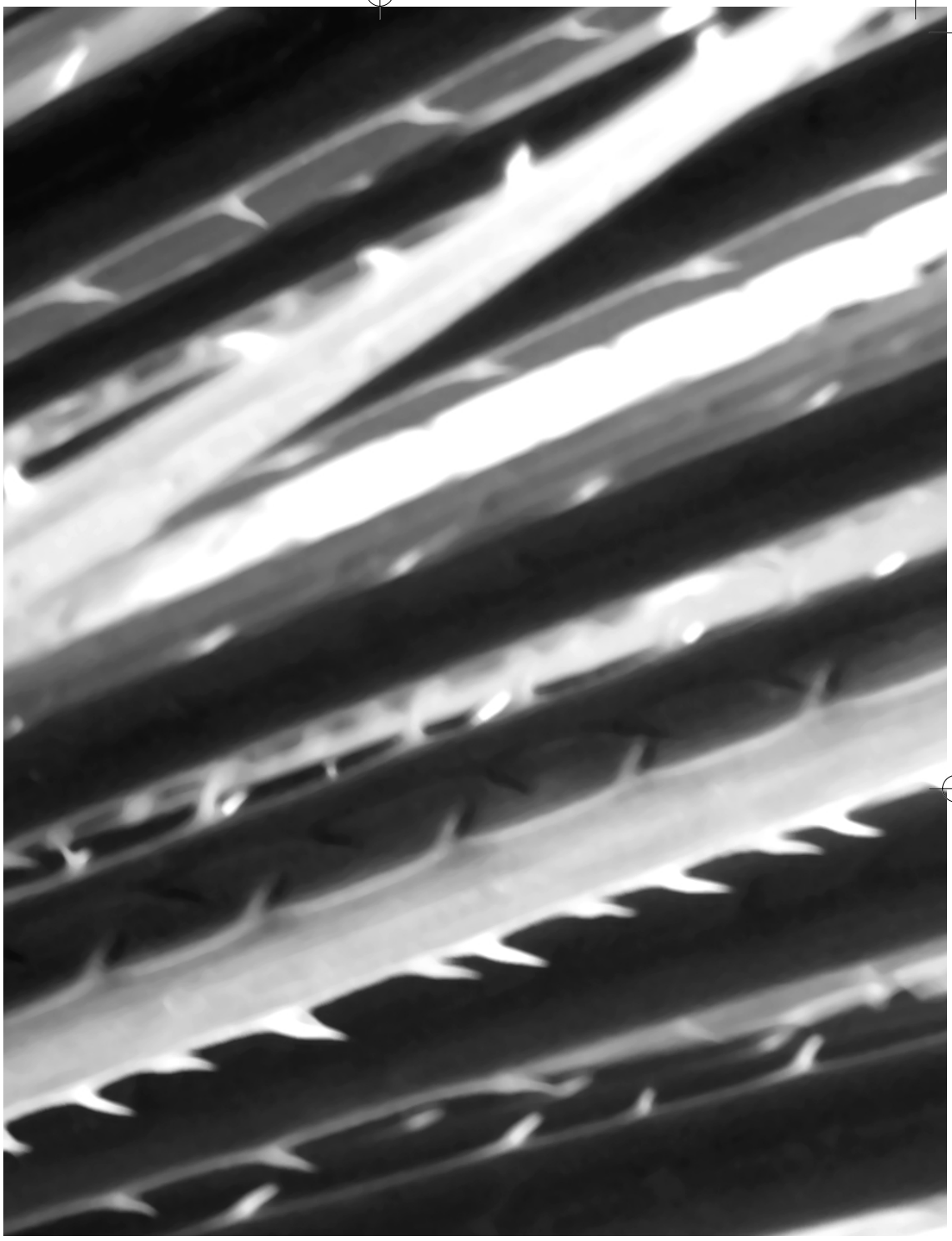
1. Ward R. Familial aggregation and genetic epidemiology of blood pressure. In: BM LJB, ed. *Hypertension: Pathophysiology, Diagnosis and Management.* New York: Raven Press; 1990:811-1000.

2. Samani NJ. Genome scans for hypertension and blood pressure regulation. *Am J Hypertens.* 2003;16:167-71.
3. Shifman S, Darvasi A. The value of isolated populations. *Nat Genet.* 2001;28:309-10.
4. Peltonen L, Palotie A, Lange K. Use of population isolates for mapping complex traits. *Nat Rev Genet.* 2000;1:182-90.
5. Wright AF, Carothers AD, Pirastu M. Population choice in mapping genes for complex diseases. *Nat Genet.* 1999;23:397-404.
6. Service SK, Lang DW, Freimer NB, Sandkuijl LA. Linkage-disequilibrium mapping of disease genes by reconstruction of ancestral haplotypes in founder populations. *Am J Hum Genet.* 1999;64:1728-38.
7. Te Meerman GJ, Van der Meulen MA, Sandkuijl LA. Perspectives of identity by descent (IBD) mapping in founder populations. *Clin Exp Allergy.* 1995;25 Suppl 2:97-102.
8. Te Meerman GJ, Van der Meulen MA. Genomic sharing surrounding alleles identical by descent: effects of genetic drift and population growth. *Genet Epidemiol.* 1997;14:1125-30.
9. Vaessen N, Heutink P, Houwing-Duistermaat JJ, Snijders PJ, Rademaker T, Testers L, Batstra MR, Sandkuijl LA, van Duijn CM, Oostra BA. A genome-wide search for linkage-disequilibrium with type 1 diabetes in a recent genetically isolated population from the Netherlands. *Diabetes.* 2002;51:856-9.
10. Schut AFC, Aulchenko YS, Deinum J, van Rijn MJE, Sayed-Tabatabaei FA, Rivadeneira F,Croes EA, Zillikens MC, Pols HAP, Witteman JCM, Oostra BA, van Duijn CM. Genetic and environmental contributions to blood pressure variance in an extended pedigree of a Dutch founder population. Submitted.
11. 1999 World Health Organization-International Society of Hypertension Guidelines for the Management of Hypertension. Guidelines Subcommittee. *J Hypertens.* 1999;17:151-83.
12. 2003 European Society of Hypertension-European Society of Cardiology guidelines for the management of arterial hypertension. *J Hypertens.* 2003;21:1011-53.
13. van Gent CM, van der Voort HA, de Bruyn AM, Klein F. Cholesterol determinations. A comparative study of methods with special reference to enzymatic procedures. *Clin Chim Acta.* 1977;75:243-51.
14. Neeley WE. Simple automated determination of serum or plasma

glucose by a hexokinase-glucose-6 -phosphate dehydrogenase method. *Clin Chem.* 1972;18:509-15.
15. Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care.* 2003;26 Suppl 1:S5-20.
16. Obesity. Preventing and Managing the Global Epidemic. Report of WHO Consultation on Obesity. Geneva; 1998.
17. NHLBI Obesity Education Initiative Expert Panel on the Evaluation, and Treatment of Overweight and Obesity in Adults. Clinical Guidelines on the Identification of overweight and obesity in adults - the evidence report. *Obes Res.* 1998;6:51S-209S.
18. Schelleman H, Klungel OH, Kromhout D, de Boer A, Stricker BH, Verschuren WM. Prevalence and determinants of undertreatment of hypertension in the Netherlands. *J Hum Hypertens.* 2004;18:317-24.
19. Kannel WB. Blood pressure as a cardiovascular risk factor: prevention and treatment. *JAMA.* 1996;275:1571-6.
20. Kannel WB. Risk stratification in hypertension: new insights from the Framingham Study. *Am J Hypertens.* 2000;13:3S-10S.
21. Rudan I, Smolej-Narancic N, Campbell H, Carothers A, Wright A, Janicijevic B, Rudan P. Inbreeding and the genetic complexity of human hypertension. *Genetics.* 2003;163:1011-21.
22. Narancic NS, Rudan I. Endogamy and variation in blood pressure levels in Croatian island isolates.
J Physiol Anthropol Appl Human Sci. 2001;20:85-94.
23. Van der Meulen MA, te Meerman GJ. Haplotype sharing analysis in affected individuals from nuclear families with at least one affected offspring. *Genet Epidemiol.* 1997;14:915-20.
24. Houwen RH, Baharloo S, Blankenship K, Raeymaekers P, Juyn J, Sandkuijl LA, Freimer NB. Genome screening by searching for shared segments: mapping a gene for benign recurrent intrahepatic cholestasis. *Nat Genet.* 1994;8:380-6.
25. Nikali K, Suomalainen A, Terwilliger J, Koskinen T, Weissenbach J, Peltonen L. Random search for shared chromosomal regions in four affected individuals: the assignment of a new hereditary ataxia locus. *Am J Hum Genet.* 1995;56:1088-95.
26. Kristjansson K, Manolescu A, Kristinsson A, Hardarson T, Knudsen H, Ingason S, Thorleifsson G, Frigge ML, Kong A, Gulcher JR, Stefansson K. Linkage of essential hypertension to chromosome 18q.

