Dissecting the Genetics of Stroke

Marie Josee Elisabeth van Rijn

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Het ontrafelen van de erfelijkheid van een beroerte

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Life does not put things in front of you
that you are unable to handle

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Chapter 4.2

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Chapter 4.3

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Chapter 5.1

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Chapter 6.2

van Rijn MJE, van Duijn CM, Slooter AJC. The Impact of genetic testing on complex diseases. Common ischaemic stroke as an example. *Eur J Epidemiol*, 2005;20:383-8.

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Chapter 1

General introduction

GENETIC EPIDEMIOLOGY

Genetic epidemiology studies genetic determinants of disease as well as the joint effects of these genes and environmental factors. As extensive information has become available about the human genome, the field of genetic epidemiology has expanded rapidly. The haploid genome consists of about 3 billion basepairs, distributed among 23 chromosomes. These basepairs code up to 20,000 genes. Although a number of phenotypes may be determined by a single gene, for the great majority, a few hundred genes contribute to their variation.[1] The DNA of two distantly related individuals is 99.9% identical.[2] Still, there will be important variation in sequence between these unrelated individuals, usually indicated as polymorphisms. Some classes of DNA sequence variants are CA-repeats, microsatellites and single nucleotide polymorphisms (SNPs). SNPs are most abundant, their number exceeding 10 million, and they are thought to account for around 90% of genetic variation.[3] Recently, copy number variations (CNVs) of DNA sequences have been mapped.[4,5] CNVs range in size from kilobases to megabases and can consist of deletions, insertions or duplications. The role of CNVs will be established in future genetic studies.

Many genes involved in monogenic disorders have been identified during the last decade.[6] The past decade, genetic epidemiology studies have been focussing more and more on complex diseases, such as diabetes mellitus, hypertension and stroke. Complex diseases are not caused by single genes, but rather by several interacting genes and environmental factors[7] and exert a significant impact on human health because of their high population incidence.

Gene discovery is far more difficult for complex traits. Two principal methods of discovering genes involved in complex diseases are linkage analysis and association studies. Linkage analysis can identify regions of the genome that contain genes possibly predisposing to a disease. This analysis is based on the principle that loci that are close together, segregate together more often than loci that are further apart, as a recombination event will occur more frequent between distant loci with a probability of 0.5. The rationale of linkage is that deviation from this probability indicates linkage of a disease to a locus. Association analysis aims to detect an overpresentation of a genetic variant in a disease by comparing genotypes between a series of patients and controls. Most studies published have used the candidate gene approach for this analysis. A new approach is genome-wide association, which uses dense maps of single nucleotide polymorphism (SNP) markers. Candidate genes have been selected on the basis of their function. In contrast, genome-wide linkage and association analyses may be used to explore the full genome for new genes. For complex diseases, which are explained by common variants and small effects, association studies are most powerful. Linkage studies are powerful for traits explained by rare variants with large effects.

Studying the genetic epidemiology of a complex disease is not an easy task, as many genes and environmental factors may play a role, harbouring only a small risk when studied as single risk factors. Most likely, these determinants will interact with one another increasing the

risk of the disease, but also the complexity. One way to overcome this complexity is to study monogenic disorders and intermediate phenotypes. A second approach is to study a trait in genetically isolated populations or animals. In animals, the phenotype can often be measured more accurately and aggressively (biopsies). In isolated populations, traits are usually more homogeneous compared to outbred populations due to selection and inbreeding.

STROKE AS A COMPLEX DISEASE

In this thesis, I focussed on stroke and its determinants. Stroke is a leading cause of death and disability in the Western world.[8] It is caused by a disrupt disturbance of circulation in the brain, leading to neurological deficits. Stroke can be divided into two main subtypes: ischaemic stroke (80%) and haemorrhagic stroke (20%). Ischaemic stroke follows after a complete occlusion of a cerebral artery. This may be caused by an intra- or extracranial atherosclerotic lesions, occluding the vessel lumen or a distal vessel by the formation of an embolus. Such an embolus can also have a cardiac origin. Symptoms already occur after 10 seconds of hypoxia. Haemorrhagic stroke is caused by rupturing of a brain vessel. This may be the result of a trauma, rupturing of an aneurysm or vascular malformation, or rupturing of small penetrating arteries. Not only the aetiology of stroke is heterogeneous. Mortality rates after haemorrhagic stroke are much higher compared with ischaemic stroke.

Risk factors for stroke include hypertension, smoking, diabetes mellitus, hypercholesterolemia, obesity as well as cardiac risk factors like atrial fibrillation and recent myocardial infarction.[9] Of these, hypertension is the most important modifiable risk factor.[10] A family history of stroke has also been reported a risk factor, and twin studies have shown a nearly fivefold increase in the prevalence of stroke among monozygotic compared with the dizygotic twin pairs, indicating a genetic susceptibility to stroke.[11-13] Although much is known about the classical risk factors of stroke, the genetic aetiology of stroke remains largely unknown.

Animal studies

Genetic studies in animals may help understand the genetics of the disease in humans. The stroke-prone spontaneously hypertensive rat (SHRSP) is a rat strain characterized by a very high frequency of cerebrovascular events as compared to stroke-resistant spontaneously hypertensive rats.[14] This rat strain has been used to study the genetics of stroke and areas on chromosomes 1,4 and 5 were found to be associated with stroke.[15] From this, the gene encoding atrial natriuretic peptide (ANP) was identified and showed functional and regulatory differences between the stroke-prone and the stroke-restistant rat strain.[16] This gene was found to be associated with stroke in human studies as well.[17,18] The role of salt-sensitive hypertension, which was later associated with cardiovascular events in humans,[19] was first obtained in the

Dahl rat model, in which the aldosterone synthase genes were identified.[20,21] The angiotensin converting enzyme (ACE) and angiotensinogen, two other polymorphisms that encode for proteins of the renin-angiotensin-aldosterone system (RAAS), were also studied in SHRSP.[22,23] Finally, mouse models detected a role of the endothelial nitric oxide synthase (eNOS) gene in the degree of cerebral ischaemia after stroke.[24]

Monogenic forms of diseases in humans

Mendelian disorders associated with ischaemic stroke, may be the result of several different pathophysiological mechanisms, including cardioembolism, hematological disorders, mitochondrial disorders, ion channel disorders and connective tissue disorders.[12,25] Many genes involved in these mendelian disorders have been identified.[12] One of the monogenetic forms studied most intensely is cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy (CADASIL), which is a small vessel disease. The disease locus was assigned to chromosome 19q12, and *Notch3* was identified as the causal gene.

For haemorrhagic stroke, the genes encoding amyloid precursor protein, cystatin C and type II intergral transmembrane protein, were found to cause amyloid angiopathy, which in turn causes early-onset haemorrhagic stroke.[25] The *Tie-2* gene was found to cause familial venous malformation disorders, as its product is related to signalling pathways affecting vascular remodelling and assembly.[25,26] These malformations are fragile and lead to haemorrhagic stroke when they rupture.

Intermediate phenotypes

Studying intermediate phenotypes of complex diseases can make the outcome of a study more homogeneous, as it focuses on a specific pathohysiological pathway. Also, it enables us to study the genetic epidemiology of the complex disease as a continuous trait. For stroke, three such intermediate phenotypes are white matter hyperintensities, carotid atherosclerosis and blood pressure. White matter hyperintensities have been associated with stroke[27,28] and were found to have high heritability estimates of greater than 50%.[29] Recently, evidence for significant linkage was found at 4cM on chromosome 4, indicating that a gene in this region may be influencing white matter hyperintensity volume.[30] One gene in this region was huntingtin, responsible for Huntington disease.

As most strokes are ischaemic and of them, the majority results from carotid atherosclerosis, studying the genetics of ischaemic stroke through atherosclerosis can provide valuable information about this type of stroke. Carotid atherosclerosis can be measured as carotid intima media thickness (IMT) or plaques, and both were found predictors of stroke.[31,32] Heritability estimates for IMT and plaques range between 20%-60% and several genes have been associ-

ated with these traits.[33-36] Linkage of IMT to chromosome 2 and 12 has been found,[37,38] but most likely there are a large number of genes involved.

As blood pressure is a strong risk factor, it can also be used in the search for genes involved in stroke. Hypertension and stroke have found to coaggregate strongly within families, suggesting overlapping genetic factors.[39-41] Some genes known to be involved in blood pressure variation are genes in the RAAS, alpha-adducin and G-protein.[42-45]Additionally, genes underlying the risk factors for hypertension and stroke (e.g., obesity, diabetes, high cholesterol levels), might form a common genetic basis.[46]

Genetically isolated populations

Isolated populations originate from a small number of founders and are characterised by minimal migration. Founder effects and genetic drift increase the genetic homogeneity in these isolates, creating a powerful setting to study the genetics of complex diseases. Furthermore, individuals selected from such a population are likely to share a more common environment and cultural background diminishing the bias from environmental factors. Last, detailed genealogic data is usually available, facilitating the construction of extended pedigrees.[47]

Recently, two genes associated with stroke have been identified by a genome wide search within multiple families derived from the isolated population of Iceland. First, the gene encoding phosphodiesterase 4D (*PDE4D*) was identified on chromosome 5q12.[48] The *PDE4D* protein plays a role in the regulation of cAMP levels. *PDE4D* significantly increased combined cardiogenic and carotid stroke. Second, the gene encoding 5-lipoxygenase activating protein (FLAP) was identified on chromosome 13q12-13.[49] It was found to increase the risk of both myocardial infarction and stroke, presumably through leukotrienen, which are proinflammatory lipid mediators.[50,51]

GENES ASSOCIATED WITH STROKE

In contrast to the work of deCODE (see previous paragraph), most studies so far have used the candidate gene approach in order to find an association between genes and stroke. Findings of candidate gene studies have been difficult to replicate and show conflicting results. Table 1 shows an overview of all genes previously associated with stroke. Some findings were replicated, however, most genes were not confirmed.

One approach to overcome this problem is to conduct a meta-analysis of all findings. Table 2 shows the genes that were found to be significantly associated with stroke in a meta-analysis. The integrin alpha C807T polymorphism was also studied in a meta-analysis, but these results were not significant (OR for stroke for the TT genotype=1.24 (95% CI: 0.66-2.31).[52] From table 2 is clear is that the risks are very small with odds ratios varying from 1.21 to 1.73.

Table 1. Genes studied in relation to stroke

Single study	Confirmed by 1 study	Confirmed by >1 study
Factor XII	Factor VII	Fibrinogen
Glutathione S-transferase omega-1	ENOS	Factor XIII
Apo A5	NOS3	GpIIb/IIIa
Stromelysin-1	Interleukin-4	GpIa/Iia
Adiponectin	KLOTHO	Lipoprotein lipase
TGF-beta	Protein Z	Interleukin-1
AGER	Angiotensinogen	Interleukin-6
Growth Hormone (Receptor)	Angiotensin II type 1 receptor	ALOX5AP
Lymphotoxin-alpha	Atrial natriuretic peptide	TNF-alpha
Estrogen Receptor alpha	Alpha-adducin	Tissue-type plasminogen activator
G-protein beta3	Serine protease inhibitor alpha(1)-antichymotrypsin	Atrial natriuretic peptide
Toll-like receptor 4		Paraoxonase 1
Cyclooxygenase 2		
Growth arrest-specific gene 6		
Kallikrein		
P-selectin		
von Willebrand Factor		

Table 2. Genes studied in relation to ischaemic stroke in a meta-analyses leading to a significant pooled risk

Genes	Polymorphism	N cases	N controls	Results OR (95% CI)	P-value
Angiotensin Converting Enzyme[53]	Insertion/ Deletion	2990	11305	D allele 1.21 (1.08-1.35)	<0.01
Apolipoprotein E[54]	Apoε2/ε3/ε4	926	890	Apoε4 allele 1.73 (1.34-2.23)	<0.001
5,10-methylenetetrahydrofolate reductase[55]	C677T	6110	8760	TT genotype 1.37 (1.15, 1.64)	<0.001
Factor V Leiden[53]	Factor V Leiden	4588	13789	Factor V Leiden 1.33 (1.12-1.58)	0.001
Prothrombin[53]	G20210A	3082	7131	A allele 1.44 (1.11-1.86)	0.006
Human platelet antigen type 2[53]	Thr145Met	564	962	Met allele 1.55 (1.14-2.11)	0.006
Plasminogen activator inhibitor 1[53]	Promoter 4G/5G I/D	842	1189	4G/5G 1.47 (1.13-1.92)	0.004
Phosphodiesterase 4D[56]	SNP 87	3808	4377	No ORs reported	0.002
	SNP 83				0.003
	SNP 41				0.003

N=total number, OR=odds ratio

Much is still unknown about this complex disease that has a major impact on public health. Understanding more about the genetic pathway of stroke might be of crucial importance in the future, as with increasing life expectancy, the incidence of stroke will tend to grow. This concerns both our understanding of aetiology as well as prognosis of stroke.

SCOPE OF THIS THESIS

In this thesis we aim to unravel the genetics of stroke by studying stroke as well as intermediate phenotypes and risk factors for stroke. Studies were performed in both isolated and outbred populations. First, we performed two heritability studies estimating the proportion of variance explained by genetic factors for carotid-femoral pulse wave velocity, carotid intima media thickness and carotid artery plaques (**Chapter 2.1**), and four blood pressure traits (**Chapter 2.2**). **Chapter 3** describes the results of two candidate gene studies in a genetically isolated population. **Chapter 3.1** shows the association between the phosphodiesterase 4D gene (*PDE4D*) and ischaemic stroke, and also describes the familial aggregation of ischaemic stroke. In **Chapter 3.2** the results are shown on the analysis between the gene encoding the 5-lipoxygenase-activating protein (*ALOX5AP*) and ischaemic stroke, as well as markers of the metabolic syndrome. **Chapter 4** encompasses candidate gene studies performed in the Rotterdam Study and the Rotterdam Scan Study. A polymorphism in the promoter region of the insulin-like growth factor I gene (*IGF-I*) was studied in relation to stroke and survival after stroke (**Chapter 4.1**). Polymorphisms of the alpha-adducin gene (*ADD1*, **Chapter 4.2**) and the angiotensinogen (*AGT*) and angiotensin II type 1 receptor (*AT1R*, **Chapter 4.3**) gene, were studied in relation to blood pressure, atherosclerosis, cardiovascular and cerebrovascular disease. **Chapter 5** shows the preliminary results of a genome wide association analysis on four blood pressure traits, performed in a genetically isolated population. Finally, we discuss the findings of this study in the context of what is known of stroke, and describe the possible impact of genetic testing on complex disease with stroke as the example (**Chapter 6**).

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Chapter 2

Heritability studies

Chapter 2.1

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

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

INTRODUCTION

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

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

Genetic factors play a major role in alterations of arterial wall function and structure.[10] Prior findings, however, on the extent to which genetic factors may explain the variance of these traits (heritability) are highly variable and limited in number. Heritability estimates (h^2) of carotid IMT published thus far range widely from 0.21 to 0.92,[11,12] but most of the studies reported heritabilities around 0.30 and 0.40.[13-17] To our knowledge, only two studies on the heritability of carotid plaques were performed, one yielding a non significant heritability[18] and the other an estimate of 0.23,[19] and no heritability estimate for PWV has been published to date.