Hypertension. 2002;39:1044-9.

27. Angius A, Petretto E, Maestrale GB, Forabosco P, Casu G, Piras D, Fanciulli M, Falchi M, Melis PM, Palermo M, Pirastu M. A new essential hypertension susceptibility locus on chromosome 2p24-p25, detected by genomewide search. *Am J Hum Genet.* 2002;71:893-905.



5. GENERAL DISCUSSION

Introduction

The most optimistic view on the genetics of hypertension is that a relatively small number of common polymorphisms in a number of genes are capable of increasing susceptibility to hypertension. This view is represented in the common disease/common variant hypothesis, which suggests that genes underlying common diseases existed within the founding population of contemporary humans.[1-3] As Homo sapiens expanded outwards from Africa and around the globe, ancient gene variations were also distributed globally. This would mean the existence of a shared, pre-existing set of susceptibility genes for hypertension in the global population. Consequently, the heterogeneity underlying hypertension may be smaller than generally assumed. If the common disease/common variant hypothesis holds, the same evolutionary ancient susceptibility alleles should be detectable in different populations around the world.

An opposing view is that disease susceptibility arose independently in various distinct populations. This implies that blood pressure alleles come from a larger number of younger, less frequent and more population-specific alleles: the common disease/rare variant hypothesis.[4,5] If blood pressure indeed is influenced by a large number of young rare alleles, they will be hard to find, especially when their effects on blood pressure are small and influenced by other alleles or environmental factors. Consequently, hypertension-susceptibility genes may be so numerous and their interactions so varied and context-dependent that there is a unique profile of alleles influencing blood pressure for each population.

Despite these daunting perspectives we accepted the major challenge of finding these alleles and initiated a search for the genetic basis of human hypertension and its cardiovascular sequelae. The results of this search are presented in this thesis. The first part of the thesis describes the findings of the genetic association studies that we conducted in the Rotterdam Study. We assessed the role of two candidate genes in relation to hypertension and three cardiovascular complications of hypertension: atherosclerosis, left ventricular hypertrophy and heart failure. The second part of this thesis encompasses two studies on the heritability of blood pressure and familial aggregation of hypertension in a Dutch genetically isolated population.

In this chapter I will discuss the main findings and place them in a broader perspective. I will address the role of candidate gene studies and the advantages of genetically isolated populations in the search of cardiovascular risk genes. Finally, I will discuss the future prospects of the studies conducted within the framework of this thesis and give a general perspective on the future of genetic research on human hypertension.

Candidate gene studies

Historically, association studies have been the workhorse of studies investigating the genetic basis of complex disease. The main advantage of these studies is the ability to

detect genes with small effects (relative risks), which we expect to be the main contributors to the genetics of complex diseases such as hypertension.[2,6]

The Angiotensin Converting Enzyme Insertion/Deletion Polymorphism

The ACE I/D polymorphism is by far the most studied candidate gene in relation to cardiovascular disease.[7,8] This polymorphism is located in an intronic region of the ACE gene and is most likely a neutral marker in strong linkage disequilibrium (LD) with one or more unknown functional variants located close to or in the ACE gene.[9,10] The ACE I/D polymorphism has consistently been associated with increased serum ACE activity.[11] Findings regarding the ACE gene and blood pressure levels or risk of hypertension, however, have been very inconsistent.[12-17] The fact that the effects of genes are likely to be influenced by environmental factors may explain part of these conflicting findings.[18,19] Genotype-phenotype relations may depend on the environmental background of the population under study. Context-dependent effects of the ACE gene have been reported by Turner et al., who observed that the relation between this gene and blood pressure was dependent on gender, age, and measures of body size.[20]

Previous reports of smoking-dependent effects of the ACE gene in relation to endothelial function and coronary atherosclerosis, motivated us to study the relation between the ACE I/D polymorphism, hypertension and carotid atherosclerosis stratified by smoking status.[21,22] We hypothesized that smoking and the ACE I/D polymorphism might influence blood pressure and the development of carotid atherosclerosis by modulating vasomotor tone and endothelial function. Indeed, persons who smoke and carry at least one copy of the D-allele had significantly higher systolic blood pressure, an increased risk of hypertension and increased carotid intima media thickness. In non- and former smokers, no effect of the ACE genotype was observed. Our findings were strengthened by the observation that both smoking and the ACE genotype significantly influenced serum ACE activity.[23] Thus, ACE activity may be an intermediate phenotype, responsible for the additive effects of the ACE I/D polymorphism and smoking on the vasculature. In a third study we observed that the presence of the D-allele increases the risk of heart failure in hypertensive persons, but not in normotensive persons. This suggests that an effect of the ACE I/D polymorphism on cardiac function may only become clinically relevant under specific haemodynamic conditions in which the cardiac growth machinery is already activated. The results of our studies indicate that the magnitude of the effects of the ACE I/D polymorphism on the risk of cardiovascular disease depends on the presence or absence of other (environmental) risk factors, such as smoking or hypertension.

Insulin-like Growth Factor-I Promoter Polymorphism

Insulin-like growth factor-I (IGF-I) is an important mediator in pathophysiological response to increased blood pressure in the vasculature.[24] In addition, IGF-I is involved in the hypertrophic response of the heart to increased arterial load and mediates tissue

repair and cell proliferation in the ischaemic heart.[25,26] Recently, we found that non-carriers of a 19 CA repeat microsatellite polymorphism (192-bp allele), 1 kb upstream to the IGF-I gene, have 20 % lower levels of circulating IGF-I, an increased risk of diabetes mellitus and lower birthweight.[27,28] As low IGF-I levels have been associated with an increased risk of cardiovascular disease[29-31], this prompted us to study the relation between the IGF-I polymorphism, hypertension, atherosclerosis and left ventricular hypertrophy. We observed an increased carotid intima media thickness and increased aortic pulse wave velocity in hypertensive persons who did not carry a copy of the 192-bp allele. In the second study, we observed that non-carriers of this 192-bp allele showed an increased risk of left ventricular hypertrophy.

With respect to the functionality of this polymorphism, it should be noted that Frayling et al. observed an opposite relation between the IGF-I polymorphism and serum IGF-I levels, and could not confirm our previous associations with height, diabetes mellitus and birthweight. He therefore debated the functional relevance of this polymorphism.[32] Nevertheless, the IGF-I gene is a good candidate gene for blood pressure and cardiac function because of the important role of IGF-I in normal and pathologic cardiovascular physiology.[24,33] In favor of involvement of the IGF-I gene in cardiac function and blood pressure regulation, Nagy et al. reported significant linkage of polymorphic microsatellite markers at IGF-I gene loci to systolic blood pressure and echocardiographically determined cardiac dimensions in a sibpair analysis.[34] Further identification of allelic variants within the IGF-I gene that can be tested in association analyses, eventually coupled with protein expression studies, may confer more insight into the role of this gene in the development of hypertension and its complications.

Validity of genetic association studies

Inconsistent results and absence of reproducibility have compromised the validity of genetic association studies for the detection of genetic variants contributing to complex diseases.[35] Lack of reproducibility is generally attributed to inadequate statistical power, phenotypic and genotypic complexity, and population stratification.[35-38]

The underlying effect-size to be detected in genetic association studies will typically be small as for complex diseases such as hypertension many polymorphisms each with small additive effects are likely to exist. Therefore large case-control samples are needed in order to detect a modest genetic effect. For example, to replicate the initial findings by Jeunemaitre et al. who observed an increased frequency of the 235T allele of the angiotensinogen gene in hypertensive cases compared to normotensive controls, at least 400 cases and 400 controls will be needed in order to have 80 % power to detect a difference of 8 % in the frequency of this allele between hypertensive cases and controls.[39]

As underlined in the discussion on our findings regarding the ACE I/D polymorphism, knowledge about specific environmental risk factors, which may interact with genetic

risk factors, in different study populations is of great importance when initiating or replicating a genetic association study. Gene-environment interactions may give rise to different relationships between genotypic and phenotypic variation in different study populations, and lead to inconsistent outcomes between different genetic association studies.