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

MATERIALS AND METHODS

Study population

Subjects were participants of the Erasmus Rucphen Family (ERF) study. This is an ongoing large family based study, which began in June 2002 in a genetically isolated population located in the southwest of The Netherlands. This population was founded in the middle of the 18th century by approximately 150 individuals and was isolated until the last few decades. An extensive genealogical database including over 63,000 individuals is available for this population.

For the ERF study, 20 couples were selected that had at least six children baptised in the community church between 1880 and 1900. All living descendants of the selected couples and their spouses (n≈2500) were invited to participate in the study. The pedigree members were not selected based on any disease status. This study is based on the first 930 participants for whom complete phenotypic data were available at the time of the analyses. The Medical

Ethics Committee of the Erasmus Medical Centre Rotterdam approved the study and informed consent was obtained from all participants.

Data collection

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

Lipids and glucose levels were measured from the fasting blood sample, according to standard procedures.[20,21] Height and weight were measured with the participant in light underclothing and body mass index (kg/m^2) was computed. Blood pressure was measured twice on the right arm in a sitting position after at least five minutes rest, using an automated device (OMRON 711). The average of the two measures was used in the analyses. Mean arterial pressure (MAP) was calculated as 1/3 systolic blood pressure + 2/3 diastolic blood pressure.

During the cardiovascular assessment, carotid-femoral PWV was measured by means of an automatic Complior® SP device with the subjects in a supine position. The time delay between the rapid upstroke from the base point of simultaneously recorded pulse wave curves in the carotid and the femoral arteries were assessed and the distance between the carotid and the femoral arteries was measured over the surface of the body with a tape measure. PWV was calculated as the ratio between the distance travelled by the pulse wave and the time delay, and expressed in meters per second.

A duplex scan ultrasonographic investigation of the carotid arteries was made using a 7.5 MHz linear array transducer (ATL, Ultramark IV). Three optimal still images were recorded on the videotapes from each artery site (common carotid, bifurcation and internal carotid arteries at the right and left sides). Measurements of IMT were performed offline using the still images. We used measurements of common carotid IMT in this study. The interfaces of the far and near wall of the distal common carotid artery are marked by an automated method over a length of 10 mm, and maximum IMT is measured on the three still images of the near and far wall from both the left and right arteries. The mean value of these measurements is used in the analyses. When a plaque was present at the 10 mm measurement site, IMT was measured at the region closest to the plaque.

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

Statistical analyses

General characteristics were compared between men and women, using student *t*-test for continuous variables and chi-square test for dichotomous variables, with SPSS 11.0 for Windows. Inbreeding coefficients were calculated using the PEDIG software[22] based on a pedigree of the total population. The inbreeding coefficient equals the probability that two identical alleles at a given locus in an individual are identical by descent.

For the heritability analyses, we first performed univariable and multivariable regression analyses using SPSS. All covariates that were significant at the 0.10 level in the multivariable analysis were retained in the final model for heritability estimation. In order to obtain a normal distribution of the regression residuals for the traits under study, we used the natural logarithm transformation of (PWV–3) and (IMT–3). Since MAP is shown to strongly influence arterial stiffness,[23] it was used instead of systolic and diastolic blood pressures in the analyses related to PWV. In the multivariable regression models for IMT and plaque score, the beta coefficient for diastolic blood pressure is reported from a model without systolic blood pressure to avoid the multicolinearity effect. Both variables were used in the final analyses.

A variance component maximum likelihood method implemented in the SOLAR 2.1.2 software package[24] was used to partition the phenotypic variance of PWV, IMT, and plaque score into their additive genetic and environmental elements. The contribution of genetic factors to these traits was then estimated as the heritability, defined as the proportion of variance (after correction for covariates) explained by additive genetic components. Heritability estimates were calculated using a model with only age and gender and a full model with all significant covariates from the regression analyses. We also presented the contribution of the genetic factors to the total variance of each trait in different models, calculated as: [(1 – proportion of variance explained by covariates) × heritability estimate]. Significance was determined by likelihood ratio tests.

RESULTS

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

Table 2 shows the association between risk factors and PWV in univariable and multivariable models. In the univariable model, the association between gender and the outcome variables was adjusted for age, and the association between age and the outcomes was adjusted for gender. All other associations were adjusted for both age and gender. In the multivariable model, the only factors that significantly determined PWV were age, gender, MAP, LDL cho-

Table 1. Demographic characteristics of the study population

Variable	Men (n=376)	Women (n=554)
Age (years)	53.85±13.71	51.05±14.36*
Body mass index (kg/m^2)	27.50±4.06	26.70±4.81*
Systolic BP (mmHg)	145.14±20.24	138.93±22.86*
Diastolic BP (mmHg)	82.25±10.42	78.82±9.66*
Mean arterial pressure (mmHg)	103.21±12.34	98.86±12.61*
LDL cholesterol (mmol/L)	3.73±0.97	3.74±1.04
HDL cholesterol (mmol/L)	1.13±0.32	1.37±0.36*
Triglycerides (mmol/L)	1.59±1.02	1.29±0.63*
Fasting glucose (mmol/L)	4.94±1.05	4.63±1.04*
Current smoking (%)	33.69	48.36*
No college education (%)	90.37	90.73
Inbreeding (%)	78.72	77.08
Pulse wave velocity (m/s)	10.55±2.49	9.48±2.06*
Common carotid IMT ($mm \times 10^{-1}$)	9.15±2.18	8.31±1.92*
Carotid plaque score (median and interquartile range)	3 (1, 7)	3 (0, 5)*

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

Table 2. Univariable and multivariable regression analyses of PWV*

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

Beta: regression coefficient; HDL: high density lipoprotein; LDL: low density lipoprotein; PWV: pulse wave velocity; SE: standard error.
* Natural logarithm transformation was performed to obtain a normal distribution of the residuals.

lesterol, and heart rate. These factors were included in the multivariable adjusted model for the heritability analyses. Since fasting glucose levels have been associated with PWV,[25] this variable was also included in the final model.

Table 3. Univariable and multivariable regression analyses of IMT*

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

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

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

† Estimated from a multivariable model without systolic blood pressure

Table 4. Univariable and multivariable regression analyses of plaque score

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

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

* Estimated from a multivariable model without systolic blood pressure.

Table 3 presents the results of the univariable and multivariable regression analyses for IMT. The multivariable model showed that only triglycerides, low education, and inbreeding coefficient were not significantly associated with IMT. For plaque score, diastolic blood pres-

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

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

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

sure, triglycerides, glucose levels, low education, heart rate, and inbreeding coefficient were not significantly associated in the multivariable model (Table 4).

The estimated components of variance for PWV, IMT, and plaque score are presented in Table 5. All estimates of heritability were statistically significant. Adjusted for age and gender, the heritability estimate was 0.36 for PWV, indicating that the additive effects of genes account for 36% of the variation in PWV that is not explained by age and gender. With 0.45 of the variance being explained by age and gender, genetic factors account for $[(1.0 - 0.45) \times 0.36] \approx 0.20$ of the total variance in the PWV trait. After further adjustment for additional covariates, the estimate of heritability was 0.26, meaning that $[(1.0 - 0.53) \times 0.26] \approx 0.12$ of the total variance of the PWV trait is explained by genetic factors. Since the other risk factors used as covariates in the model may be in part genetically determined, this result indicates that 12% of the total variance of PWV is explained by unknown genetic factors that contribute to PWV variance independently of the covariates in the model. For IMT, the heritability was 0.41 in the first model and 0.35 in the second model, suggesting that genetic factors explain ≈ 0.16 and ≈ 0.12 of the total variance of IMT in the first and the second models, respectively. Heritability estimates of plaque score were 0.28 and 0.21, corresponding to ≈ 0.17 and ≈ 0.12 of the total variance explained by genetic factors.

DISCUSSION

In this large family based study in an isolated population, we investigated the contribution of genetic and environmental factors to carotid-femoral pulse wave velocity, common carotid intima media thickness, and carotid plaque score. Our study indicated that after adjusting for

appropriate risk factors, the additive effects of genes explain significant proportions of the variability in the function and structure of the arterial wall in this population.

To our knowledge, the present study is the first to report on the heritability of PWV, which mainly measures aortic stiffness. In the Rotterdam Study, we previously showed that arterial stiffness is strongly associated with common carotid intima media thickness, severity of plaques in the carotid artery, and severity of plaques in the aorta.[6] The associations between CVD risk factors and PWV that we observed in this isolate were in line with previous findings.[26] In this study, we investigated to what extent genetic factors contribute to the variation of PWV in this population and if this is comparable to the contribution of genetic factors to the variance of atherosclerosis measurements; carotid IMT and plaque score. For PWV, we found a heritability of 0.36. This estimate is quite similar to the heritability of IMT in our study. After adjustment for appropriate cardiovascular risk factors, the PWV heritability was reduced to 0.26. Although this reduction in the heritability estimate is greater than that observed for other traits in our study, we also observed a greater increase in the proportion of variance associated with the covariates. Some of these covariates are genetically mediated themselves, such as blood pressure (presented in this analysis as MAP).

Duggirala *et al*[12] reported a considerably higher heritability of carotid artery IMT (h^2=0.92) among a small sample (46 sibships of various sizes) from Mexico City; however, the authors suggested that their findings should be interpreted with caution because utilizing small sample of sibships only might have inflated their heritability estimate. In contrast, the findings from the present study are similar to those in previous reports, suggesting that genetic factors account for 0.30 to 0.40 of IMT variation in families, after adjustment for traditional CVD risk factors.[14-17] In this study, we also considered the genetic basis of another marker of subclinical atherosclerosis, the carotid plaque score. The observed associations between CVD risk factors and plaque score in our study were comparable to previous findings.[19,27] We found a heritability estimate of 0.28, which decreased to 0.21 after adjusting for the established cardiovascular risk factors. These heritability estimates were fairly similar to an earlier published study[19] performed in the San Antonio Family Heart Study, also using a randomly ascertained study population. Moskau *et al*,[18] who reported no significant heritability of plaque score, however, utilized a limited sample size of families that were ascertained by a parent affected with manifest atherosclerosis. It makes those results not directly comparable to results obtained in randomly selected, healthy populations.

It should be noted that direct comparison of the heritability estimates from the present study with those obtained from other studies is problematic. Different study designs, adjustments for covariates, and population specific environmental contributions to the phenotypic variance might result in different heritabilities even when the genetic variance estimates in the different populations are similar. In the present study, we used a huge single pedigree, the largest pedigree used so far to study heritability estimates. Previously published studies were mainly based on sib-pair analyses or multiple families selected on the basis of disease

status with relatively small sizes. In contrast, the members of our extended pedigree represent a random sample of our study population and were not ascertained through persons with a specific disease status. This allows us to make inferences about these heritabilities at the population level. At the same time, it is important to realise that the genetic contribution to a specific trait may not be constant between populations, even when they inherited the same genetic make-up. For instance, genes involved in salt sensitivity will not express in a population with low salt intake. Presence or absence of an environmental factor (diet, physical activity, life style, etc.) may indicate whether or not a certain genetic make-up plays an important role in the variability of a trait.

Inbreeding coefficient was not significantly related to any of the traits under the study. Excessive inbreeding may lead to a lower heritability due to an increase in homozygosity. However, we do not expect an effect of inbreeding on heritability estimates in this population, since this is a young isolated population and fluctuations for common genetic variants (>1%) in this population are therefore small.[28] The inclusion of covariates that are known to aggregate in families may have affected our results. Indeed, some of the covariates that we included are themselves genetically mediated, for example blood pressures and plasma lipid levels. Including such variables in the heritability calculations could reduce the heritability estimates whenever there are pleiotropic effects of genes on the covariates and the phenotypic measures under study. The heritability estimates derived from a model adjusted for age and gender indicate to what extent the genetic factors (directly or through other covariates) contribute to part of the variance of the trait unexplained by age and gender. On the other hand, estimated heritabilities from a fully adjusted model represent only the contribution of the genes that are acting independently of the considered covariates.

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

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Chapter 2.2

Heritability of blood pressure traits and the genetic contribution to blood pressure variance explained by four blood pressure related genes

Objective - To study the heritability of four blood pressure traits and the proportion of variance explained by four blood pressure related genes.

Methods - All participants are members of an extended pedigree from a Dutch genetically isolated population. Heritability and genetic correlations of systolic (SBP), diastolic (DBP), mean arterial (MAP) and pulse pressure (PP) were assessed using a variance components approach (SOLAR). Polymorphisms of the alpha-adducin (*ADD1*), angiotensinogen (*AGT*), angiotensin II type 1 receptor (*AT1R*) and G protein ß3 (*GNB3*) genes were typed.

Results - Heritability estimates were significant for all four blood pressure traits, ranging between 0.24-0.37. Genetic correlations between SBP, DBP and MAP were high (0.93-0.98), and low between PP and DBP (0.05). The *ADD1* polymorphism explained 0.3 % of the variance of PP (p=0.07), and the polymorphism of *GNB3*, explained 0.4 % of the variance of SBP (p=0.02), 0.2 % of MAP (p=0.05) and 0.3 % of PP (p=0.06).

Conclusion - Genetic factors contribute to a substantial proportion of blood pressure variance. In this study, the effect of polymorphisms of *ADD1*, *AGT*, *AT1R* and *GNB3* explained a very small proportion of the heritability of blood pressure traits. As new genes associated with blood pressure will be localised in the future, their effect on blood pressure variance should be calculated.

INTRODUCTION

It has long been recognized that genetic factors play a crucial role in blood pressure regulation. Twin, adoption and nuclear family studies indicated that a substantial proportion of systolic (SBP) and diastolic blood pressure (DBP) variance is due to the effect of genes.[1-8] Heritability estimates, however, range widely between different study populations, depending heavily on the type of relative pairs used. Heritability estimates for systolic and diastolic blood pressure vary around 60% in twin studies and around 25% in nuclear family studies.[1,3,5,6,9] Scarcer studies on heritability of mean arterial pressure (MAP) and pulse pressure (PP) produced estimates which vary from 35 to 60%.[10-12] 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 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.

A number of genes may explain part of the heritability of blood pressure. Genes involved in salt-sensitivity and the renin-angiotensin system (RAS), are known to play a role in blood pressure variance.[13] Until now, no studies have addressed the extent to which these genes explain the heritability of blood pressure.

In the present study, we aimed to assess to what extent genes influence blood pressure variance in 1006 inhabitants of a genetically isolated community in the Southwest part of the Netherlands. They were all related to each other in one extended pedigree, making it less likely that familial effects play a role. Heritability was estimated for four quantitative blood pressure traits: SBP, DBP, MAP and PP. Also, we estimated what proportion of blood pressure variance could be explained by the following polymorphisms: Gly460Trp of *ADD1*, M235T of angiotensinogen (*AGT*), C573T of the angiotensin II type 1 receptor (*AT1R*) and rs2301339 G/A of *GNB3*.