Population stratification is a problem that may arise in genetic association studies when the study population consists of a mixture of two or more subpopulations with different allele frequencies and disease risks. Large differences in the prevalence of hypertension and the frequency of the angiotensinogen 235T allele between the African population and the Caucasian population, have generated inconsistent results regarding the association between this allele and hypertension.[39-41] To overcome the problem of population stratification a few strategies can be followed. First, cases and controls should be carefully matched by demographic background. Another approach may be the use of anonymous genetic markers to detect population substructures.[42] In family-based association studies the usage of a pseudo case-controls group, based on transmitted and non-transmitted parental alleles, is a frequently used strategy, better known as the transmission disequilibrium test (TDT).[43,44] However, confounding by population substructure is unlikely to be a serious problem in most association studies, as heterogeneity of study populations is usually not very extensive.[36,45]

In order to increase the validity of candidate gene studies several stringent criteria have been proposed.[46] The ideal features of a genetic association study include: "Large sample sizes, small p-values, reported associations that make biological sense and alleles that affect the gene product in a physiological meaningful way. In addition they should include an initial study as well as an independent replication, the association should be observed both in family-based and population-based studies". In my opinion the Rotterdam Study meets the above-mentioned prerequisites and has provided us with a suitable study population and study design for genetic association studies. The Rotterdam Study is a very large population-based study, including over 8000 participants, increasing the chance to detect modest genetic effects with sufficient power. The prospective nature of this study reduces the presence of selection bias, which may confound genetic associations. The careful collection of extensive phenotypic information on all participants offers the opportunity to study the pleiotropic effects of candidate genes and take into account environmental factors influencing genotype-phenotype relations. Finally, the Rotterdam Study provided us with an ethnically homogenous study population as 98 % of the participants are Caucasian and they all live in the same Rotterdam suburb. In addition, the family-based ERF study offers a select opportunity to replicate initial findings of the Rotterdam Study in a linkage analysis.

Genetic research in an isolated population

An alternative approach to testing candidate genes for hypertension is to find "new" genes responsible for blood pressure regulation in a genome-wide search. Although scanning of the entire genome in search for linkage has been very successful for monogenic disorders, linkage approaches have been far from successful for complex genetic diseases. Two of the largest genome scans for blood pressure completed to date, unfortunately, were not able to provide us with more optimistic results. The US National Institute of Health funded Family Blood Pressure Programs (NIH-FBPP), which genotyped a total of 6245 individuals in four multi-center networks, did not find significant linkage of blood pressure to a single locus.[47-52] This program comprises the data of four large studies (GenNet, GENOA, HyperGEN and SAPHIRe), each initially set up independently. Pooling of the individual results of these studies did not enhance any of the initial findings, most likely due to the different populations and study designs that were used in the four studies.[51] The Medical Research Council funded British Genetics of Hypertension study (BRIGHT study), including 3599 hypertensive patients was able to identify only one single locus on chromosome 6 that attained significant genome-wide linkage.[53] Many other, smaller studies that performed genome-wide searches for hypertension or blood pressure in various study populations have provided us with many loci possibly involved in blood pressure regulation, arguing in favor of the common disease/rare variant hypothesis.[54,55] Nevertheless, some regions on both chromosome 2[56-59] and 6[56,60,61] have shown (suggestive) linkage to blood pressure in different study populations, which supports the common disease/common variant hypothesis. However, the presence of many alleles possibly influencing blood pressure in outbred study populations severely complicates the search for susceptibility genes.

In order to reduce this problem of genetic heterogeneity, population isolates are increasingly used in attempts to map genes underlying complex diseases.[62,63] Chromosomal and mitochondrial DNA of people derived form a population isolates shows significant less genetic diversity than that of humanity as whole.[64,65] Reduced genetic diversity in these populations is the result of a small number of founders, the occurrence of population bottlenecks and a stochastic process called genetic drift. By definition, genetically isolated populations are founded by a small number of individuals. Many isolated populations have experienced a sudden decrease in size (a population bottleneck), in the form of famine, infectious disease epidemics and war. Rapid population expansion when a population rebounds from a bottleneck increases the occurrence of genetic drift, which further reduces genetic diversity. Furthermore, inhabitants of most isolated communities share a common environmental and cultural background, which reduces the amount of environmental "noise" confounding genetic analyses of complex diseases.

Finally, in genetically isolated populations genealogical records in the form of birth, death and marriage registries are often available, which are indispensable for the reconstruction of large pedigrees. For the GRIP study population, we now have a large pedigree database available including over 63.000 individuals, who all live or have lived in this community.

As a consequence of genetic drift and founder effects, patients in a genetically isolated population are likely to have inherited disease susceptibility from a common ancestor. In our study this might imply that all 229 hypertensive patients, who were connected in one extended pedigree, have hypertension because they share the same susceptibility genes, introduced into the pedigree by a common ancestor 10 generations ago. Since adjacent markers on a chromosome are often transmitted together, our hypertensive patients who have inherited the same disease susceptibility genes are also likely to share considerable parts of DNA surrounding the disease gene. This property has been exploited statistically in order to increase the power to detect susceptibility loci.[66-68] Two genome screens for hypertension performed in genetically isolated populations identified significant "genome-wide" linkage on chromosome 2p and 18q.[69,70]

We observed significant heritability estimates for blood pressure, ranging between 25 and 40 %, and substantial familial aggregation among hypertensive patients in this community, which provide a good rationale to further explore the genetic background of blood pressure in this population.

Future perspectives

In this thesis the search for cardiovascular risk genes has focused on two important areas in genetic epidemiological research: the study of candidate genes and the study of genetically isolated populations in order to map new susceptibility genes for hypertension.

I believe candidate gene studies in the form of "high quality" association studies, which have adapted stringent criteria with respect to sample size, phenotypic and genotypic assessment and biological plausibility, will continue to play an important role in future research on the genetics of hypertension. Moreover, the creation of the Single Nucleotide Polymorphism (SNP) database will in the near future lead to large-scale, genome-wide association studies.[2,71,72] This approach entails the study of variations in (candidate) genes on a large scale, i.e. conducting high density genome scans that are dependent on linkage disequilibrium.[71] A large scale SNP-discovery project has already led to the identification of 1.5 million SNPs, the most common form of human genetic variation.[71,73,74] Between 50.000 and 100.000 of these SNPs lie within coding and adjacent regions and some will alter gene function and protein expression.[72] Genome-wide association studies using evenly spaced SNP markers are now being contemplated in population-based case-control studies, in which they compare the genotypes of a large

number of polymorphic markers between subjects with the trait (e.g. hypertension) and controls.[75,76] Although high throughput SNP genotyping methods have been developed, genotyping many individuals for about 100.000 SNPs remains a daunting task and is not yet feasible.

An approach that is perhaps more feasible will come from the identification of haplotype blocks, based on linkage disequilibrium (LD) between considerable parts of the human genome, in which a small number of common haplotypes represent 90 % of all chromosomal variation.[77] This suggests that 6 to 8, or even less, carefully selected "tag" SNPs may explain the vast majority of genetic variation within each of these haplotypes.[78] Thus a careful selection of SNP markers, which represent not only genes but also the common haplotypes of genes may offer the possibility to detect disease association in a genome-wide scan.[79,80] Especially in young genetically isolated populations, such as the GRIP population, where haplotype blocks might be considerably longer due to extended LD between marker and disease alleles, this may proof a powerful tool for the identification of susceptibility genes.[68,81] The resources needed for the identification of gene variants that cause susceptibility for hypertension, including the availability of a large number of SNP markers, advanced technology for high throughput, rapid and low cost genotyping and definition of the extent and nature of LD in the human population, are therefore eagerly awaited.

The use of genetically isolated populations in the search of susceptibility genes for complex diseases has emerged over the last few years. The general assumption is that in these populations affected individuals have a tendency to share ancestral haplotypes and a hopefully common disease mutation.[82] The value of the GRIP population in our search of susceptibility genes for hypertension will be two-fold. First, the extended pedigree structure of the hypertensive patients in our study offers the opportunity to identify candidate regions in linkage analysis and use haplotype mapping to identify risk haplotypes and hopefully pinpoint a disease locus. We will then test the relevance of these risk haplotypes and/or susceptibility loci in the general population, in a case-control setting within the Rotterdam study. Second, the family-based structure of the ERF-study offers the opportunity to identify quantitative trait loci (QTLs) for blood pressure in future genome wide scans.