MATERIAL AND METHODS

Setting

Analyses were performed on phenotypic data collected from Dutch inhabitants of a genetically 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 program aims to identify genetic risk factors in the development of complex disorders.[14] The study was approved by the Medical Ethics Committee of Erasmus Medical Centre Rotterdam. Written informed consent was obtained from all participants.

Participants

Genealogical records demonstrated that almost all of the inhabitants of this isolated population could be traced back to about 150 individuals who founded this community around 1750. For years, minimal inward migration and considerable population growth characterised this population. About 20,000 inhabitants are now scattered over eight adjacent villages. Genealogical information on this population was reconstructed using church and the municipality records and is currently available in the form of a large pedigree-database including over 63,000 individuals.

For the ERF study, twenty couples that had at least 6 children baptised in the community church between 1880-1900 were identified with the help of genealogical records. All living descendants of these couples and their spouses were invited to participate in the study. With the use of the pedigree-database, these families could be linked to one founder couple in a large, complex pedigree.

Data collection

Participants were invited for a series of clinical examinations at our research centre, located within the community. 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 analysis. MAP (1/3 SBP + 2/3 DBP) and PP (SBP-DBP) were calculated. Hypertension was defined as a DBP of 90 mmHg or higher and/or a SBP of 140 mmHg or higher and/or use of anti-hypertensive medication indicated for the treatment of hypertension.[15,16] Height and weight were measured with the participant dressed in light under clothing and body mass index (BMI) was calculated (kg/m^2). Finally, a research physician obtained information on medical history, medication use, smoking and alcohol use in a personal interview.

At the start of the clinical examinations, fasting blood samples were drawn for the extraction of DNA and measurement of lipids, glucose, plasma creatinine, and plasma albumin levels according to a standardized procedure.[17,18] 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 to 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.[19]

Data collection started in June 2002 and was finished in February 2005. In this study, we focused on the first 1006 participants for whom complete phenotypic, genotypic and genealogical information was available.

Genotyping

We genotyped the following polymorphisms: *ADD1* Gly460Trp, the *AGT* M235T, the *AT1R* C573T and the *GNB3* rs2301339 G/A. Genotyping was performed using TaqMan allelic discrimination Assays-By-Design (Applied Biosystems, Foster City, CA).[20] For *ADD1*, the forward primer sequences was 5'-GAGAAGACAAGATGGCTGAACTCT-3' and reverse primer sequence 5'-GTCTTCGACTTGGGACTGCTT-3'. The minor groove binding probes were 5'-VIC-CATTCTGCCCTTCCTC-NFQ-3' and 5'-FAM-ATTCTGCCATTCCTC-NFQ-3'. We used the reverse strand design for this polymorphism. Forward primer sequences were 5'-GGTTTGCCTTACCTTGGAAGTG-3' and 5'-TGTGCTTTCCATTATGAGTCCCAAA-3' for *AGT* and *AT1R* respectively. Reverse primer sequences were 5'-GCTGTGACAGGATGGAAGACT-3' and 5'-CAGAAAAGGAAACAGGAAACCCAGTATA-3'for *AGT* and *AT1R* respectively. The minor groove binding probes were 5'-VIC-TGGCTCCCATCAGG-NFQ-3' and 5'-FAM-CTGGCTCCCGTCAGG-NFQ-3' for *AGT*. The minor groove binding probes were 5'-VIC-CTATCGGGAGGGTTG-NFQ-3' and 5'-FAM-CTATCGGAAGGGTTG-NFQ-3' for *AT1R*. We used the reverse strand design for this polymorphism. For *GNB3*, the forward primer sequences was 5'-GGCAGGGCTGCTTCTCA-3' and reverse primer sequence 5'-GCAAGCCGCTGCTCTCA-3'. The minor groove binding probes were 5'-VIC-AAACCAAGGAAGGGACA-NFQ-3' and 5'-FAM-ACCAAGGGAGGGACA-NFQ-3'. The assays utilized 5 nanograms of genomic DNA and 5 micro liter reaction volumes. The amplification and extension protocol was as follows: an initial activation step of 10 min at 95 °C preceded 40 cycles of denaturation at 95 °C for 15 s. and annealing and extension at 50 °C for 60 s. Allele-specific fluorescence was then analysed on an ABI Prism 7900HT Sequence Detection System with SDS v 2.1 (Applied Biosystems, Foster City, CA). Based on the analysis of blind duplicates, there was a 98% concordance in genotyping of *ADD,* 99.4% of *AGT* and 100% for *AT1R* and *GNB3*.

Statistical analysis

Baseline characteristics were compared using univariate ANOVA or χ^2 statistics. Univariate analyses of variance were used to assess the relation between the *ADD1* Gly460Trp, the *AGT* M235T, the *AT1R* C573T and the *GNB3* rs2301339 G/A polymorphisms and blood pressure traits (SPSS 11.0). A variance component maximum likelihood approach, implemented in the SOLAR software package, was used to estimate heritability and genetic correlations for SBP, DBP, MAP and PP.[21] Heritability (h^2) was estimated as the ratio of the variance of the trait explained by additive polygenic effects to the total phenotypic variance of the trait. We identified significant (environmental) covariates for each blood pressure trait in order to estimate

the contribution of environmental factors to blood pressure variance. Significant effects of each covariate were tested using a likelihood ratio test with 1 *df* (SPSS 11.0). Covariates that were included in the final model, were significant at the 0.10 level. These were age, sex, total cholesterol, HDL cholesterol, glucose levels, alcohol intake, anti-hypertensive medication, and BMI. In order to satisfy distributional assumptions, SBP, DBP, MAP and PP were natural log transformed to ensure normally distributed residuals. Heritability models included age, sex, anti-hypertensive medication, BMI, total cholesterol, HDL cholesterol, alcohol intake and glucose levels as covariates.

Bivariate analysis was performed to estimate the genetic and environmental correlations between the four blood pressure traits.[22,23] The phenotypic correlations between the blood pressure traits were then calculated by the following formula[24,25]:

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

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. To test whether a given correlation between two blood pressure traits was significantly different from zero, the likelihood of a model in which this correlation was constrained to zero was compared 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.

Next, we assessed the proportion of variance explained by the following polymorphisms: Gly460Trp of *ADD1*, M235T of *AGT* and C573T of *AT1R* and rs2301339 G/A of *GNB3*. The significance of these polymorphisms was tested using the likelihood ratio test, where the likelihood of a model including the polymorphism is estimated, then compared with the likelihood of a model without the polymorphism. Twice the difference in the natural In-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.[26] In the output files, the proportion of variance explained by all covariates is presented. The absolute increase in this proportion by adding the polymorphism to the model, equals the proportion of variance explained the polymorphism.

RESULTS

In total, 1,006 participants were available for analysis, including 907 first-degree relative pairs, 659 second-degree relative pairs and 2370 third-degree relative pairs.

Table 1 presents the general descriptives of the total study population stratified by sex. The mean age was 56 years for men and 54 years for women, but as participants were ascertained from three generations, the age range was very broad (18-92 years). Most characteristics were higher in males, with the exception of HDL cholesterol and smoking.

In table 2, the heritability estimates for SBP, DBP, MAP and PP are presented. Heritability estimates for SBP, DBP, MAP and PP were significant ($p<0.001$), and ranged from 0.24 for PP to 0.37 for DBP.

Table 1. General characteristics of the study population

Number	Men (n=407)	Women (n=597)	p-value
Age (yrs)	55.7 ± 14.4	53.5 ± 15.6	0.02
SBP (mmHg)	145.7 ± 20.3	139.5 ± 22.4	<0.001
DBP (mmHg)	82.6 ± 10.4	78.7 ± 9.6	<0.001
MAP (mmHg)	103.6 ± 12.4	98.6 ± 12.5	<0.001
Pulse pressure (mmHg)	63.2 ± 16.0	59.8 ± 18.0	<0.01
Anti-hypertensive medication (%)	29.1	22.4	0.02
Total-cholesterol – mmol/l	5.5 ± 1.1	5.6 ± 1.2	0.11
HDL-cholesterol – mmol/l	1.1 ± 0.3	1.4 ± 0.4	<0.001
LDL-cholesterol – mmol/l	3.7 ± 1.0	3.7 ± 1.0	0.94
Triglycerides – mmol/l	1.6 ± 1.0	1.3 ± 0.6	<0.001
Glucose – mmol/l	5.0 ± 1.1	4.6 ± 1.1	<0.001
Diabetes Mellitus – %	7.3	6.4	0.61
Hyperlipidemia – %	35.7	27.5	<0.01
BMI – kg/m^2	27.7 ± 4.2	26.8 ± 4.8	<0.01
Current smokers – %	33.2	48.2	<0.001
Alcohol use – units/week	8.4 ± 12.8	1.7 ± 4.0	<0.001

Values are unadjusted and presented as percentage or mean ± SD, SBP=systolic blood pressure, DBP=diastolic blood pressure, MAP= mean arterial pressure, BMI=body mass index.

Table 2. Heritability estimates of SBP, DBP, MAP and PP

Phenotype	n	H^2 ± se	p-value
SBP	1006	0.34 ± 0.08	<0.001
DBP	1006	0.37 ± 0.09	<0.001
MAP	1006	0.40 ± 0.08	<0.001
PP	1006	0.24 ± 0.08	<0.001

H^2=heritability, SBP=systolic blood pressure, DBP=diastolic blood pressure,
MAP=mean arterial pressure, PP= pulse pressure. Heritability analyses are adjusted for age, sex, anti-hypertensive medication, body mass index, total cholesterol, HDL cholesterol, alcohol intake and glucose levels.

Table 3. Phenotypic, genetic and environmental correlations between SBP, DBP, MAP and PP

	ρP	ρG	ρE
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

ρP=phenotypic correlation, ρG=genetic correlation, ρE=environmental correlation, SBP=systolic blood pressure, DBP=diastolic blood pressure, MAP=mean arterial pressure, PP= pulse pressure
*All correlations are significant with p<0.001, except for ρP and ρG between DBP and PP.

Table 3 shows the results of the bivariate analyses. All correlations were positive and significant, with the exceptions of the phenotypic and genetic correlations 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 significant and very high (0.93-0.98), whereas the genetic correlation between PP and DBP was not significant and low (0.05). Environmental correlations ranged widely, from 0.19 for DBP-PP to 0.89 for SBP-MAP.

Figure 1 shows that SBP was significantly higher in AG carriers of *GNB3* compared with AA carriers (p=0.003). SBP was also higher in GG carriers, however this was not significant (p=0.06). MAP was also significantly higher in AG carriers (p=0.02) and PP was higher in both AG (p=0.002) and GG carriers (p=0.05, data not shown). No significant differences were found between the other genotype groups and SBP, DBP, MAP or PP. From figure 1, we choose the following models for the analyses presented in table 4; *ADD1* dominant model, *AGT* recessive model, *AT1R* dominant model and *GNB3* dominant model.

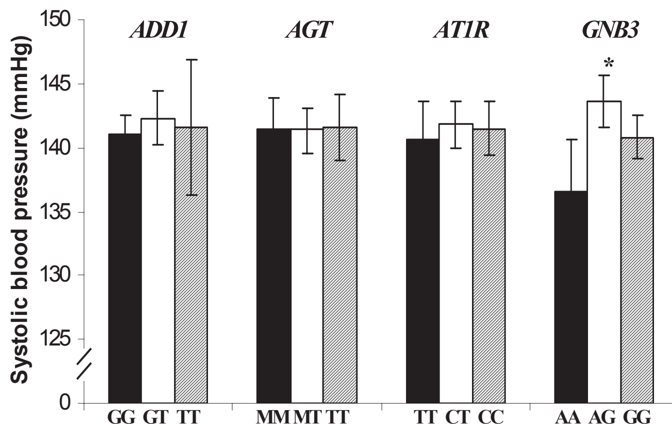

Figure 1. Mean systolic blood pressure levels in four different genotype groups.
*p<0.05 compared with the reference group AA, adjusted for age and sex.

Table 4. Proportion of variance of SBP, DBP, MAP and PP explained by all covariates and four blood pressure genes

Phenotype	Proportion of variance explained by								
	All covariates	**ADD1**		**AGT**		**AT1R**		**GNB3**	
	(%)	(%)	*p-value*	(%)	*p-value*	(%)	*p-value*	(%)	*p-value*
SBP	27.8	0.2	0.16	<0.1	0.45	<0.1	0.93	0.4	0.02
DBP	11.0	0.1	0.78	0.2	0.84	0.1	0.95	0.3	0.24
MAP	21.5	<0.1	0.54	0.1	0.73	0.1	0.99	0.2	0.05
PP	24.5	0.3	0.07	<0.1	0.09	<0.1	0.15	0.3	0.06

ADD1: Alpha-adducin Gly460Trp, *AGT*: Angiotensinogen M235T, *AT1R*: Angiotensin II type 1 receptor C573T, *GNB3*: G-Protein rs2301339 G/A polymorphism, SBP=systolic blood pressure, DBP=diastolic blood pressure, MAP=mean arterial pressure, PP= pulse pressure. All analyses are adjusted for age, sex, anti-hypertensive medication, body mass index, total cholesterol, HDL cholesterol, alcohol intake, glucose levels and pedigree structure.

Table 4 shows the proportion of variance in SBP, DBP, MAP and PP explained by all covariates and by the polymorphisms in *ADD1*, *AGT*, *AT1R* and *GNB3*. Age and sex explained 25.8% of SBP, 7.4% of DBP, 18.4% of MAP and 24.0% of PP (data not shown). Adding anti-hypertensive medication, BMI, total cholesterol, HDL cholesterol, alcohol intake and glucose levels to the model only slightly increased the proportion of variance explained by all covariates (with a maximum of 4%), suggesting that age and sex explain most of the blood pressure variance. The Gly460Trp polymorphism of *ADD1* explained 0.3 % of the variance of PP (p=0.07). The rs2301339 G/A polymorphism of *GNB3*, explained 0.4 % of the variance of SBP (p=0.02), 0.2 % of MAP (p=0.05) and 0.3 % of PP (p=0.06). The other 2 polymorphisms also explained less than 1% of the variance of the blood pressure traits, but none were significant.

DISCUSSION

In this study, heritability estimates for blood pressure traits were highly significant and varied between 24 and 40%. Also, high phenotypic, genetic and environmental correlations between SBP, DBP, MAP and PP were found. The proportion of variance explained by covariates was mostly determined by age and sex. In the single gene analysis, a small but significant proportion of the variance of SBP and MAP was determined by the rs2301339 G/A polymorphism of *GNB3*.

The strength of our study lies within its population-based nature, embedded in a family-based study design. The members of our extended pedigree therefore represent a random sample of our study population and were not ascertained through persons with extreme blood pressure values.

All of our heritability estimates were significant and ranged between 0.25 for SBP and 0.37 for DBP. They are within the 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. Other than age and sex, BMI, fasting glucose levels and alcohol intake were the most important covariates influencing blood pressure in this population.

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. In contrast, the genetic correlation between PP and DBP was absent. This suggests the existence of an independent set of genes influencing PP and DBP variance. This is concordance with a previous finding suggesting that DBP and PP map to different loci.[30] Pulse pressure is an important measure of arterial stiffness and a strong predictor of cardiovascular morbidity and mortality, independent of blood pressure.[31] 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.

Previous studies found associations between polymorphisms in *ADD1*, *AGT*, *AT1R, GNB3* and blood pressure, hypertension and salt-sensitivity.[13,32-34] None of these studies, however, addressed the proportion of blood pressure variance that these genes explain. In this study, only the rs2301339 G/A polymorphism of *GNB3* significantly explained a portion of the variance of SBP and MAP. This proportion however, was less than 1 %.

To calculate the association between the four polymorphisms and blood pressure traits, we used the measured genotype approach, comparing the likelihood of a model including the polymorphism, with the likelihood of a model without the polymorphism, using the SOLAR software package. This approach was chosen because of the complex nature of our pedigree, which contains numerous loops. The SOLAR software package was able to analyse the complete pedigree, without breaking any of the loops.

In conclusion, our study shows that polymorphisms of *ADD1*, *AGT*, *AT1R* and *GNB3*, explain a very small part of the heritability of blood pressure traits. Given the high heritability estimates, still many genes remain to be identified.

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Chapter 3

Candidate gene studies in a genetically isolated population

Chapter 3.1

Familial aggregation, the PDE4D gene and ischaemic stroke in a genetically isolated population

Objective – To study the familial aggregation of ischaemic stroke and the association between the *PDE4D* gene and ischaemic stroke.
Methods – This study was performed in an isolated population in the Netherlands, where we identified 91 patients with ischaemic stroke. Ischaemic stroke was sub classified in large- and small-vessel infarction. We calculated kinship and inbreeding coefficients and genotyped all patients for 3 single nucleotide polymorphisms (SNPs) in the *PDE4D* gene.
Results –The proportion of related pairs was higher in patients with ischaemic stroke (68.8 %) compared with controls (30.7 %, p<0.001). For large-vessel infarction, the proportion of related pairs was higher (71 %) compared with small-vessel infarction (62.8 %, p<0.001). Familial aggregation was strongest for early-onset (onset <45 years) patients. All stroke groups were significantly more inbred compared with controls. In inbred individuals, the C allele of SNP45 increased the risk of small-vessel infarction 4.8 times (95% CI: 1.1-22.3) compared with controls (p=0.04). The T allele of SNP39 increased the risk of small-vessel infarction 6.3 times (95% CI: 1.4-28.7) compared with controls (p=0.02). No associations were found for large-vessel stroke.
Conclusions – There was familial aggregation of ischaemic stroke and a difference in degree of familial clustering between stroke subtypes. We also found that the *PDE4D* gene is significantly associated with small-vessel infarction in inbred individuals.

INTRODUCTION

One risk factor for ischaemic stroke is a positive family history of stroke,[1,2] suggesting an underlying genetic susceptibility, which is also supported by animal studies.[3,4] A recent meta-analysis reported major heterogeneity between different genetic epidemiological studies of ischaemic stroke.[2] While there is evidence for differences in the degree of familial clustering in subtypes of ischaemic stroke,[5-7] few studies have considered ischaemic stroke phenotype in detail.

Recently, a genomic screen was conducted in multiple affected families with stroke from the isolated Icelandic population where extensive genealogic information is available. This led to the identification of the risk gene encoding phosphodiesterase 4D (*PDE4D*).[8] However, this study was criticized for its lack of accurate phenotype definition.[9] Furthermore, it is not known whether this gene confers a risk in other ethnic groups.

Most studies of familial aggregation have been based on family history. We had the opportunity to study familial aggregation of ischaemic stroke in an isolated population for which we had access to genealogic data. We studied the familial aggregation of ischaemic stroke, ischaemic stroke subtypes and early-onset ischaemic stroke and performed an analysis of the gene encoding *PDE4D* in an isolated population in the Netherlands.

METHODS

Study population

Our study is part of the research program "Genetic Research in Isolated Populations (GRIP), which studies various complex diseases within a recently isolated population in the Southwest of the Netherlands, in the region between Roosendaal and Breda. The village was founded in the middle of the 18th century by approximately 150 individuals and was isolated until the last few decades. The population is characterized by rapid growth and has now expanded to 20.000 inhabitants. The Medical Ethical Committee of the Erasmus Medical Centre Rotterdam approved the study protocol.

Participants

Patients were recruited by the general practitioners who are working in this population. They selected all users of anti-platelet drugs or anticoagulants and from them, selected all patients who had had a stroke. Furthermore, only patients for whom neuro-imaging information was available and who were seen by a neurologist were selected. A total of 151 patients were selected and invited to participate and 107 patients gave informed consent (response rate of 71

%). Next, clinical information was obtained from medical records of the general practitioners, neurologists, cardiologists and rehabilitation physicians. The diagnosis of ischaemic stroke was verified independently by two neurologists with expertise in neurovascular diseases (JCvS, LJK) and was based on all available information, including the re-examination of neuro-imaging findings. 16 patients were excluded, as the diagnosis of ischaemic stroke could not be verified and another condition had caused the stroke-like symptoms, e.g. haemorrhagic stroke, epilepsy or migraine. All ischaemic stroke patients were sub classified as large-vessel infarction, small-vessel infarction, or unspecified stroke. A stroke was classified as large-vessel infarction if a lesion on CT or MRI greater than 1.5 cm was present or clinical findings included cortical impairment, brainstem or cerebellar dysfunction. A stroke was classified as small-vessel infarction if a lesion on CT or MRI smaller than 1.5 cm was present or CT /MRI was normal or the traditional clinical lacunar syndrome was present with no evidence of cortical dysfunction. If symptoms and imaging results were inconsistent with both large- and small-vessel infarction, stroke was classified as unspecified.

We did not specify stroke with a possible cardio embolic source, as we did not have cardiac imaging information available for re-examination. EKG information was available (through the medical records), however, many high-risk cardiac sources are missed with only EKG information. Early-onset stroke was defined as stroke with an age at onset ≤ 45 years. In case of discrepancy, the final diagnosis was established in a consensus meeting.

The controls were individuals living in the same isolated population without a history of stroke or TIA. First, 200 spouses of patients participating in an ongoing study in the same isolate, the Erasmus Rucphen Family study (ERF) were selected for comparing general characteristics between patients and controls and for studying the association between stroke and the Icelandic SNPs. Second, for every ischaemic stroke group, a random sample with the same size, age and sex matched, was drawn from the genealogic database. We did 1000 replicas of this sampling. This was done for the calculations on kinship and inbreeding.

Measurements and definitions

Information on medical history, medication use and lifestyle was obtained from all participants by means of interviews, as was height and weight. Blood was drawn for DNA analysis and clinical chemistry. Blood pressure was measured twice with the participant in the sitting position using an automated device. Hypertension was defined as a diastolic blood pressure of ≥ 100 mm Hg or a systolic blood pressure of ≥ 160 mm Hg or use of antihypertensive medication used for the treatment of high blood pressure (grades 2 and 3 of the 1999 World Health Organization criteria).[10] Total serum cholesterol and HDL cholesterol were determined with an automated enzymatic procedure.[11] Glucose levels were measured by the glucose hexokinase method.[12] Hyperlipidemia was defined as total serum cholesterol (mmol/L)) ≥ 6.5 - ≤ 9.0 if cholesterol/HDL cholesterol ratio (chol/HDL) ≥ 5.0 or total serum cholesterol < 6.5 if chol/HDL

> 8.0 or total serum cholesterol ≥ 9.0 or use of cholesterol-lowering medication. Diabetes mellitus was defined as random serum glucose level (mmol/L) ≥ 11.1 in non-fasting blood or ≥ 7.0 in fasting blood (American Diabetes Association criteria) or use of glucose-lowering medication.

Intima-media thickness (IMT) of the carotid artery and total number of plaques were assessed by duplex scan ultrasonography. This was done with a 7.5-MHz linear-array transducer (ATL Ultra-Mark IV). Measurements of IMT were performed offline from the still images recorded on videotape.[13] IMT was measured at the common carotid artery (CCA IMT) and was measured over an average distance of 10 mm. We used the average of the measurements of three still images of both the left and right arteries. CCA IMT was determined as the mean of the maximum IMT of near and far wall measurements of both the left and right arteries. We defined plaques as focal widening of the vessel wall with protrusion into the lumen. 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 12 (left and right sided, near and far wall common carotid arteries, bifurcation, and internal carotid arteries).

Genealogy

The names, dates and places of births of family members were collected and extended up to 22 generations using municipal registers and data from a large genealogy database that holds genealogical information on more than 70,000 individuals from the GRIP region.

Genealogic relationships between two individuals can be expressed as the pair wise kinship coefficient (K). This is the probability that a randomly drawn allele from one person is identical by descent with a randomly drawn allele at the same locus of another person. Three stroke patients were ascertained through another stroke patient, because they were related to each other. These patients were excluded from the kinship analyses. The probability that two alleles in one individual are identical by descent is expressed as the inbreeding coefficient (I). This coefficient represents the degree of consanguinity between the parents of this individual.

Laboratory analysis

We genotyped three single nucleotide polymorphisms (SNPs), SNP83, SNP45 and SNP39. These SNPs were chosen as they were found to be significantly associated with stroke in the Icelandic population 8. For SNP41, a primer could not be designed. SNP83 and SNP45 are located in the gene encoding phosphodiesterase 4D (*PDE4D*), SNP39 is located in the overlapping gene, prostate androgen-regulated transcript (PART1). Both genes are located on chromosome 5q12. Genotyping of SNP83, SNP45 and SNP39 was performed using Taq-

Man allelic discrimination Assays-By-Design (Applied Biosystems, Foster City, CA).[14] Forward primer sequences were 5′-CATCCTGTATAGGTATGAGTCCTGCTA-3′, 5′-GTGAACAAAAGTATT-GCTGCCATCA-3′ and 5′-GGGCTCCCCAGATTCTTCTTTG-3′ and reverse primer sequences were 5′-AACTAATATCAGATTGGAAGGATCTGCTG-3′, 5′-CAAAACTGAGAGCAAGCAGCAAATA-3′ and 5′-GGGATGGCACACAAAAAGATTTAGT-3′. The minor groove binding probes were 5′-VIC-TG-GATAAACCACATTTT-NFQ-3′ and 5′-FAM-CTGGATAAACCACGTTTT-NFQ-3′ for SNP83 (for this SNP, we used the reverse strand design), 5′-VIC-CTCCTGTTACTGTGCCC-NFQ-3′ and 5′-FAM-CTCCTGTTATTGTGCCC-NFQ-3′ for SNP45 and 5′-VIC-CCTAACCTCCCTCCC-NFQ-3′ and 5′-FAM-CCTATCCTCCCTCCC-NFQ-3′ for SNP39. The assays utilized 5 nanograms of genomic DNA and 5 micro liter reaction volumes. The amplification and extension protocol was as follows: an initial activation step of 10 min at 95 °C preceded 40 cycles of denaturation at 95 °C for 15 s. and annealing and extension at 50 °C for 60 s. Allele-specific fluorescence was then analysed on an ABI Prism 7900HT Sequence Detection System with SDS v 2.1 (Applied Biosystems, Foster City, CA). Based on the analysis of blind duplicates, there was a 100% concordance in genotyping of all three SNPs.

Statistical analysis

General characteristics were compared between patients and controls using univariate ANOVA for continuous variables and χ^2 statistics for dichotomous variables with SPSS 11.0 for Windows. We calculated pair wise K and I using PEDIG software[15] based on a pedigree of the total population, consisting of 74461 patients. We studied whether K and I in stroke patients deviate from what is to be expected by comparing it to a random sample of controls, living in the same isolated population. For every ischaemic stroke group, a random group with the same size, age and sex matched, was drawn from the genealogic database. We did 1000 replicas of this sampling in order to obtain a null distribution of K and I. Then, we used empirical p-values to test whether the estimates of K and I we obtained in patients, deviated from the null distribution we derived from bootstrapping. We used χ^2 statistics to compare the distribution of K and I between different stroke groups. Next, we performed binary logistic regression (SPSS 11.0) to calculate the odds ratio for stroke for SNP83, SNP45 and SNP39. We used Bonferroni to adjust for multiple testing. We used the software package PHASE, version 2.1.1, to estimate haplotypes from the genotypic data of SNP83, SNP45 and SNP39.[16]

RESULTS

We found 91 patients with ischaemic stroke. Of the stroke patients, 43 (47 %) were classified as having large-vessel infarction, 39 (43 %) as small-vessel infarction and 9 (10 %) remained unspecified. Of the 91 patients, 13 (14 %) had an early-onset of ischaemic stroke (age

Table 1. General characteristics for all ischaemic stroke patients, stroke subtypes and controls

	Large-Vessel Infarction (n=43)	Small-Vessel Infarction (n=39)	Early-Onset Stroke (n=13)	Late-Onset Stroke (n=78)	All Ischaemic Stroke (n=91)	Controls (n=200)
Age, yrs	65.3 ± 11.4 *	61.5 ± 13.1 *	42.8 ± 6.3	67.7 ± 8.8	64.1 ± 12.2 *	56.8 ± 11.6
Sex, % male	55.8	61.5 *	23.1	65.4 *	59.3 *	40.0
Hypertension, %	74.4 *	71.8 *	30.8	78.9 *	71.9 *	39.6
Hyperlipidemia, %	76.2 *	55.3 *	41.7	67.5 *	64.0 *	30.5
Diabetes Mellitus	19.0 *	23.7 *	0.0	25.0 *	21.6 *	7.0
Current smoking, %	44.2	51.3	69.2 *	43.6	47.3	41.0
BMI, kg/m^2	28.2 ± 4.5	27.1 ± 4.8	25.6 ± 7.1	27.8 ± 4.1	27.5 ± 4.6	27.6 ± 4.9
CCA IMT, mm	1.11 ± 0.3 *	0.93 ± 0.2	0.74 ± 0.1	1.10 ± 0.2 *	1.03 ± 0.2 *	0.85 ± 0.2
Total number of plaques	7.3 ± 4.0 *	4.9 ± 3.9	1.2 ± 2.8 *	7.6 ± 3.7 *	5.9 ± 4.2 *	4.5 ± 3.5

All values are unadjusted means ± SD or percentages. * Significantly different from controls ($p<0.05$). CCA IMT=Intima-media thickness of the common carotid artery; Total number of plaques=Total number of sites with plaques in the carotid artery ranging from 0-12.

at onset ≤ 45 years). Patients were significantly older and significantly more often male when compared with the 200 controls (Table 1). There were significantly more stroke patients with hypertension, hyperlipidemia and diabetes mellitus. Also, the CCA IMT and total number of plaques in the carotid artery were significantly higher among patients compared with controls (Table 1). These differences remained significant after adjusting for age and sex. Table 1 also shows the differences between subgroups of ischaemic stroke and controls. Patients with large-vessel infarction, small-vessel infarction and late-onset stroke, had significantly more hypertension, hyperlipidemia and diabetes mellitus compared with controls. As expected, large-vessel stroke patients had significantly higher CCA IMT and plaque score compared with controls. After adjusting for age and sex, this remained significant. The same was found for late-onset stroke.

We also compared among patients those with large- and small-vessel infarction and early- and late-onset stroke. After adjusting for age and sex, CCA IMT and plaque score were significantly higher in patients with large-vessel compared with small-vessel infarction.