To close, whether we will be able to find all genetic pieces for the complex puzzle of blood pressure regulation, to be honest, I do not know. What I do know is that considerable progress is being made and promising results are emerging continuously. The advance of genome-wide association testing with the identification of haplotype blocks in the human genome and the gene-mapping opportunities of genetically isolated populations are bound to confer more insight into the genetics of hypertension and its cardiovascular sequelae. Identification of genetic variants involved in the development of

hypertension will offer many opportunities to investigate the physiological, biochemical and therapeutic aspects of blood pressure control. Moreover, the high prevalence of hypertension in the general population alone warrants a decisive search for genetic factors in blood pressure regulation, in the hope that one-day advances made in genetic research will translate into improvement of human health.

References

1. Lander ES. The new genomics: global views of biology. *Science.* 1996;274:536-9.
2. Risch N, Merikangas K. The future of genetic studies of complex human diseases. *Science.* 1996;273:1516-7.
3. Collins A, Lonjou C, Morton NE. Genetic epidemiology of single-nucleotide polymorphisms. *Proc Natl Acad Sci U S A.* 1999;96:15173-7.
4. Pritchard JK. Are rare variants responsible for susceptibility to complex diseases? *Am J Hum Genet.* 2001;69:124-37.
5. Weiss KM, Terwilliger JD. How many diseases does it take to map a gene with SNPs? *Nat Genet.* 2000;26:151-7.
6. Jones HB. The relative power of linkage and association studies for the detection of genes involved in hypertension. *Kidney Int.* 1998;53:1446-8.
7. Butler R. The DD-ACE genotype and cardiovascular disease. *Pharmacogenomics.* 2000;1:153-67.
8. Schunkert H. Polymorphism of the angiotensin-converting enzyme gene and cardiovascular disease. *J Mol Med.* 1997;75:867-75.
9. Villard E, Tiret L, Visvikis S, Rakotovao R, Cambien F, Soubrier F. Identification of new polymorphisms of the angiotensin I-converting enzyme (ACE) gene, and study of their relationship to plasma ACE levels by two-QTL segregation-linkage analysis. *Am J Hum Genet.* 1996;58:1268-78.
10. Tiret L, Rigat B, Visvikis S, Breda C, Corvol P, Cambien F, Soubrier F. Evidence, from combined segregation and linkage analysis, that a variant of the angiotensin I-converting enzyme (ACE) gene controls plasma ACE levels. *Am J Hum Genet.* 1992;51:197-205.
11. Rigat B, Hubert C, Alhenc-Gelas F, Cambien F, Corvol P, Soubrier F. An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. *J Clin Invest.* 1990;86:1343-6.

12. Morise T, Takeuchi Y, Takeda R. Angiotensin-converting enzyme polymorphism and essential hypertension. *Lancet.* 1994;343:125.
13. O'Donnell CJ, Lindpaintner K, Larson MG, Rao VS, Ordovas JM, Schaefer EJ, Myers RH, Levy D. Evidence for association and genetic linkage of the angiotensin- converting enzyme locus with hypertension and blood pressure in men but not women in the Framingham Heart Study. *Circulation.* 1998;97:1766-72.
14. Schmidt S, van Hooft IM, Grobbee DE, Ganten D, Ritz E. Polymorphism of the angiotensin I converting enzyme gene is apparently not related to high blood pressure: Dutch Hypertension and Offspring Study. *J Hypertens.* 1993;11:345-8.
15. Johnson AG, Simons LA, Friedlander Y, Simons J, Davis DR, MaCallum J. I/D polymorphism of the angiotensin-converting enzyme gene does not predict isolated systolic or systolic-diastolic hypertension in the elderly. *J Hum Hypertens.* 1996;10:167-9.
16. Fuentes RM, Perola M, Nissinen A, Tuomilehto J. ACE gene and physical activity, blood pressure, and hypertension: a population study in Finland. *J Appl Physiol.* 2002;92:2508-12.
17. Stefansson B, Ricksten A, Rymo L, Aurell M, Herlitz H. Angiotensin-converting enzyme gene I/D polymorphism in malignant hypertension. *Blood Press.* 2000;9:104-9.
18. Hamet P. Environmentally-regulated genes of hypertension. *Clin Exp Hypertens.* 1996;18:267-78.
19. Hamet P, Pausova Z, Adarichev V, Adaricheva K, Tremblay J. Hypertension: genes and environment. *J Hypertens.* 1998;16:397-418.
20. Turner ST, Boerwinkle E, Sing CF. Context-dependent associations of the ACE I/D polymorphism with blood pressure. *Hypertension.* 1999;34:773-8.
21. Hibi K, Ishigami T, Kimura K, Nakao M, Iwamoto T, Tamura K, Nemoto T, Shimizu T, Mochida Y, Ochiai H, Umemura S, Ishii M. Angiotensin-converting enzyme gene polymorphism adds risk for the severity of coronary atherosclerosis in smokers. *Hypertension.* 1997;30:574-9.
22. Butler R, Morris AD, Burchell B, Struthers AD. DD angiotensin-converting enzyme gene polymorphism is associated with endothelial dysfunction in normal humans. *Hypertension.* 1999;33:1164-8.
23. Sayed-Tabatabaei FA, Schut AF, Hofman A, Bertoli-Avella AM, Vergeer J, Witteman JC, van Duijn CM. A study of gene-environment

interaction on the gene for angiotensin converting enzyme: a combined functional and population based approach.
J Med Genet. 2004;41:99-103.

24. Diez J. Insulin-like growth factor I in essential hypertension [clinical conference]. *Kidney Int.* 1999;55:744-59.
25. Wickman A, Isgaard J, Adams MA, Friberg P. Inhibition of nitric oxide in rats. Regulation of cardiovascular structure and expression of insulin-like growth factor I and its receptor messenger RNA.
J Hypertens. 1997;15:751-9.
26. Buerke M, Murohara T, Skurk C, Nuss C, Tomaselli K, Lefer AM. Cardioprotective effect of insulin-like growth factor I in myocardial ischemia followed by reperfusion.
Proc Natl Acad Sci U S A. 1995;92:8031-5.
27. Vaessen N, Heutink P, Janssen JA, Witteman JC, Testers L, Hofman A, Lamberts SW, Oostra BA, Pols HA, van Duijn CM. A polymorphism in the gene for IGF-I: functional properties and risk for type 2 diabetes and myocardial infarction. *Diabetes.* 2001;50:637-42.
28. Vaessen N, Janssen JA, Heutink P, Hofman A, Lamberts SW, Oostra BA, Pols HA, van Duijn CM. Association between genetic variation in the gene for insulin-like growth factor-I and low birthweight.
Lancet. 2002;359:1036-7.
29. Spallarossa P, Brunelli C, Minuto F, Caruso D, Battistini M, Caponnetto S, Cordera R. Insulin-like growth factor-I and angiographically documented coronary artery disease.
Am J Cardiol. 1996;77:200-2.
30. Juul A, Scheike T, Davidsen M, Gyllenborg J, Jorgensen T. Low serum insulin-like growth factor I is associated with increased risk of ischemic heart disease: a population-based case-control study.
Circulation. 2002;106:939-44.
31. Janssen JA, Stolk RP, Pols HA, Grobbee DE, Lamberts SW. Serum total IGF-I, free IGF-I, and IGFB-1 levels in an elderly population: relation to cardiovascular risk factors and disease.
Arterioscler Thromb Vasc Biol. 1998;18:277-82.
32. Frayling TM, Hattersley AT, McCarthy A, Holly J, Mitchell SM, Gloyn AL, Owen K, Davies D, Smith GD, Ben-Shlomo Y. A putative functional polymorphism in the IGF-I gene: association studies with type 2 diabetes, adult height, glucose tolerance, and fetal growth in U.K. populations. *Diabetes.* 2002;51:2313-6.