Genealogic research showed that 50 patients (55.0%) could be linked to one common ancestor within 7 to 9 generations (Figure 1). We show the genealogic lineages of these patients based on the shortest connection with this common ancestor. In reality, numerous connections exist between these patients via multiple ancestors.

To study the degree of relationship between all 91 ischaemic stroke patients, we calculated pair wise kinship coefficients (K) for every possible pair in the ischaemic stroke group. We also calculated K for every possible pair in 1000 randomly drawn control samples, each containing 91 controls. We made 3 categories for K: ½ – ½ 6 = related within 3 generations, < ½ 6 = related within more than 3 generations, 0=not related. The frequencies of pairs in each category were calculated. For controls, the frequencies are the means of the 1000 samples (Table 2). The pro-

Chapter 3.1

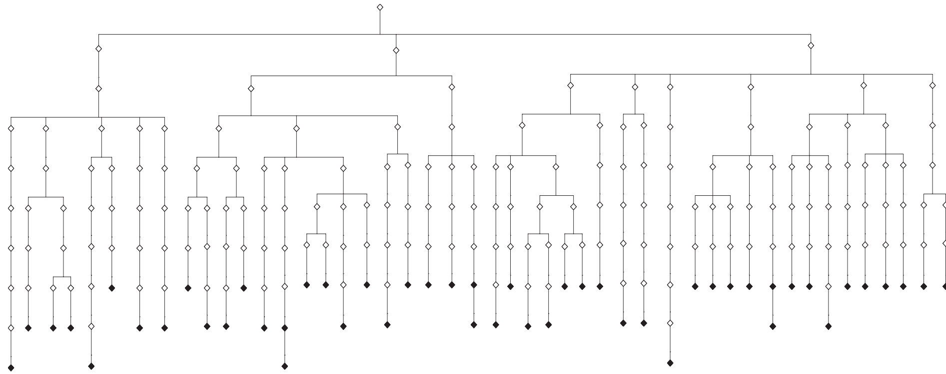

Figure 1. Pedigree of 50 stroke patients from the genetically isolated population

portion of patients that is connected to a common ancestor (K>0) was higher for the overall stroke group (68.8%) compared with controls (30.7%, p<0.001). For all other ischaemic stroke subgroups, we also calculated K and drew 1000 samples of age and sex matched controls with the same size to calculate K for controls. For all ischaemic stroke subgroups, K>0 was significantly higher compared with controls (Table 2, the distribution of K in the control samples is not shown.). For early-onset stroke, all possible pairs were related. The proportion of closely and remotely related pairs was significantly higher for all ischaemic stroke groups compared with controls.

Table 2 also shows that among patients with large-vessel infarction, there was a higher proportion of related pairs (71.0%) than among those with small-vessel infarction (62.8%, p<0.001). The distribution of closely related pairs was also different between patients with large- and small-vessel infarction (p<0.05), as was the distribution of remotely related pairs (p<0.001). For early-onset ischaemic strokes, there were no unrelated pairs while 35.2% of the late-onset pairs was unrelated (p<0.001). The proportion of moderately related pairs was also different from late-onset pairs (p<0.001).

The proportion of ischaemic stroke patients that is inbred (I>0) is 65.9% (Table 3). This higher compared with controls (27.5%, p<0.01). Also for all other stroke groups, the proportion

Table 2. Distribution of kinship coefficients (K) for all ischaemic stroke patients, stroke subtypes and controls

Kinship Coefficient (K)	Large-Vessel Infarction	Small-Vessel Infarction	Early-Onset Ischaemic Stroke	Late-Onset Ischaemic Stroke	All Ischaemic Stroke	Controls
$½ – ½^6$	2.3 (18) *	4.5 (33) *	5.1 (4) **	2.7 (76) *	3.3 (124) *	0.5 (18)
$< ½^6$	68.7 (536) *	58.3 (432) *	94.9 (74) **	60.6 (1682) *	65.5 (2451) *	30.2 (1130)
0	29.0 (226) *	37.2 (276) *	0.0 (0) **	36.7 (1017) *	31.2 (1166) *	69.3 (2592)
Total number of pairs	780	741	78	2775	3741	3741

All values are percentages of the total number of pairs with absolute numbers in between brackets. *p<0.001, **p<0.01 compared with controls.

Table 3. Distribution of inbreeding coefficients (I) for all ischaemic stroke patients, stroke subtypes and controls

Inbreeding Coefficient (I)	Large-Vessel Infarction	Small-Vessel Infarction	Early-Onset Ischaemic Stroke	Late-Onset Ischaemic Stroke	All Ischaemic Stroke	Controls
½ – ½ [6]	7.0 (3)	5.1 (2)	7.7 (1)	6.4 (5) *	6.6 (6) **	2.2 (2)
< ½ [6]	62.8 (27) *	59.0 (23) *	76.9 (10) *	56.4 (44) *	59.3 (54) **	25.3 (23)
0	30.2 (13) *	35.9 (14) *	15.4 (2) *	37.2 (29) *	34.1 (31) **	72.5 (66)
Total number	43	39	13	78	91	91

All values are percentages of the total number of individuals with absolute numbers in between brackets. *p<0.001, **p<0.01 compared with controls.

Table 4. Genotype distribution of 3 SNPs in the *PDE4D* gene for all ischaemic stroke patients, stroke subtypes and controls.

	SNP 83			SNP 45			SNP 39		
	TT	CT	CC	TT	CT	CC	AA	AT	TT
Controls, n (%)	32 (17.0)	86 (45.8)	70 (37.2)	5 (2.6)	47 (24.6)	139 (72.8)	3 (1.6)	59 (30.7)	130 (67.7)
Patients, n (%)	17 (19.3)	37 (42.1)	34 (38.6)	5 (5.6)	19 (21.3)	65 (73.1)	3 (3.4)	23 (26.1)	62 (70.5)
-LV, n (%)	6 (14.3)	18 (42.9)	18 (42.9)	2 (4.8)	13 (31.0)	27 (64.3)	2 (4.8)	13 (31.0)	27 (64.2)
-SV, n (%)	8 (21.6)	17 (45.9)	12 (32.4)	0 (0.0)	8 (21.6)	29 (78.4)	0 (0.0)	8 (21.6)	29 (78.9)
-EO, n (%)	3 (23.1)	8 (61.5)	2 (15.4)	1 (7.7)	3 (23.1)	9 (69.2)	1 (7.7)	3 (23.1)	9 (69.2)
-LO, n (%)	14 (18.7)	29 (38.7)	32 (42.6)	4 (5.3)	16 (21.0)	56 (73.7)	2 (2.7)	20 (26.7)	53 (70.6)

LV=Large-vessel infarction, SV=Small-vessel infarction, EO=Early-onset stroke, LO=Late-onset stroke

of inbred patients was higher compared with controls (p<0.001, the distribution of I for all other control groups is not shown). Inbreeding was highest in early-onset stroke patients. In this group, 84.6% is inbred to some extent. There were no differences in inbreeding between patients with large- and small-vessel infarction, or early- and late-onset patients (Table 3).

88 Patients and 190 controls were genotyped for SNP83, SNP45 and SNP39 of the *PDE4D* and *PART1* gene. In 3 patients and 10 controls, genotyping failed or DNA was not available. Genotype distributions in patients and controls did not deviate from Hardy Weinberg equilibrium (Table 4). Overall, we did not find an association between any of the Icelandic SNPs and the risk of stroke. However, when analysing only inbred individuals (Table 5), we found that the C allele of SNP45 increased the risk of small-vessel infarction 4.8 times (95% CI: 1.1-22.3) compared with controls (p=0.04, adjusted for age and sex in a recessive model). When compared with large-vessel infarction, this risk was 9.7 (95% CI: 1.8-52.4, p<0.01 adjusted for age and sex). In inbred individuals, the T-allele of SNP39 increased the risk of small-vessel infarction 6.3 times (95% CI: 1.4-28.7) compared with controls (p=0.02 adjusted for age and sex in a recessive model). This risk was 9.4 (95% CI: 1.7-51.7) when compared with large-vessel infarction (p=0.01, adjusted for age and sex). After adjusting for multiple testing using Bonferroni, only the odds ratios that were calculated using large-vessel infarction as the reference group, remained borderline significant (p=0.05 for SNP45, 0.06 for SNP39).

Table 5. Genotype distribution of 3 SNPs in the *PDE4D* gene in inbred individuals for all ischaemic stroke patients, stroke subtypes and controls.

	SNP 83			SNP 45			SNP 39		
	TT	CT	CC	TT	CT	CC	AA	AT	TT
Controls, n (%)	25 (18.0)	62 (44.6)	52 (37.4)	4 (2.8)	35 (24.6)	103 (72.5)	3 (2.1)	45 (31.5)	95 (66.4)
Patients, n (%)	10 (17.5)	24 (42.1)	23 (40.4)	2 (3.4)	14 (24.1)	42 (72.4)	1 (1.8)	14 (24.6)	42 (73.6)
-LV, n (%)	5 (17.2)	11 (37.9)	13 (44.8)	2 (6.7)	11 (36.7)	17 (56.7)	1 (3.4)	11 (37.9)	17 (58.7)
-SV, n (%)	4 (17.4)	12 (52.2)	7 (30.4)	0 (0.0)	2 (8.7)	21 (91.3)†£	0 (0.0)	2 (8.7)	21 (91.3)*‡
-EO, n (%)	2 (18.2)	7 (63.6)	2 (18.2)	1 (9.1)	2 (18.2)	8 (72.7)	1 (9.1)	2 (18.2)	8 (72.7)
-LO, n (%)	8 (17.4)	17 (37.0)	21 (45.7)	0 (0.0)	12 (26.1)	34 (73.9)	0 (0.0)	12 (26.1)	34 (73.9)

LV=Large-vessel infarction, SV=Small-vessel infarction, EO=Early-onset stroke, LO=Late-onset stroke
†RR=4.8 (95% CI: 1.1-22.3) compared with controls, p=0.04 adjusted for age and sex (recessive model).
£RR=9.7 (95% CI: 1.8-52.4) compared with large-vessel infarction, p<0.01 adjusted for age and sex (recessive model).
*RR=6.3 (95% CI: 1.4-28.7) compared with controls, p=0.02 adjusted for age and sex (recessive model).
‡RR=9.4 (95% CI: 1.7-51.7) compared with large-vessel infarction, p=0.01 adjusted for age and sex (recessive mode)

We also estimated haplotypes from the genotypic data of SNP83, SNP45 and SNP39. There were no significant differences between haplotypes in overall stroke patients and controls or between sub types of ischaemic stroke patients and controls. When only selecting inbred patients, there was a non-significant increase in the frequency of the TCT haplotype (T allele of SNP83, C allele of SNP45 and T allele of SNP39) in patients with small-vessel infarction compared with controls. Next, we constructed haplogenotypes. Of interest was the haplogenotype that refers to individuals who carry both C alleles of SNP45 and both T alleles of SNP39, regardless of the alleles of SNP83. We will further indicate this haplogenotype as xCT_xCT. xCT_xCT was present in 22 patients with small-vessel infarction (88.0%) compared with 94 controls (65.7%, p=0.03).

DISCUSSION

We studied the familial aggregation of ischaemic stroke and ischaemic stroke subtypes in a genetically isolated population in the Netherlands. We calculated the pair wise kinship coefficient and found evidence for familial aggregation of ischaemic stroke as the proportion of related pairs in patients with ischaemic stroke was significantly higher compared with the proportion in controls. Furthermore, we found that familial aggregation was stronger for large-vessel compared with small-vessel infarctions. However, familial aggregation was strongest for early-onset patients (age at onset ≤ 45 years). A second finding is that inbreeding is higher in all stroke groups compared with controls. This suggests that recessive genes might play a role in the development of stroke (subtypes). In line with this finding, we found that 2 of the 3 Icelandic SNPs of the *PDE4D* gene increased the risk of small-vessel infarction significantly in inbred individuals.

The strength of our study is the use of genealogic data instead of family history. Family history relies on information and memory of individuals, which often leads to inadequate information. Also, a major obstacle in dissecting the genetics of ischaemic stroke is the weak familial aggregation, which may only become evident when studying distant relatives of an affected individual. This is only possible when extensive genealogic data are available, as is the case in our study population. It also offered the opportunity to study familial aggregation in subtypes of ischaemic stroke.

Since we studied ischaemic stroke in a genetically isolated population, we cannot be sure that our findings can be generalized to other populations. However, since our population is of more recent isolation, the genetic make-up may more closely resemble that of the general population.[17]

We only invited patients to participate that were seen by a neurologist and for whom we had neuro-imaging. This may raise the question of inclusion bias. However, it is unlikely that the inclusion criteria were associated with the *PDE4D* gene or the relatedness between the patients and therefore will not have biased our results.

In our study, the number of early-onset stroke patient is relatively high (14%). This may have been the result of our inclusion criteria, as younger patients might sooner be referred to a hospital than elderly patients. Also, this may have been the result of inbreeding, which was associated with stroke in this population and was especially high in early-onset patients. Patients with early-onset ischaemic stroke did not differ much from the other stroke groups, with the exception of smoking, which was highest for patients with early onset stroke. Ischaemic stroke may be explained by homocysteinuria, which is a risk factor for ischaemic stroke, in 2 of the 13 early onset patients. For all other patients, no rare causes could be found.

Ischaemic stroke is heterogeneous and can be grouped into different subtypes, all with a (partly) different pathogenesis. It is also known that ischaemic stroke in the young is different from that in the elderly.[18] These diverse pathways are very likely to be under different genetic influences. Still, most genetic epidemiological studies have failed to differentiate between these phenotypes, which might have led to the many inconsistent results found so far.[1,2] In our study, the percentage of pairs that could not be connected to a common ancestor was significantly different between patients with large- or small-vessel infarctions compared with controls. This is in line with previous studies that family history of stroke is a risk factor for both large- and small-vessel infarction independently.[5,6] We showed that the frequency of pairs related to a common ancestor is significantly higher for large-vessel compared with small-vessel infarction. Family history studies that did differentiate between stroke subtypes have described different relative risks for large- and small-vessel stroke, but there is no consistency in which of the subtypes has stronger familial aggregation.[5-7] It has previously been described that a positive family history of stroke is a stronger risk factor for early-onset than for late-onset ischaemic stroke patients.[5,7] which is in concordance with our findings.

We did not differentiate stroke with a possible cardio embolic source as we only had EKG information, which is not sufficient for identification of all high-risk cardiac sources. From the medical records we could identify 14 patients with a history of myocardial infarction (> 6 months before the stroke), atrial fibrillation, endocarditis or mitralis valve prolapse. The proportion of these patients that is connected to a common ancestor was 58.2%, which was higher compared with controls (30.7%, $p<0.05$). Compared with large-vessel infarction (71%), this was lower ($p<0.01$). There were no significant differences when compared with small-vessel infarction. A recent meta-analysis also found that non-cardio embolic stroke was more heritable compared with cardio embolic stroke.[7] However, these findings should be interpreted with caution as not all high-risk cardiac sources might have been identified in all patients.

As we identified a significant difference in familial aggregation between subtypes of ischaemic stroke, this might imply that different genes are involved in these subtypes. Indeed, we found an association between SNP45 and SNP39 of the *PDE4D* and *PART1* gene and small-vessel infarction. A point of consideration is that this was only found in inbred patients and inbreeding itself increases the risk of being homozygous for a certain allele. However, the effect of inbreeding on these 2 SNPs was only found for small-vessel infarction. For all other groups, the frequency of the CC and TT genotype did not change when only selecting inbred patients, suggesting that this is not a false positive finding. There were no significant differences between the inbred and the outbred population with respect to sex, smoking, hypertension, hyperlipidemia or diabetes mellitus. However, systolic and diastolic blood pressure, were significantly higher in inbred compared with outbred subjects. This might explain the finding that the *PDE4D* gene was only associated with small-vessel infarction in the inbred population. Excluding the 14 previously described patients with a possible cardiac source did not alter our findings on the association between the *PDE4D* gene and small-vessel stroke as only 3 of these patients were initially sub typed as small-vessel infarction.

In Iceland, the C allele of SNP45 and the T allele of SNP39 were the risk alleles for stroke. This is confirmed in our study. We also found the haplogenotype xCT_xCT, indicating individuals with both C alleles of SNP45 and both T alleles of SNP39, regardless of the alleles of SNP83, to be associated with stroke. In Iceland, SNP45 and SNP39 were part of block B in which there was strong linkage disequilibrium (LD). SNP83 was not part of this block. This is in concordance with our finding that only the alleles of SNP45 and SNP39 are of interest when analysing haplotypes, regardless of SNP83, as LD is less strong between SNP83 and SNP45, and SNP83 and SNP39. As we only typed SNPs and no macrosatellite markers and no primer could be designed for SNP41, we could not analyse haplotypes AX, GO and GX as defined by decode.[8]

In the Icelandic population, SNP45 and SNP39 were found to be associated with combined cardiogenic and carotid stroke, while we detected an increased risk for small-vessel infarction. *PDE4D* degrades second messenger cAMP which plays a central role in signal transduction.[19] *PDE4D* isoforms are expressed in vascular smooth muscle cells, endothelial cells, T-lymphocyte and macrophages in tissues like brain, blood vessels, kidney and the pancreas.[20,21] These

cells are all important in atherosclerosis (cardiogenic and carotid stroke), but just as well play a role in the development of small-vessel stroke. Small vessel stroke is generally thought to result from endothelial proliferation.[8] As low levels of cAMP increase proliferation of endothelial cells[22] and *PDE4D* expression lowers cAMP levels, *PDE4D* may thus increase the risk of small-vessel stroke. Further studies are needed to confirm our findings.

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Chapter 3.2

ALOX5AP is associated with ischaemic stroke in a genetically isolated population

Objective - The gene product of *ALOX5AP* plays a role in the synthesis of leukotrienes, which are proinflammatory lipid mediators. *ALOX5AP* has been associated with stroke and myocardial infarction (MI). We aimed to study the association between *ALOX5AP* and ischaemic stroke as well as risk factors of ischaemic stroke.
Methods - Our study was conducted in a Dutch genetically isolated population in which we identified 91 ischaemic stroke and 115 early onset hypertensive patients and their relatives, comprising 123 pedigrees. We used genealogic information to construct pedigrees up to 4 generations. We performed family-based association tests using the PBAT program, studying 3 polymorphisms within *ALOX5AP* as well as their haplotypes.
Results - We found that the polymorphism SG13S32 (A allele) was associated with increased systolic (p=0.02) and diastolic blood pressure (p=0.03), body mass index (p=0.004) and ischaemic stroke (p=0.003). SNP SG13S114 (T allele) was significantly associated with self-reported MI (p=0.03). Also, we found an association between the SG13S114_T/SG13S89_G/SG13S32_C (TGC) haplotype and ischaemic stroke (p=0.02) and BMI (p=0.08).
Conclusions - Our study confirms the association between *ALOX5AP* and ischaemic stroke. Our study further suggests a role of this gene in BMI and blood pressure regulation.

INTRODUCTION

ALOX5AP encodes the 5-lipoxygenase (5-LO) activating protein (FLAP), which plays a role in the leukotriene synthesis.[1] Leukotrienes, especially B4, are proinflammatory lipid mediators.[2,3] Leukotrienes are derived from arachidonic acid by the action of the enzyme 5-LO. This enzyme must be bound to FLAP to metabolize arachidonic acid.[4] The gene *ALOX5AP* was identified as a gene involved in stroke in Iceland following a genomic screen suggesting 13q12-13 as the primary region.[5] Findings of studies aiming to replicate this association in different populations, have been inconsistent.[6-11]

The 5-LO pathway was found to influence hyperlipidemia-dependent inflammation of the arterial wall in aortic aneurysms.[12] Also, 5-LO and FLAP were found to play a role in the development of pulmonary hypertension.[13,14] *ALOX5AP* is expressed in adipose tissue and its expression is associated with body weight and insuline resistance.[15] *ALOX5AP* has also been associated with the development of atherosclerosis in mice.[16] We aimed to study *ALOX5AP* in relation to stroke as well as risk factors of ischaemic stroke in a genetically isolated population in the Netherlands.

METHODS

Study population

Our study is part of the research program "Genetic Research in Isolated Populations" (GRIP), which aims to identify genetic risk factors in the aaetiology of complex diseases. The study was conducted within a recently isolated population in the Southwest of the Netherlands. The village was founded around 1750 by approximately 150 individuals and was isolated until the last few decades. Descendants of this population, about 20,000 individuals, are now scattered over 8 adjacent villages. The structure of this population has been described previously.[17] The Medical Ethical Committee of the Erasmus Medical Centre Rotterdam approved the study protocol.

Hypertension

Patients were recruited by the general practitioners (GPs) who are working in this population. The diagnosis was based on medical history and/or use of anti-hypertensive medication, which was verified by a research physician. Newly diagnosed hypertension was defined as a systolic blood pressure (SBP) ≥ 160 mmHg or a diastolic blood pressure (DBP) ≥ 100 mmHg. Participants with known causes of hypertension (e.g. kidney disease), were excluded from this study. In order to increase homogeneity of our study population and to reduce the chance of

including secondary forms of hypertension, only participants who developed hypertension before 60 years of age were included.

Ischaemic stroke

Patients were recruited by the GPs, as described previously.[18] Briefly, clinical information was obtained from medical records of the GPs, neurologists, cardiologists and rehabilitation physicians. The diagnosis of ischaemic stroke was verified independently by two neurologists and was based on all available information, including the re-examination of neuro-imaging findings. For this study, we did not specify sub types of ischaemic stroke.

Relatives

Each patient was asked to name first-degree relatives (parents, siblings, offspring) possibly willing to participate in this study. These relatives were also invited to participate.

Measurements and definitions

Smoking, medical history (including a history of MI), medication use, height and weight, were obtained from all participants by means of interviews. Body mass index (BMI) was calculated from height and weight (kg/m2). Blood pressure was measured twice with the participant in sitting position using an automated device. Blood was drawn for DNA analysis and clinical chemistry. Total serum cholesterol, HDL cholesterol and glucose levels were measured and hypertension, hypercholesterolemia and diabetes mellitus were defined as previously described.[18] The prevalence of MI was based on self-report and not verified in all participants.

Genealogy

Data on the presence of stroke and hypertension in first-, second- and third-degree relatives was collected by a family-history questionnaire. Newly diagnosed hypertension was assessed by means of blood pressure measurements. The names, dates and places of births of parents and grandparents were obtained from the participants. This information was extended up to 16 generations by means of local municipality records. For this study, small pedigrees were constructed with a maximum of 4 generations.

Genotyping

We genotyped three single nucleotide polymorphisms (SNPs), SG13S114, SG13S89 and SG13S32. These SNPs tag the haplotype indicated as HapA by deCODE, which was found to

be significantly associated with stroke and MI in the Icelandic population.[5] For SNP SG13S25, a primer could not be designed. Genotyping was performed using TaqMan allelic discrimination Assays-By-Design (Applied Biosystems, Foster City, CA).[19] Forward primer sequences were 5′-CAGATGTATGTCCAAGCCTCTCT-3′, 5′-CGGGAGGCCGTGTTTCA-3′ and 5′-CTGCTTTAGTTCTTGACCTCACCAA-3′ and reverse primer sequences were 5′-AGGTAGGTCTATGGTTGCAACATTG-3′, 5′-CAGGGAGCAAGCATTAGCAAT-3′ and 5′-GCTGGAGCAACTTCTGGAAAG-3′. The minor groove binding probes were 5′-VIC- TTGCAATTCTAATTAACC-NFQ-3′ and 5′-FAM-TTGCAATTCTATTTAACC-NFQ-3′ for SG13S14, 5′-VIC- CAGAGCGCATGTGAT-NFQ-3′ and 5′-FAM- CAGAGCGCGTGTGAT-NFQ-3′ for SG13S89 and 5′-VIC- AGGAGGAATTGCTAGATGA-NFQ-3′ and 5′-FAM- AGGAGGAATTGCTCGATGA-NFQ-3′ for SG13S32. The assays utilized 5 nanograms of genomic DNA and 5 micro liter reaction volumes. The amplification and extension protocol was as follows: an initial activation step of 10 min at 95 °C preceded 40 cycles of denaturation at 95 °C for 15 seconds and annealing and extension at 50 °C for 60 seconds. Allele-specific fluorescence was then analysed on an ABI Prism 7900HT Sequence Detection System with SDS v 2.1 (Applied Biosystems, Foster City, CA). Based on the analysis of blind duplicates, there was a 99% concordance in genotyping of all three SNPs.

Statistical analyses

Hardy-Weinberg equilibrium proportions of the 3 SNPs within *ALOX5AP* was tested using GENEPOP package.[20] The association between the 3 individual SNPs and haplotypes, and various dichotomous and continuous traits was tested using the family-based association test. For this, we used the software program PBAT, version 2.6, which can handle both nuclear families, as well as extended pedigrees (available from the PBAT web page at http://www.biostat.harvard.edu/~clange/default.htm).[21,22] An additive model was assumed. The analyses were adjusted for environmental effects. To know the effect of the transmitted allele on the traits values, a univariate FBAT test was performed for each allele. A positive Z-statistic is indicative for overtransmission, suggesting a risk effect of the allele. A negative Z-statistic is indicative for under transmission, suggesting a protective effect of the allele. The Z-statistic is only calculated in the FBAT program. Therefore, we re-analysed significant findings derived from PBAT, using FBAT version 1.5.3 (available from the FBAT web page at http://www.biostat.harvard.edu/~fbat/fbat.htm),[23,24] to estimate whether an allele was positively or negatively associated with the trait.

RESULTS

Genotype frequencies for the 3 SNPs were in Hardy-Weinberg equilibrium. Initially, 115 participants with hypertension and 91 participants with ischaemic stroke were ascertained through

the GPs. Furthermore, 379 first-degree relatives participated in this study. These individuals were part of 123 pedigrees, extending from 1 to 4 generations. The largest pedigrees consisted of 24 individuals. For ten patients, no relatives were available.

Table 1 shows the general characteristics for stroke and hypertensive patients and relatives. Overall, stroke patients had the highest age with a mean of 64.7 years. Within this group, 72.5 % were hypertensive, while within the group of hypertensive patients, only 9.6 % were also diagnosed with stroke. Compared with the relatives, total cholesterol levels were significantly lower in stroke patients, which may be the result of the significantly higher use of lipid lowering medication. All other characteristics were significantly higher in stroke patients compared with relatives, with the exception of DBP. Age, SBP, DBP, use of blood pressure and lipid lowering medication, and prevalence of stroke were all significantly higher in hypertensive patients compared with relatives. Between stroke and hypertensive patients, all variables differed significantly with the exception of MI, smoking and BMI.

The association between SNPs SG13S114, SG13S89 and SG13S32 and several continuous traits are shown in table 2. No associations were found for SNPs SG13S114 or SG13S89. However, in most families SG13S89 was not informative. SNP SG13S32 was significantly associated with SBP, DBP and BMI after adjusting for age, sex, environmental correlations and blood pressure lowering medication (for SBP and DBP only). The association with total cholesterol was significant after adjusting for age, sex and environmental correlation (p=0.03), but did not

Table 1. General characteristics

	Stroke patients (n=91)	Hypertensive patients (n=115)	Relatives (n=381)
Age, yrs	64.7 ± 11.9 *	55.8 ± 10.3 *	50.4 ± 15.1
Sex, % male	59.3 **	44.3	45.1
SBP, mmHg	146.9 ± 22.6 *	153.4 ± 20.7 *	137.1 ± 18.8
DBP, mmHg	77.1 ± 14.3	84.3 ± 13.2 *	75.8 ± 12.6
Blood pressure lowering medication, %	65.8 *	87.4 *	20.6
Hypertension, %	72.5 *	100.0 *	23.5
BMI, kg/m^2	27.5 ± 4.6 **	28.0 ± 4.7 *	26.2 ± 4.5
Current smoking, %	47.3 **	42.0	36.4
Total cholesterol, mmol/l	5.2 ± 1.2 *	5.8 ± 1.1	5.6 ± 1.1
Lipid lowering medication, %	52.3 *	24.3 *	8.9
Hypercholesterolemia, %	62.6 *	30.4	23.5
Serum glucose, mmol/l	6.3 ± 2.3 *	5.6 ± 1.5	5.5 ± 1.8
Glucose lowering medication, %	17.6 *	6.8	3.4
Diabetes mellitus, %	20.9 *	8.7	4.5
Stroke, %	100.0 *	9.6 *	0.8
History of MI, %	13.2 *	5.2	4.5

*p<0.01, **p<0.05 compared with relatives. All values are unadjusted means ± SD or total numbers (percentages). SBP=systolic blood pressure, DBP=diastolic blood pressure, BMI=bodymass index, MI=myocardial infarction

Table 2. Association between SNPs of *ALOX5AP* and several continuous triats

SNP	Phenotype	Allele	N informative families	P-value
SG13S114	Systolic blood pressure	T	28	0.58
		A	27	0.73
SG13S89		G	10	0.71
		A	7	0.55
SG13S32		C	34	0.01
		A	35	0.01
SG13S114	Diastolic blood pressure	T	28	0.69
		A	27	0.84
SG13S89		G	10	0.52
		A	7	0.68
SG13S32		C	34	0.02
		A	35	0.03
SG13S114	BMI	T	28	0.47
		A	27	0.44
SG13S89		G	10	0.75
		A	7	0.41
SG13S32		C	34	0.006
		A	35	0.004
SG13S114	Total cholesterol	T	28	0.67
		A	27	0.45
SG13S89		G	10	0.47
		A	7	0.71
SG13S32		C	34	0.17
		A	35	0.17
SG13S114	Glucose	T	28	0.50
		A	27	0.42
SG13S89		G	10	0.77
		A	7	0.65
SG13S32		C	34	0.58
		A	35	0.51

P-value adjusted for age, sex, environmental correlations and additionally for blood pressure lowering medication (SBP and DBP), for lipid lowering medication (total cholesterol) and glucose lowering medication (glucose). BMI=body mass index.

remain significant after additional adjustment for cholesterol lowering medication (see table 2). There was a borderline significant association between SNP SG13S32 and glucose levels, adjusting for age, sex and environmental correlations (p=0.08), which disappeared when adjusting for the use of glucose lowering medication (see table 2). When re-analysing the traits using the FBAT program, we found for all traits, that the A allele was overtransmitted and therefore was associated with an increased risk, while the C allele was undertransmitted.