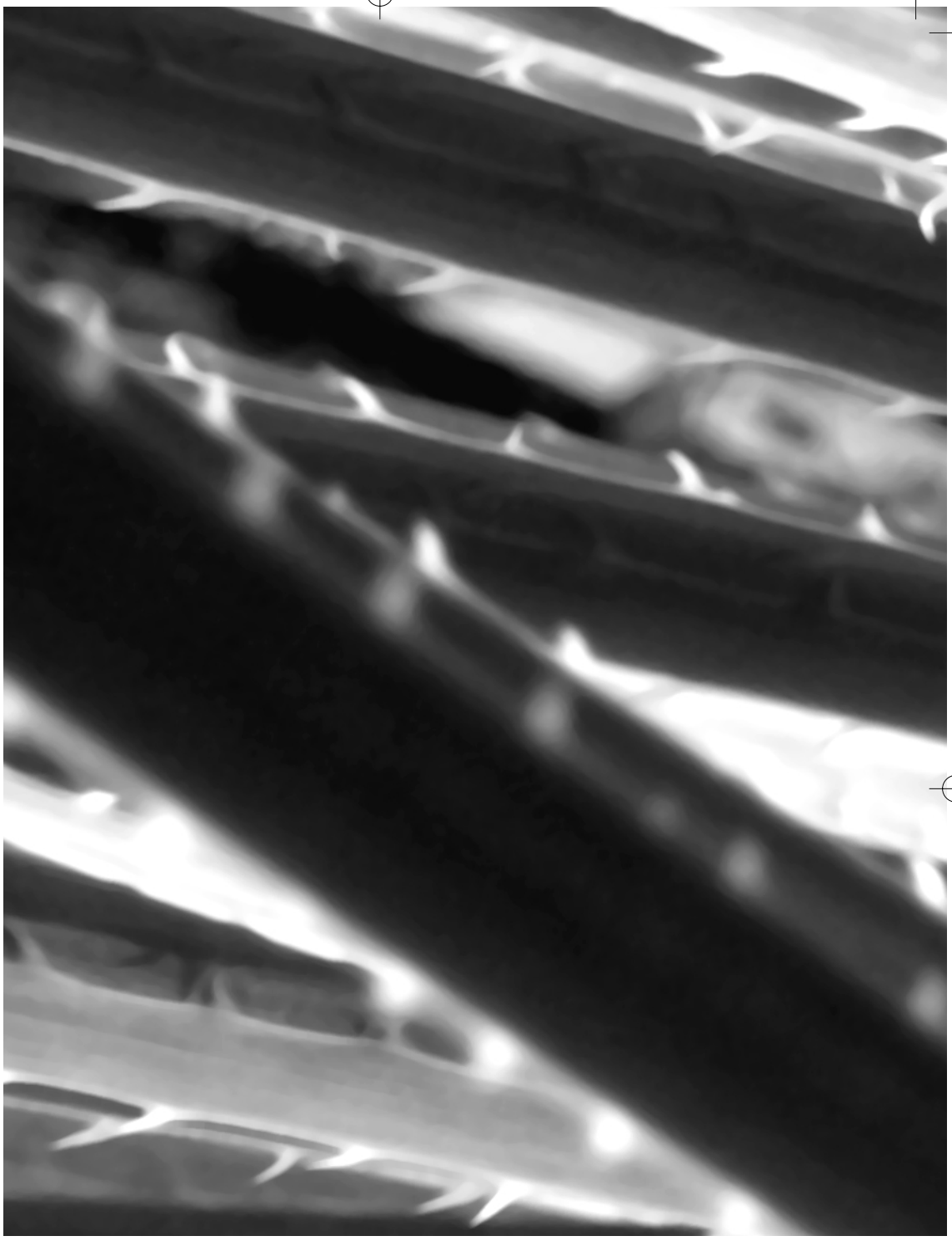
33. Sowers JR. Insulin and insulin-like growth factor in normal and pathological cardiovascular physiology. *Hypertension.* 1997;29:691-9.
34. Nagy Z, Busjahn A, Bahring S, Faulhaber HD, Gohlke HR, Knoblauch H, Rosenthal M, Muller-Myhsok B, Schuster H, Luft FC. Quantitative trait loci for blood pressure exist near the IGF-1, the Liddle syndrome, the angiotensin II-receptor gene and the renin loci in man. *J Am Soc Nephrol.* 1999;10:1709-16.
35. Colhoun HM, McKeigue PM, Davey Smith G. Problems of reporting genetic associations with complex outcomes. *Lancet.* 2003;361:865-72.
36. Cardon LR, Palmer LJ. Population stratification and spurious allelic association. *Lancet.* 2003;361:598-604.
37. Gambaro G, Anglani F, D'Angelo A. Association studies of genetic polymorphisms and complex disease. *Lancet.* 2000;355:308-11.
38. Ioannidis JP, Ntzani EE, Trikalinos TA, Contopoulos-Ioannidis DG. Replication validity of genetic association studies. *Nat Genet.* 2001;29:306-9.
39. Jeunemaitre X, Soubrier F, Kotelevtsev YV, Lifton RP, Williams CS, Charru A, Hunt SC, Hopkins PN, Williams RR, Lalouel JM, et al. Molecular basis of human hypertension: role of angiotensinogen. *Cell.* 1992;71:169-80.
40. Hata A, Namikawa C, Sasaki M, Sato K, Nakamura T, Tamura K, Lalouel JM. Angiotensinogen as a risk factor for essential hypertension in Japan. *J Clin Invest.* 1994;93:1285-7.
41. Rotimi C, Morrison L, Cooper R, Oyejide C, Effiong E, Ladipo M, Osotemihen B, Ward R. Angiotensinogen gene in human hypertension. Lack of an association of the 235T allele among African Americans. *Hypertension.* 1994;24:591-4.
42. Pritchard JK, Rosenberg NA. Use of unlinked genetic markers to detect population stratification in association studies. *Am J Hum Genet.* 1999;65:220-8.
43. Ewens WJ, Spielman RS. The transmission/disequilibrium test: history, subdivision, and admixture. *Am J Hum Genet.* 1995;57:455-64.
44. Spielman RS, McGinnis RE, Ewens WJ. Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM). *Am J Hum Genet.* 1993;52:506-16.
45. Wacholder S, Rothman N, Caporaso N. Population stratification in

epidemiologic studies of common genetic variants and cancer: quantification of bias. *J Natl Cancer Inst.* 2000;92:1151-8.
46. Freely associating. *Nat Genet.* 1999;22:1-2.
47. Multi-center genetic study of hypertension: The Family Blood Pressure Program (FBPP). *Hypertension.* 2002;39:3-9.
48. Ranade K, Hinds D, Hsiung CA, Chuang LM, Chang MS, Chen YT, Pesich R, Hebert J, Chen YD, Dzau V, Olshen R, Curb D, Botstein D, Cox DR, Risch N. A genome scan for hypertension susceptibility loci in populations of Chinese and Japanese origins. *Am J Hypertens.* 2003;16:158-62.
49. Thiel BA, Chakravarti A, Cooper RS, Luke A, Lewis S, Lynn A, Tiwari H, Schork NJ, Weder AB. A genome-wide linkage analysis investigating the determinants of blood pressure in whites and African Americans. *Am J Hypertens.* 2003;16:151-3.
50. Rao DC, Province MA, Leppert MF, Oberman A, Heiss G, Ellison RC, Arnett DK, Eckfeldt JH, Schwander K, Mockrin SC, Hunt SC. A genome-wide affected sibpair linkage analysis of hypertension: the HyperGEN network. *Am J Hypertens.* 2003;16:148-50.
51. Province MA, Kardia SL, Ranade K, Rao DC, Thiel BA, Cooper RS, Risch N, Turner ST, Cox DR, Hunt SC, Weder AB, Boerwinkle E. A meta-analysis of genome-wide linkage scans for hypertension: the National Heart, Lung and Blood Institute Family Blood Pressure Program. *Am J Hypertens.* 2003;16:144-7.
52. Kardia SL, Rozek LS, Krushkal J, Ferrell RE, Turner ST, Hutchinson R, Brown A, Sing CF, Boerwinkle E. Genome-wide linkage analyses for hypertension genes in two ethnically and geographically diverse populations. *Am J Hypertens.* 2003;16:154-7.
53. Caulfield M, Munroe P, Pembroke J, Samani N, Dominiczak A, Brown M, Benjamin N, Webster J, Ratcliffe P, O'Shea S, Papp J, Taylor E, Dobson R, Knight J, Newhouse S, Hooper J, Lee W, Brain N, Clayton D, Lathrop GM, Farrall M, Connell J. Genome-wide mapping of human loci for essential hypertension. *Lancet.* 2003;361:2118-23.
54. Morris BJ, Benjafield AV, Lin RC. Essential hypertension: genes and dreams. *Clin Chem Lab Med.* 2003;41:834-44.
55. Samani NJ. Genome scans for hypertension and blood pressure regulation. *Am J Hypertens.* 2003;16:167-71.
56. Krushkal J, Ferrell R, Mockrin SC, Turner ST, Sing CF, Boerwinkle E. Genome-wide linkage analyses of systolic blood pressure using high-

ly discordant siblings. *Circulation.* 1999;99:1407-10.
57. Rice T, Rankinen T, Province MA, Chagnon YC, Perusse L, Borecki IB, Bouchard C, Rao DC. Genome-wide linkage analysis of systolic and diastolic blood pressure: the Quebec Family Study. *Circulation.* 2000;102:1956-63.
58. Atwood LD, Samollow PB, Hixson JE, Stern MP, MacCluer JW. Genome-wide linkage analysis of blood pressure in Mexican Americans. *Genet Epidemiol.* 2001;20:373-82.
59. Rice T, Rankinen T, Chagnon YC, Province MA, Perusse L, Leon AS, Skinner JS, Wilmore JH, Bouchard C, Rao DC. Genomewide linkage scan of resting blood pressure: HERITAGE Family Study. Health, Risk Factors, Exercise Training, and Genetics. *Hypertension.* 2002;39:1037-43.
60. Allayee H, de Bruin TW, Michelle Dominguez K, Cheng LS, Ipp E, Cantor RM, Krass KL, Keulen ET, Aouizerat BE, Lusis AJ, Rotter JI. Genome scan for blood pressure in Dutch dyslipidemic families reveals linkage to a locus on chromosome 4p. *Hypertension.* 2001;38:773-8.
61. Hunt SC, Ellison RC, Atwood LD, Pankow JS, Province MA, Leppert MF. Genome scans for blood pressure and hypertension: the National Heart, Lung, and Blood Institute Family Heart Study. *Hypertension.* 2002;40:1-6.
62. Aulchenko YS, Vaessen N, Heutink P, Pullen J, Snijders PJ, Hofman A, Sandkuijl LA, Houwing-Duistermaat JJ, Edwards M, Bennett S, Oostra BA, van Duijn CM. A genome-wide search for genes involved in type 2 diabetes in a recently genetically isolated population from the Netherlands. *Diabetes.* 2003;52:3001-4.
63. Wright AF, Carothers AD, Pirastu M. Population choice in mapping genes for complex diseases. *Nat Genet.* 1999;23:397-404.
64. Kittles RA, Perola M, Peltonen L, Bergen AW, Aragon RA, Virkkunen M, Linnoila M, Goldman D, Long JC. Dual origins of Finns revealed by Y chromosome haplotype variation. *Am J Hum Genet.* 1998;62:1171-9.
65. Sajantila A, Salem AH, Savolainen P, Bauer K, Gierig C, Paabo S. Paternal and maternal DNA lineages reveal a bottleneck in the founding of the Finnish population. *Proc Natl Acad Sci U S A.* 1996;93:12035-9.
66. Te Meerman GJ, Van der Meulen MA. Genomic sharing surrounding

alleles identical by descent: effects of genetic drift and population growth. *Genet Epidemiol.* 1997;14:1125-30.
67. Te Meerman GJ, Van der Meulen MA, Sandkuijl LA. Perspectives of identity by descent (IBD) mapping in founder populations. *Clin Exp Allergy.* 1995;25 Suppl 2:97-102.
68. Service SK, Lang DW, Freimer NB, Sandkuijl LA. Linkage-disequilibrium mapping of disease genes by reconstruction of ancestral haplotypes in founder populations. *Am J Hum Genet.* 1999;64:1728-38.
69. Kristjansson K, Manolescu A, Kristinsson A, Hardarson T, Knudsen H, Ingason S, Thorleifsson G, Frigge ML, Kong A, Gulcher JR, Stefansson K. Linkage of essential hypertension to chromosome 18q. *Hypertension.* 2002;39:1044-9.
70. Angius A, Petretto E, Maestrale GB, Forabosco P, Casu G, Piras D, Fanciulli M, Falchi M, Melis PM, Palermo M, Pirastu M. A new essential hypertension susceptibility locus on chromosome 2p24-p25, detected by genomewide search. *Am J Hum Genet.* 2002;71:893-905.
71. Collins FS, Guyer MS, Charkravarti A. Variations on a theme: cataloging human DNA sequence variation. *Science.* 1997;278:1580-1.
72. Botstein D, Risch N. Discovering genotypes underlying human phenotypes: past successes for mendelian disease, future approaches for complex disease. *Nat Genet.* 2003;33 Suppl:228-37.
73. Venter JC, et al. The sequence of the human genome. *Science.* 2001;291:1304-51.
74. Sachidanandam R, Weissman D, Schmidt SC, Kakol JM, Stein LD, Marth G, Sherry S, Mullikin JC, Mortimore BJ, Willey DL, Hunt SE, Cole CG, Coggill PC, Rice CM, Ning Z, Rogers J, Bentley DR, Kwok PY, Mardis ER, Yeh RT, Schultz B, Cook L, Davenport R, Dante M, Fulton L, Hillier L, Waterston RH, McPherson JD, Gilman B, Schaffner S, Van Etten WJ, Reich D, Higgins J, Daly MJ, Blumenstiel B, Baldwin J, Stange-Thomann N, Zody MC, Linton L, Lander ES, Altshuler D. A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. *Nature.* 2001;409:928-33.
75. John S, Shephard N, Liu G, Zeggini E, Cao M, Chen W, Vasavda N, Mills T, Barton A, Hinks A, Eyre S, Jones KW, Ollier W, Silman A, Gibson N, Worthington J, Kennedy GC. Whole-genome scan, in a complex disease, using 11,245 single-nucleotide polymorphisms: comparison with microsatellites. *Am J Hum Genet.* 2004;75:54-64.
76. Carlson CS, Eberle MA, Kruglyak L, Nickerson DA. Mapping complex

disease loci in whole-genome association studies. *Nature*. 2004;429:446-52.
77. Consortium TiH. The international HapMap project. *Nature*. 2003;426:789-796.
78. Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B, Higgins J, DeFelice M, Lochner A, Faggart M, Liu-Cordero SN, Rotimi C, Adeyemo A, Cooper R, Ward R, Lander ES, Daly MJ, Altshuler D. The structure of haplotype blocks in the human genome. *Science*. 2002;296:2225-9.
79. Akey J, Jin L, Xiong M. Haplotypes vs single marker linkage disequilibrium tests: what do we gain? *Eur J Hum Genet*. 2001;9:291-300.
80. Stephens JC, Schneider JA, Tanguay DA, Choi J, Acharya T, Stanley SE, Jiang R, Messer CJ, Chew A, Han JH, Duan J, Carr JL, Lee MS, Koshy B, Kumar AM, Zhang G, Newell WR, Windemuth A, Xu C, Kalbfleisch TS, Shaner SL, Arnold K, Schulz V, Drysdale CM, Nandabalan K, Judson RS, Ruano G, Vovis GF. Haplotype variation and linkage disequilibrium in 313 human genes. *Science*. 2001;293:489-93.
81. Shifman S, Darvasi A. The value of isolated populations. *Nat Genet*. 2001;28:309-10.
82. Peltonen L, Palotie A, Lange K. Use of population isolates for mapping complex traits. *Nat Rev Genet*. 2000;1:182-90.



6.SUMMARY

Hypertension, or elevated arterial blood pressure is a substantial public health problem, affecting about 25 % of the adult population. This disorder is a major risk factor for common causes of morbidity and mortality including stroke, myocardial infarction, heart failure, and end-stage renal disease. Despite the morbid consequences of hypertension, its pathogenesis remains to be fully elucidated. Difficulties in defining the causes of hypertension from physiological studies alone have motivated studies to search for genetic factors in the etiology of hypertension. Identification of genes influencing blood pressure variation may confer more insight into the physiological mechanisms underlying blood pressure variation and reveal pathways and targets for therapeutical intervention. In recent years, several genes responsible for rare Mendelian forms of hypertension have been identified.

In Chapter 1, a general introduction is given on the epidemiology, physiology and genetics of hypertension. Chapter1.2 describes the methodological aspects of genetic epidemiological research in the field of hypertension. An outline is given on the basic principles of genetic epidemiological studies.

Chapter 2 and 3 present the results of several candidate genes studies performed in the Rotterdam Study, a population-based cohort study. We studied an insertion/deletion polymorphism in the angiotensin-converting enzyme gene in relation to blood pressure and hypertension in chapter 2.1. In smokers we found a significantly higher systolic blood pressure in DD-carriers of the ACE genotype compared to II-carriers. The risk of hypertension was significantly increased in smokers who carried one or two copies of the D-allele. In former and non-smokers no effect of the ACE gene on blood pressure was observed. In chapter 2.2 we observed that the ACE I/D polymorphism and smoking were the main determinants for plasma ACE activity. Persons who smoke and carry at least one copy of the D-allele had significantly increased carotid intima media thickness (IMT) and slightly more carotid plaques. This relationship again was not present in former and non-smokers. The studies described in chapter 2.1 and 2.2 underline the importance of gene-environment interactions in the study of candidate genes for hypertension and atherosclerosis. In chapter 2.3 we found an increased risk of heart failure in hypertensive persons compared to normotensive persons that was dependent on the presence of the D-allele of the ACE I/D polymorphism. The incidence rate of heart failure in hypertensive persons increased with the number of D-alleles present. Hypertensive persons did not have a significantly increased risk of heart failure compared to normotensive persons, unless they carried one or two copies of the D-allele. This suggests that the ACE I/D polymorphism plays a modifying role in the development of heart failure in persons with hypertension.