Table 3. Association between SNPs of *ALOX5AP* and several dichotomous triats

SNP	Phenotype	Allele	N informative families	P-value
SG13S114	Hypertension	T	28	0.79
		A	27	0.88
SG13S89		G	10	0.95
		A	7	0.58
SG13S32		C	33	0.71
		A	34	0.53
SG13S114	Ischaemic stroke	T	27	0.16
		A	26	0.16
SG13S89		G	9	0.78
		A	6	0.47
SG13S32		C	29	0.003
		A	30	0.003
SG13S114	Hypercholesterolemia	T	28	0.74
		A	27	0.90
SG13S89		G	10	0.33
		A	7	0.21
SG13S32		C	34	0.52
		A	35	0.52
SG13S114	DM	T	28	0.80
		A	27	0.56
SG13S89		G	10	0.68
		A	7	0.16
SG13S32		C	34	0.47
		A	35	0.47
SG13S114	MI	T	28	0.03
		A	27	0.03
SG13S89		G	10	1.00
		A	7	1.00
SG13S32		C	34	0.30
		A	35	0.30

P-value adjusted for age, sex and environmental correlations. DM=diabetes mellitus, MI=myocardial infarction.

Table 4. Haplotype analyses for common haplotypes of combination of SNPs SG13S114, SG13S89 and SG13S32 for ischaemic stroke and BMI.

Haplotype	Phenotype	Frequency	N informative families	P-value
T/G/C	Ischaemic stroke	0.43	45	0.02
T/G/A		0.28	44	0.10
A/G/A		0.16	29	0.28
A/G/C		0.07	20	0.73
A/A/C		0.04	12	0.88

P-value adjusted for age, sex, and environmental correlations.

Table 3 shows that after adjusting for age and sex, SNP SG13S32 was strongly associated with ischaemic stroke. The A allele was overtransmitted, suggesting this allele was associated with an increased risk of ischaemic stroke, while the C allele was undertransmitted. Hypertension, hypercholesterolemia and diabetes mellitus were not significantly associated with any of the 3 SNPs of *ALOX5AP*. SNP SG13S114 was significantly associated with MI (p=0.03).

The haplotype SG13S114_T/SG13S89_G/SG13S32_C (TGC) was significantly associated with ischaemic stroke (p=0.02), as shown in table 4. This haplotype was undertransmitted. The TGA haplotype tended to be overtransmitted (p=0.10). The TGA haplotype was also borderline significantly associated with BMI (p=0.08), but no significant evidence was found for an association with blood pressure (data not shown).

DISCUSSION

In 123 hypertension and stroke families ascertained in a Dutch genetically isolated population, we found that the A allele of SNP SG13S32 was significantly associated with SBP, DBP, BMI and ischaemic stroke. We further found a significant association between the SG13S114_T/SG13S89_G/SG13S32_C (TGC) haplotype and ischaemic stroke.

Our findings are based on a relatively small series of patients and relatives. The power of this study is further reduced by the fact that some markers were not very informative (e.g. SNP SG13S89 was only informative in 6-10 families). Yet, we were able to find an association between *ALOX5AP* and blood pressure, BMI and stroke. The strength of our study lies within the design, as it is embedded in an isolated population. Individuals selected from such a population are likely to share a more common genetic origin of disease as well as a common environmental background.[25] Furthermore, the FBAT approach we used provides a test of linkage and association, which boosts the statistical power. This statistic is unaffected by population admixture.

ALOX5AP was significantly associated with MI, stroke and periferal arterial occlusive disease in the Icelandic population.[5] The associated haplotype (HapA) was defined by SNPs SG13S25 (allele G), SG13S114 (allele T), SG13S89 (allele G) and SG13S32 (allele A). DeCODE replicated their findings on ischaemic stroke in a Scottish population, whereas the association with MI could not be replicated within a British cohort.[5,9] Other studies have also found conflicting results.[6-8,10] Our study showed that a single SNP within *ALOX5AP*, SG13S32 was significantly associated with ischaemic stroke and, in line with the findings by deCODE, that the A allele was the risk allele. We found a significant association between ischaemic stroke and the haplotype SG13S114_T/SG13S89_G/SG13S32_C (TGC). It was shown, that this haplotype was undertransmitted, indicating a protective effect, as opposite to the risk effect of the TGA haplotype described by deCODE. The TGA haplotype was also overtransmitted in stroke patients in our study population, however, this finding was not significant. We also found an association

between SNP SG13S114 and MI, however, this finding should be interpreted with caution as MI was based on self-report.

The association of *ALOX5AP* with stroke and MI might be the result of the involvement of FLAP and 5-LO within atherosclerotic lesions and carotid intima media thickness, as reported earlier.[26,27] We have studied several risk factors, which have been associated with stroke. Hypertension is the most important risk factor, but obesity, high glucose levels and hyperlipidemia also increase the risk of stroke.[28-32] Furthermore, all of these risk factors have been associated with inflammation.[33-37] In line with the positive association between stroke and *ALOX5AP* and the previously reported relation with inflammation, we found a significant association between *ALOX5AP* and SBP, DBP and BMI. Total cholesterol and glucose levels were also (borderline) significantly associated with SNP SG13S32, however, after adjusting for medication use, this finding did not reach statistical significance. The strongest association in terms of p-value was that of *ALOX5AP* and BMI. As BMI is a determinant of blood pressure,[38] one may argue that the association to blood pressure is secondary to the association of *ALOX5AP* and BMI. An association between *ALOX5AP* and body weight, has been previously reported.[15]

In summary, in this study we confirmed the findings by deCODE on the relation between *ALOX5AP* to stroke, and perhaps MI. Furthermore, we extended their findings by showing an association between *ALOX5AP* and blood pressure as well as BMI.

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Chapter 4

Candidate gene studies in an outbred population

Chapter 4.1

Insulin-like growth factor I promoter polymorphism, risk of stroke and survival after stroke. The Rotterdam Study.

Objective – Low levels of Insulin-like growth factor-I (IGF-I) predispose to atherosclerosis and may therefore increase the risk of stroke. Low levels have also been found to influence the outcome of cardiovascular and cerebrovascular disease. A polymorphism in the promoter region of the Insulin-like Growth Factor-I (*IGF-I*) gene influences IGF-I levels. Non-carriers of the 192-bp allele have lower levels of *IGF-I* compared with 192-bp allele carriers. We studied the *IGF-I* polymorphism in relation to the risk of stroke and survival after stroke.
Methods - We studied 6808 subjects of the Rotterdam Study, who were followed for the occurrence of stroke and death after stroke. Subjects were grouped according to the 192-bp allele of *IGF-I* into non-carriers, heterozygotes and homozygotes. The risk of stroke and survival after stroke was studied using Cox regression analysis, adjusting for age and sex, with homozygotes for the wild-type allele as the reference.
Results – Non-carriers had a relative risk of 0.8 (95% CI: 0.6-1.0) for the occurrence of any stroke and 0.7 (95% CI: 0.5-1.0) for ischaemic stroke. For non-carriers, the relative risk of death after any stroke was 1.5 (95% CI: 1.0-2.2). After an ischaemic stroke, this relative risk was 1.5 (95% CI: 0.9-2.6) and after a haemorrhagic stroke 5.2 (95% CI: 1.3-21.5).
Conclusions – Our study suggests that *IGF-I* is a significant determinant of survival after stroke.

INTRODUCTION

Insulin-like growth factor I (IGF-I) appears to be involved in the development of atherosclerosis and cardiovascular disease because of its role in tissue repair and cell proliferation.[1-4] Also, IGF-I induces the synthesis of elastin and prevents apoptosis of vascular smooth muscle cells.[5-7] Therefore, low levels might be a risk factor for stroke. Expression of IGF-I is increased after hypoxic injury, in regions with neuronal loss[8] and animal studies have found that administration of IGF-I reduces infarct volume and improves neurologic function after ischaemia.[8-10] This suggests that IGF-I levels may be involved in outcome after stroke. Indeed, it was found that IGF-I levels are lower after cerebral ischaemia,[11,12] and low levels are associated with a poor outcome after ischaemic stroke[11] as well as myocardial infarction.[13]

Earlier we have found that a polymorphism in the promoter region of the *IGF-I* gene, located on chromosome 12 q, is associated with plasma IGF-I levels. Non-carriers of the 192-bp (wild-type) allele of this polymorphism, have lower plasma IGF-I levels compared with 192-bp allele carriers.[14] Also, non-carriers of the 192 bp allele were found to have an increased risk of myocardial infarction[14] and an increase in mean intima-media thickness of the common carotid arteries and mean pulse wave velocity.[15] We studied the association between the *IGF-I* promoter polymorphism and the risk of stroke, as well as survival after stroke, for any stroke, ischaemic stroke and haemorrhagic stroke.

METHODS

Study population

The present study was performed as part of the Rotterdam Study, an ongoing prospective population-based cohort study on chronic and disabling diseases in the elderly.[16] The medical ethics committee of Erasmus Medical Centre, Rotterdam, approved the study. Participants gave written informed consent and permission to retrieve information from treating physicians. A total of 7983 subjects participated in this study (response of 78 %), all aged 55 years and over at baseline. In 7012 (68.5 %) persons, *IGF-I* genotyping was successfully performed. For this, we used frozen serum samples collected at baseline. The success rate of genotyping was 87.8 %. No DNA was available for 948 subjects and there was a genotyping failure in 23 subjects. Serum IGF-I levels were assessed in a randomly selected subgroup of 406 subjects, as described earlier.[17] Participants with a stroke before baseline were excluded from the analyses (n=258). Our study population therefore comprised 6808 persons (85 %). The sample appeared to be random, as we did not find differences between participants with and without a known *IGF-I* genotype with regard to demographic or cardiovascular characteristics.

Measurements and diagnosis of stroke

Baseline data were collected from 1990 until 1993. Measurements and definitions of body mass index (BMI), hypertension, cholesterol levels and diabetes mellitus are described elsewhere.[18] A previous stroke was determined during the baseline interview by asking 'did you ever suffer from a stroke, diagnosed by a physician?' Medical records of subjects who answered 'yes' were checked in order to verify the diagnosis.[19]

Follow-up started at baseline and lasted until 1 January 2002 for the present study. Once subjects enter the Rotterdam Study, they are continuously monitored for major events through automated linkage of the study database with files from general practitioners and the municipality. Also nursery home physician's files are scrutinized. For reported events, additional information (including brain images) is obtained from hospital records. Trained research physicians reviewed information on all possible strokes and transient ischaemic attacks. An experienced stroke neurologist (P.J.K.) verified all diagnoses. Subarachnoid haemorrhages and retinal strokes were excluded. Follow-up was completed until January 1, 2001 for 97.1% of all potential person years.[20] Ischaemic strokes were diagnosed when a patient had typical symptoms and a CT or MRI that was made within 4 weeks ruled out other diagnoses or when indirect evidence (deficit limited to 1 limb or completely resolved within 72 hours, atrial fibrillation in the absence of anticoagulants) pointed at an ischaemic nature of the stroke. Haemorrhagic stroke was diagnosed when a relevant haemorrhage was shown on CT or MRI scan, or when the subject permanently lost consciousness or died within hours after onset of focal signs. If a stroke did not match these criteria, it was classified as unspecified. Neuro-imaging was available for 61 % of all events. For ischaemic stroke, this was 91 % and for haemorrhagic stroke 78 %. Ischaemic stroke was sub classified into large-vessel disease, small-vessel disease and stroke due to a cardio-embolic source using the TOAST criteria.[21]

Information on mortality was obtained from the GPs and hospitals and also regularly from the municipal health authorities in Rotterdam. Two research physicians independently coded all events according to the International Classification of Diseases, 10th edition (ICD-10).[22] We defined cardiovascular mortality as death from ischaemic heart disease (I20-25), ventricular fibrillation (I49), congestive heart failure (I50), cerebrovascular disease (I60-I67), peripheral artery disease (I70-74), hypertensive heart disease (I10-I15), disorders of aorta, arteries, arterioles and capillaries (I70-79) or mesenteric atherosclerosis (K55), sudden cardiac death (I46) or sudden death unknown (R96). The *IGF-I* gene promoter polymorphism was genotyped as described previously, without knowledge of the clinical diagnosis.[14]

Data analysis

Hardy-Weinberg equilibrium of the *IGF-I* promoter polymorphism genotypes was tested using GENEPOP package.[23] Baseline characteristics were compared using univariate ANOVA or

χ^2 statistics. Cox proportional hazards regression analysis was used to assess the relative risk of stroke, as well as the relative risk of death after stroke, with the homozygous carriers of the wild-type allele (192-bp) as the reference group (SPSS 11.0). We tested the proportional hazard assumption using the log-log survival curve (SPSS 11.0) and the Goodness-of-Fit test using S-PLUS 6.0. All analyses were adjusted for age and sex.

RESULTS

All *IGF-I* genotype and allele proportions were in Hardy-Weinberg equilibrium. Table 1 shows the baseline characteristics of the study population stratified by *IGF-I* genotype. Serum total IGF-I levels were significantly lower in non-carriers of the 192-bp allele compared with homozygous and heterozygous carriers (p<0.001). No significant differences were observed between the genotype groups and age, sex or other cardiovascular risk factors.

During a mean follow-up of 8 years, we observed 637 incident stroke cases in our study population. We classified 357 as ischaemic stroke, 59 as haemorrhagic stroke and 221 as unspecified. We had data on the *IGF-I* genotype for 551 (86.5%) cases. This corresponds to the proportion of successfully performed genotyping in overall subjects (87.8%). Table 2 shows the absolute numbers of strokes and risk estimates for each genotype group for any, ischaemic, haemorrhagic and unspecified stroke. Non-carriers were found to have a risk of 0.8 (95% CI: 0.6-1.0, p=0.05) for the occurrence of any stroke and 0.7 (95% CI: 0.5-1.0, p=0.07) for ischaemic stroke, compared with homozygote carriers of the 192-bp allele. In this table, we

Table 1. Baseline characteristics of the study population stratified by IGF-1 genotype

	IGF-1		
	192/192	**192/-**	**-/-**
Number of subjects	2985	3014	809
Age	69.5 ± 9.2	69.3 ± 9.2	69.5 ± 9.4
Sex – n (% male)	1191 (39.9)	1202 (39.9)	310 (38.3)
SBP (mm Hg)	139.1 ± 22.1	139.3 ± 22.5	139.0 ± 22.3
DBP (mm Hg)	73.6 ± 11.4	73.9 ± 11.8	73.3 ± 11.4
Hypertension n (%)	981 (33.8)	998 (34.2)	256 (32.7)
Diabetes – n (%)	287 (9.6)	316 (10.5)	74 (9.1)
Current smoking – n (%)	656 (22.7)	676 (23.3)	159 (20.2)
BMI (kg/m2)	26.3 ± 3.8	26.2 ± 3.6	26.2 ± 3.7
Total cholesterol (mmol/l)	6.6 ± 1.3	6.6 ± 1.2	6.6 ± 1.2
HDL cholesterol (mmol/l)	1.3 ± 0.4	1.3 ± 0.4	1.4 ± 0.4
Serum total IGF-I levels (nmol/l)*	18.5 ± 7.0 (n=130)	18.4 ± 7.8 (n=136)	14.6 ± 5.8 (n=140)†

All values are presented as unadjusted means ± standard deviation or total numbers (percentages). *Determined in a randomly drawn subgroup. †Significantly different from homozygous and heterozygous carriers (p<0.001).