Chapters 3.1 and 3.2 present our findings regarding a promoter polymorphism in the IGF-I gene in relation to early signs of atherosclerosis and left ventricular hypertrophy. In chapter 3.1 we describe an association between this polymorphism in the IGF-I gene

and IMT of the carotid arteries in the general population. Further analysis revealed that the association between this polymorphism and atherosclerosis was most pronounced in hypertensive persons. Hypertensive persons who did not carry a copy of the 192-bp allele (wildtype allele) had an increased carotid IMT and higher aortic pulse wave velocity (PWV) compared to heterozygous and homozygous carriers of the 192-bp allele. The effect of this polymorphism was even stronger in persons with untreated hypertension. In normotensive persons no association was found between this polymorphism and IMT or PWV. The polymorphism we studied in the IGF-I gene most likely is a modifier of the risk for atherosclerosis in persons with hypertension. Furthermore, non-carriers of the 192-base pair polymorphism in the IGF-I gene are more susceptible to the development of left ventricular hypertrophy than individuals homozygous for this allele (chapter 3.2).

In chapter 4.1 we report the first results of the ERF study. This is a study conducted among 2500 inhabitants of a genetically isolated population in the Southwest part of the Netherlands, which aims to determine genetic risk factors in the development of complex diseases. Inhabitants of this isolated community are thought the share a more homogenous genetic background, which will facilitate the search for susceptibility genes of complex diseases such as hypertension. We estimated the heritability and genetic correlations of various blood pressure traits in the first 1000 participants of the ERF study. They were all members of one extended pedigree. Heritability estimates ranged between 0.25 and 0.40, suggesting that up to 40 % of the blood pressure variance in this population may be explained by genetic factors. Furthermore we observed high genetic correlations between systolic, diastolic and mean arterial pressure, which indicates that these traits may be influenced by the same genes. Pulse pressure was less strongly correlated with the other blood pressure traits, suggesting a partly distinct genetic background for this trait. The results of this study provide a rationale to further explore the genetic background of blood pressure in this population. Moreover, studying pulse pressure in future linkage or association analyses may lead to the identification of susceptibility genes related to different aspects of blood pressure pathophysiology, such as arterial stiffness.

In a second study, we investigated 366 hypertensive patients ascertained in the same isolated community (chapter 4.2). 229 patients could be linked to a common ancestor within 10 generations. About 50 % of the hypertensive patients received anti-hypertensive therapy and the median age-at-diagnosis was 50 years. In 80 % of the patients at least one metabolically linked risk factor was present, obesity and overweight were the most frequent ones. Hypertensive patients were more closely related than a normotensive control group. They also had higher levels of inbreeding than this control group. In addition to our findings in chapter 4.1, higher inbreeding levels among hypertensive patients and the availability of an extended pedigree offer promising statistical tools to identify new risk genes for hypertension in future genetic analyses in this population.

Finally, chapter 5 provides a discussion of the results and outlines the future perspectives of the studies described in this thesis. Special emphasis is put on the value of candidate gene studies and the advantages of genetically isolated populations in the search of cardiovascular risk genes.

SAMENVATTING

Hypertensie is een veel voorkomende aandoening waar ongeveer 25 % van de volwassen bevolking aan lijdt. Een hoge bloeddruk is een belangrijke risico factor voor het krijgen van hart- en vaatziekten zoals een beroerte of een hartinfarct en daarom een groot probleem voor de volksgezondheid. Hart- en vaatziekten vormen de belangrijkste oorzaak van ziekte en sterfte in de westerse wereld. Ondanks de ernstige gevolgen van een hoge bloeddruk, zijn de oorzaken van een hoge bloeddruk niet duidelijk. Fysiologische studies hebben geen eenduidige reden voor het ontstaan van hypertensie kunnen vinden. Dit heeft er toe geleid dat er de laatste jaren ook veel onderzoek wordt gedaan naar genetische factoren in de etiologie van hypertensie. Heel zeldzaam zijn de erfelijke vormen van hypertensie die door een mutatie in slechts één gen worden veroorzaakt. In het algemeen wordt aangenomen dat hypertensie het gevolg is van mutaties in meerdere genen in combinatie met de aanwezigheid van omgevings factoren zoals roken, een ongezond dieet of te weinig fysieke activiteit. De identificatie van genen die de bloeddruk beïnvloeden zal meer inzicht geven in de (patho)fysiologie van bloeddruk regulatie en hopelijk leiden tot de ontwikkeling van nieuwe therapeutische interventies.

In hoofdstuk 1 wordt eerst een algemene introductie gegeven over de epidemiologie, fysiologie en genetica van hypertensie. Hoofdstuk 1.2 beschrijft de methodologische aspecten van genetisch-epidemiologisch onderzoek. De basis-principes van genetisch-epidemiologische studies als mede de reeds behaalde resultaten op het gebied van hypertensie onderzoek komen aan de orde.

In de hoofdstukken 2 en 3 worden de resultaten van 5 kandidaat-gen studies in de ERGO studie, een grootschalig populatie onderzoek, beschreven. We bestudeerden een insertie/deletie variatie in het angiotensine converterende enzym (ACE) gen in relatie tot bloeddruk en het risico op hypertensie in hoofdstuk 2.1. We vonden een significant verhoogde systolische bloeddruk in rokers die de DD variant van het ACE gen dragen in vergelijking met rokers die de II variant dragen van het ACE gen. Rokers die een of twee kopieën hebben van deze D variant in het ACE gen, hebben meer kans op het krijgen van hypertensie dan rokers die twee kopieën van de I variant hebben. In niet-rokers had het ACE gen geen effect op de bloeddruk. In hoofdstuk 2.2 beschrijven we dat het ACE gen en roken de belangrijkste determinanten zijn van de plasma ACE activiteit. Personen die roken en tenminste één kopie dragen van de D variant van het ACE gen hebben een dikkere vaatwand in de halsslagaders en meer aderverkalking in deze vaatwand. Deze relatie was afwezig bij niet rokers. De studies beschreven in hoofdstuk 2.1 en 2.2 benadrukken het belang van gen-omgevings interacties wanneer kandidaat genen bestudeerd worden voor hypertensie en aderverkalking. In hoofdstuk 2.3 vonden we dat het verhoogde risico op hartfalen in personen met hypertensie afhankelijk is van de D variant in het ACE gen. De incidentie van hartfalen bij hypertensieve personen nam toe wanneer ze één of twee kopieën van de D variant hadden. In onze studie hadden

personen met hypertensie geen verhoogd risico op het krijgen van hartfalen in vergelijking met normotensieve personen, tenzij zij dragers waren van één of twee kopieën van de D variant van het ACE gen. Dit suggereert dat de insertie/deletie variatie in het ACE gen een modificerende rol speelt bij het ontstaan van hartfalen in hypertensieve personen.

Hoofdstuk 3.1 en 3.2 beschrijven de bevindingen van het insuline-afhankelijke groeifactor-I (IGF-I) gen in relatie tot atherosclerose en linker ventrikel hypertrofie (LVH) van het hart. In hoofdstuk 3.1 wordt een relatie gevonden tussen variaties in het IGF-I gen en de dikte van de vaatwand (intima media dikte, IMT) in de halsslagaders in de algemene populatie. Verdere analyses lieten zien dat deze relatie het sterkst was in hypertensieve personen. Hypertensieve personen die geen kopie van de meest voorkomende variatie in het IGF-I gen (het wildtype allel) hebben, hebben een dikkere IMT in de halsslagader en stijvere vaten, hetgeen uitgedrukt wordt in een hogere pulse wave velocity (PWV). Het effect van deze variaties in het IGF-I gen was nog sterker in hypertensieve personen die geen bloeddruk verlagende medicatie gebruikten. In normotensieve personen vonden we geen relatie tussen het IGF-I gen en IMT of PWV. Het IGF-I gen beïnvloedt waarschijnlijk het risico op atherosclerose bij personen met hypertensie. Verder observeerden we ook dat personen zonder het wildtype allel een groter risico hebben op het ontwikkelen van linker ventrikel hypertrofie van het hart (hoofdstuk 3.2).

In hoofdstuk 4 worden de eerste resultaten van de ERF studie gepresenteerd. Deze studie is uitgevoerd onder 2500 inwoners van een genetisch geïsoleerde gemeenschap in Zuidwest-Nederland. Het doel van deze studie is om genetische risico factoren te vinden die een rol spelen bij het ontstaan van complexe ziekten. Bewoners van een geïsoleerde gemeenschap delen waarschijnlijk een meer homogene genetische achtergrond hetgeen een zoektocht naar risico genen voor complexe ziekten zoals hypertensie makkelijker maakt. We hebben de erfelijkheid van verschillende bloeddruk maten berekend voor de eerste 1000 deelnemers van de ERF studie. Deze deelnemers zijn allemaal aan elkaar verwant in een grote, zeer uitgebreide stamboom. De erfelijkheid van bloeddruk varieerde tussen de 0.25 en 0.40, hetgeen wil zeggen dat tussen de 25 en 40 % van de bloeddruk variatie in deze populatie veroorzaakt wordt door genetische factoren. De genetische correlatie tussen systolische, diastolische en de gemiddelde arteriële bloeddruk was zeer hoog. Dit suggereert dat deze bloeddruk maten waarschijnlijk beïnvloed worden door dezelfde genen. De polsdruk was minder sterk gecorreleerd aan de andere bloeddruk maten, hetgeen impliceert dat deze bloeddruk maat misschien een gedeeltelijk aparte genetische achtergrond heeft. De resultaten van deze studie zijn een aansporing om de genetische aspecten van bloeddruk in deze populatie verder te onderzoeken. Het bestuderen van de polsdruk kan in toekomstige genetische linkage of associatie-analyses leiden tot de identificatie van genen die gerelateerd zijn aan verschillende aspecten van de bloeddruk pathofysiologie, zoals bijvoorbeeld de vaatstijfheid.

In een tweede studie onderzochten we 366 hypertensieve patiënten uit dezelfde genetisch geïsoleerde gemeenschap (hoofdstuk 4.2). Een groot gedeelte van deze hypertensieve patiënten (62 %) was binnen 10 generaties aan elkaar verwant via één gemeenschappelijke voorvader. Ongeveer 50 % van de patiënten werd behandeld met bloeddruk verlagende medicijnen en de mediane leeftijd waarop de diagnose hypertensie werd gesteld was 50 jaar. Tachtig procent van de patiënten had tenminste één andere metabole risico factor, het meest frequent hadden ze overgewicht. Hypertensieve patiënten bleken nauwer aan elkaar verwant dan een groep normotensieve personen uit dezelfde populatie. In de patiënten groep kwam bloedverwantschap ook vaker voor. Meer bloedverwantschap onder hypertensieve patiënten en de beschikbaarheid van een grote uitgebreide stamboom, bieden veelbelovende statistische mogelijkheden om nieuwe risicogenen voor hypertensie te ontdekken in toekomstige genetische analyses in deze populatie.

Tenslotte worden in hoofdstuk 5 de resultaten en de toekomst perspectieven van de studies beschreven in dit proefschrift besproken. Speciale nadruk is in deze discussie gelegd op de waarde van kandidaat-gen studies en de voordelen van genetisch geïsoleerde populaties in de zoektocht naar cardiovasculaire risicogenen.

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LIST OF PUBLICATIONS

Schut AFC, Janssen JAMJL, Deinum J, Vergeer JM, Hofman A, Lamberts SW, Oostra BA, Pols HAP, Witteman JCM, van Duijn CM. Polymorphism in the promoter region of the insulin-like growth factor I gene is related to carotid intima-media thickness and aortic pulse wave velocity in subjects with hypertension. *Stroke*. 2003 Jul;34(7):1623-1627.

Schut AFC, Sayed-Tabatabaei FA, Witteman JC, Avella AM, Vergeer JM, Pols HA, Hofman A, Deinum J, van Duijn CM. Smoking-dependent effects of the angiotensin-converting enzyme gene insertion/deletion polymorphism on blood pressure. *J Hypertens.* 2004 Feb;22(2):313-319.

Sayed-Tabatabaei FA, Schut AFC, Hofman A, Bertoli-Avella AM, Vergeer JM, Witteman JCM, van Duijn CM. A study of gene-environment interaction on the gene for angiotensin converting enzyme: a combined functional and population based approach. *J Med Genet.* 2004 Feb;41(2):99-103.

Bleumink GS, Schut AFC, Sturkenboom MCJM, Janssen JAMJL, Witteman JCM, van Duijn CM, Hofman A, Stricker BHCh. Absence of the 192-bp allele in a promoter polymorphism of the IGF-I gene is associated with increased risk of left ventricular hypertrophy. Accepted *Heart.*

Schut AFC, Bleumink GS, Stricker BHCh, Hofman A, Witteman JCM, Pols HAP, Deckers JW, Deinum J, van Duijn CM
Angiotensin-converting enzyme insertion /deletion polymorphism and risk of heart failure in hypertensive subjects. Submitted.

Schut AFC, CM van Duijn. Genetic epidemiology of hypertension. Submitted.

Schut AFC, Aulchenko YS, Deinum J, van Rijn MJE, Sayed-Tabatabaei FA, Rivadeneira F, Croes EA, Zillikens MC, Pols HAP, Witteman JCM, Oostra BA, van Duijn MC. Genetic and environmental contributions to blood pressure variance in an extended pedigree of a Dutch genetically isolated population. Submitted.

Schut AFC, van Rijn MJE, Deinum J, Zillikens MC, Snijders PJLM, Pols HAP, Witteman JCM, Oostra BA, van Duijn CM. Hypertension in a Dutch genetically isolated population. Submitted.

Sayed-Tabatabaei FA, Schut AFC, Arias Vásquez A, Bertoli-Avella AM, Hofman A, Witteman JCM, van Duijn CM. Angiotensin converting enzyme gene polymorphism and cardiovascular morbidity and mortality: the Rotterdam Study. Accepted *J Med Genet*.

Arias Vásquez A, Sayed-Tabatabaei FA, Schut AFC, Hofman A, Bertoli-Avella AM, Vergeer JM, Aulchenko YS, Witteman JCM, van Duijn CM. The angiotensin converting enzyme gene, smoking and mortality in a population-based study. Submitted.

Bleumink GS, Schut AFC, Sturkenboom MCJM, Deckers JW, van Duijn CM, Stricker BHCh. Genetic polymorphisms and heart failure. Accepted *Gen. Med.*

Bleumink GS, Schut AFC, Sturkenboom MCJM, van Duijn CM, Deckers JW, Hofman A, Herre Kingma J, Witteman JCM, Stricker BHCh. Mortality in patients with hypertension on angiotensin-I converting enzyme (ACE)-inhibitor treatment is influenced by the ACE Insertion/Deletion polymorphism. Submitted.

Van Rijn MJE, Slooter AJC, Schut AFC, Snijders PJLM, Kapelle LJ, Koudstaal PJ, van Swieten JC, Oostra BA, van Duijn CM. Familial aggregation of ischemic stroke in a genetically isolated population. Submitted.

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

Isaacs A, Sayed-Tabatabaei FA, Aulchenko YS, Schut AFC, Zillikens MC, Rutten WPF, Pols HAP, Witteman JCM, Oostra BA, van Duijn CM. Heritabilities and Effects of Inbreeding on Plasma Lipids in a Genetically Isolated Population: The Erasmus Rucphen Family Study. Submitted.

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Anna Schut was born on May 11, 1975 in Delft, the Netherlands. She moved to Roermond at the age of eleven, where she graduated in 1993 at the "Bischoppelijk College Schöndeln" (Gymnasium). In 1993 she started her medical studies at the Erasmus University in Rotterdam. She obtained her medical degree in 2000 (cum laude). In December 2000 started the work described in this thesis at the Genetic Epidemiology Unit of the Department of Epidemiology & Biostatistics (Prof.dr. C.M. van Duijn) in close collaboration with the Departments of Internal Medicine (Prof.dr. H.A.P. Pols) and Clinical Genetics (Prof.dr. B.A. Oostra) of the Erasmus Medical Center Rotterdam. During this period she obtained a Master of Science degree in Genetic Epidemiology at the Netherlands Institute of Health Sciences.
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