Table 2. Relative risk of stroke and stroke sub types in relation to IGF-1 genotype

	***IGF-I* genotype**					
	192/192 (n=2985)		**192/- (n=3014)**		**-/- (n=809)**	
	n	RR (95% CI)	n	RR (95% CI)	n	RR (95% CI)
All strokes	266	1.0 (reference)	229	0.9 (0.7-1.0)	56	0.8 (0.6-1.0)
Ischaemic strokes	156	1.0 (reference)	131	0.8 (0.7-1.1)	30	0.7 (0.5-1.0)
Haemorrhagic strokes	22	1.0 (reference)	25	1.1 (0.6-2.0)	6	1.0 (0.4-2.4)
Unspecified stroke	88	1.0 (reference)	73	0.8 (0.6-1.1)	20	0.8 (0.5-1.3)
Large-vessel disease	12	1.0 (reference)	13	1.1 (0.5-2.4)	5	1.5 (0.5-4.4)
Cardio-embolic stroke	26	1.0 (reference)	22	0.8 (0.5-1.5)	8	1.1 (0.5-2.5)
Small-vessel disease	21	1.0 (reference)	18	0.9 (0.5-1.6)	3	0.5 (0.2-1.7)

RR=relative risk with 95 % confidence interval, adjusted for age and sex, n=total number of cases

also show the risk of sub types of ischaemic stroke for different genotypes of *IGF-I*. We found that non-carriers had a relative risk of large-vessel disease of 1.5 (95% CI: 0.5-4.4, p=0.4), a relative risk of cardio-embolic stroke of 1.1 (95% CI: 0.5-2.5, p=0.8) and a relative risk of small-vessel disease of 0.5 (95% CI: 0.2-1.7, p=0.3). All values were adjusted for age and sex. Adjusting for additional risk factors (smoking status, diabetes mellitus, hypertension, body mass index and total cholesterol level) did not alter these results.

Figure 1 shows the cumulative survival in years after any stroke. The cumulative survival is lowest for non-carriers. Also, it is shown that the effect of the IGF-1 genotype on survival is

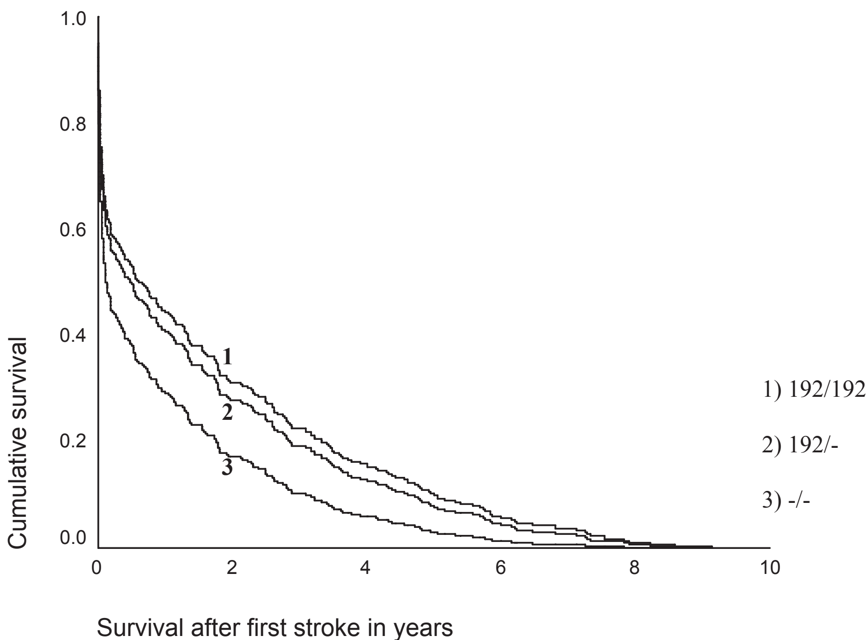

Figure 1. Cumulative survival after any stroke. This figure shows the cumulative survival in years after any stroke stratified by IGF-I genotype, 1=192/192, 2=192/-, 3=non-carriers

Table 3. Relative risk of death after stroke in relation to IGF-1 genotype

IGF-1	Any stroke		Ischaemic stroke		Haemorrhagic stroke		Unspecified	
	n	RR (95 % CI)	n	RR (95 % CI)	n	RR (95 % CI)	n	RR (95 % CI)
192/192	144	1.0 (reference)	65	1.0 (reference)	15	1.0 (reference)	88	1.0 (reference)
192/-	135	1.1 (0.9-1.4)	65	1.2 (0.9-1.8)	19	0.9 (0.4-1.9)	73	1.1 (0.8-1.7)
-/-	37	1.5 (1.0-2.2)†	17	1.5 (0.9-2.6)	5	5.2 (1.3-21.5)†	20	1.4 (0.7-2.4)

RR=relative risk of death with 95 % confidence intervals, adjusted for age and sex, n=absolute numbers of death after stroke, †p<0.05 compared with the reference group.

Table 4. Relative risk of cardiovascular death after stroke in relation to IGF-1 genotype

	Any stroke		Ischaemic stroke		Haemorrhagic stroke		Unspecified	
IGF-I	n	RR (95% CI)	n	RR (95% CI)	n	RR (95% CI)	n	RR (95% CI)
192/192	71	1.0 (reference)	32	1.0 (reference)	8	1.0 (reference)	31	1.0 (reference)
192/-	64	0.9 (0.7-1.3)	23	0.7 (0.4-1.3)	12	0.7 (0.3-1.9)	29	1.45 (0.9-2.5)
-/-	20	1.3 (0.8-2.2)	8	1.3 (0.6-2.9)	4	3.0 (0.6-14.5)	8	1.2 (0.5-2.9)

RR=relative risk of cardiovascular death with 95 % confidence intervals, adjusted for age and sex, n=absolute numbers of cardiovascular death after stroke

strongest in the first months after a stroke. The same was found for survival after ischaemic stroke, haemorrhagic stroke and unspecified stroke. Table 3 describes the absolute numbers of death after a stroke and the relative risk for each genotype. For non-carriers, we found that the relative risk of death after any stroke was 1.5 (95% CI: 1.0-2.2, p=0.03), after ischaemic stroke 1.5 (95% CI: 0.9-2.6, p=0.1) and after haemorrhagic stroke 5.2 (95% CI: 1.3-21.5, p=0.02) compared with homozygous carriers. Numbers were too small for the analyses in sub classifications of ischaemic stroke. The risk estimates remained similar after adjusting for the additional risk factors.

We also analysed the effect of *IGF-I* on survival in the overall population, with exclusion of prevalent and incident stroke (not shown). The mean survival time in the cohort (till death or end of follow-up) was 8.5 years for all 3 genotypes, adjusted for age and sex. We found a relative risk of 1.0 (95% CI: 0.9-1.1) for heterozygote carriers and of 1.0 (95% CI: 0.8-1.1) for non-carriers compared with homozygous carriers of the 192 bp allele.

Next, we analysed the effect of the *IGF-I* genotype on cardiovascular death after stroke (CV death). For any stroke, ischaemic stroke and haemorrhagic stroke, the absolute numbers of CV death were 20 (35.7 %), 8 (26.7 %) and 4 (66.7 %) in non-carriers, compared with 71 (26.7 %), 32 (20.5 %) and 8 (36.4 %) in homozygous carriers, respectively (table 4). The highest RR for CV death was 3.0 (95% CI: 0.6-14.5), which was found in non-carriers after haemorrhagic stroke. However, this finding was not significant. Also, the RR of CV death after any, ischaemic or unspecified stroke, was not significant (table 4). The proportional hazard assumption was met for all previously shown analyses.

DISCUSSION

This is the first investigation on the *IGF-I* promoter polymorphism and stroke. In this large population-based cohort study, we found a protective effect for non-carriers of the 192-bp allele of the *IGF-I* polymorphism on any and ischaemic stroke, although this was not significant. However, we found that it was a significant determinant of survival after stroke.

We cannot be sure that the ascertainment of stroke was complete. We retrieved information on stroke events through the general practitioner (GP), nursery home physician and the participant. For clinical stroke, we believe to have complete ascertainment as we screened 97 % of all GP and nursery home records for stroke events. Only if a patient did not seek any medical attention, the GP or nursery home physician would not have been informed. Sub clinical stroke however, presents without clinical symptoms. The only accurate way of verifying the completeness of stroke ascertainment would have been to make a MRI- or CT-scan of the brain for every participant. Unfortunately, we did not have the equipment available to do so in this study. However, as incompleteness of follow-up of stroke will not have been dependent on genotype, we do not believe this has biased our results.

We previously observed that non-carriers of the 192-bp allele had lower levels of *IGF-I* compared with heterozygous and homozygous carriers[14] and that non-carriers of the *IGF-I* gene were at higher risk of developing atherosclerosis and myocardial infarction.[14,15] In this study, for any and ischaemic stroke, we found a protective effect for non-carriers of the 192-bp allele, although not significant. A possible explanation for this may be that non-carriers die from a myocardial infarction before they can develop a stroke. However, when we excluded prevalent and incident MI from the analyses, the RR for any and ischaemic stroke remained lower than one. Another explanation may be that there are more causes for ischaemic stroke than large-vessel atherosclerosis, which is in most cases the sole cause of myocardial infarction. When we sub typed ischaemic stroke into large-vessel disease, small-vessel disease and cardio-embolic stroke according to the TOAST criteria,[21] we found that non-carriers had a relative risk of large-vessel disease of 1.5 (95 % CI: 0.5-4.4), a relative risk of cardio-embolic stroke of 1.1 (95% CI: 0.5-2.5) and a relative risk of small-vessel disease of 0.5 (95% CI: 0.2-1.7). These results were not significant, however, they do show that non-carriers are at higher risk of stroke due to large-vessel atherosclerosis (large-vessel and cardio-embolic stroke), while this is not the case for small-vessel stroke. This is also in concordance with earlier findings in this study population that non-carriers of the 192-bp allele had a significantly higher common carotid intima-media thickness and a higher mean pulse wave velocity compared with homozygous carriers,[15] suggesting an association between the *IGF-I* polymorphism and large-vessel atherosclerosis.

Our study shows that the *IGF-I* promoter polymorphism is a significant determinant of survival after any and haemorrhagic stroke. As our analyses on haemorrhagic stroke were only based on 5 cases, the high estimate of the relative risk of 5.20 should be interpreted with caution. We could not find an association between *IGF-I* and survival in the overall population,

which suggests that *IGF-I* is a determinant of survival specifically after stroke. The effect of *IGF-I* on survival after stroke may again be due to the association with myocardial infarction, as it is one of the early causes of death after a stroke.[24,25] In this study, we also found that the most important cause of death after a stroke was cardiovascular disease. After any stroke, 35.7 % of the non-carriers died from cardiovascular disease compared with 26.7 % of homozygous carriers. After haemorrhagic stroke, 67 % of the non-carriers died of cardiovascular disease, compared with 36.4 % of homozygous carriers. Although the RR of CV death for non-carriers was greater than one for all stroke groups, these findings were not significant.

Another explanation could be related to the influence of the 192-bp allele on *IGF-I* secretion. *IGF-I* is involved in brain development.[26] When neuronal loss occurs in the brain, levels of *IGF-I* increase.[8] It has been shown that *IGF-I* reduces infarct volume and improves neurologic function following cerebral ischaemia, as *IGF-I* protects neurons from apoptosis after diverse forms of injury.[10,27,28] Non-carriers have lower plasma IGF-I levels[14], and hence less neuroprotection, which may lead to more severe handicap and higher case fatality after ischaemic stroke. As we did not have data on handicap after stroke, we were not able to test this hypothesis.

Our findings are in line with a recent study by Denti et al. who found that low levels of IGF-I are a significant predictor of poor outcome, mainly death, after ischaemic stroke.[11] However, it was unclear if low IGF-I levels were a cause or a consequence of the poor outcome. Since we studied a gene associated with IGF-I levels, we believe to have strong evidence that low IGF-I levels are a cause and not a consequence of the disease outcome. This indicates that IGF-I might be used as a possible treatment for patients with stroke. Indeed, several animal studies have found that administration of IGF-I reduces infarct volume and improves neurologic function after ischaemia.[8-10,27]

In conclusion, in this large prospective population-based follow-up study, we found that the *IGF-I* polymorphism was a significant determinant of survival after any stroke.

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Chapter 4.2

Alpha-adducin polymorphism, atherosclerosis and cardiovascular and cerebrovascular risk

Objective - Carriers of the 460Trp allele of the α-adducin gene (*ADD1*) show higher rates of sodium reabsorption compared with homozygous carriers of the Gly460 allele and were found to have an increased risk of hypertension and cardiovascular disease. We studied the association between the Gly460Trp polymorphism and atherosclerosis, cardiovascular and cerebrovascular disease.
Methods - Intima-media thickness of the carotid artery (CCA IMT), as well as incident stroke and myocardial infarction (MI) were studied within 6471 subjects of the Rotterdam Study. Within 1018 subjects of the Rotterdam Scan Study, prevalent silent brain infarcts (SBI) and cerebral white matter lesions (WML) were studied. Subjects were grouped into 460Trp carriers (variant carriers) and homozygous carriers of the Gly460 allele (reference).
Results - CCA IMT was 0.80 mm in variant carriers compared with 0.79 mm in the reference group (p=0.04). Variant carriers had an increased risk of any stroke (HR 1.22, 95% CI: 1.02-1.45), of ischaemic stroke (HR 1.29, 95% CI: 1.02-1.63), of haemorrhagic stroke (HR 1.07, 95% CI: 0.59-1.92) and of MI (HR 1.33, 95% CI: 1.05-1.69). For any and ischaemic stroke, there was a significant interaction between the Gly460Trp polymorphism and hypertension. Variant carriers more often had a SBI (OR 1.36, 95% CI: 0.98-1.88) and had more sub cortical WML then the reference group (1.45 ml vs1.24 ml, p=0.22).
Conclusions – The Gly460Trp polymorphism is associated with atherosclerosis, cardiovascular and cerebrovascular disease, especially in hypertensive subjects.

INTRODUCTION

Hypertension is a major risk factor for atherosclerosis, cardiovascular and cerebrovascular disease.[1-4] Recently, it was found that hypertension and stroke, but also hypertension and myocardial infarction (MI) cluster within families.[5,6] This suggests that overlapping genetic factors, alone or in conjunction with environmental factors, influence susceptibility to hypertension, stroke and MI.

Adducin is a cytoskeleton protein consisting of a α- and β- subunit. It favors the binding of actin to spectrin and may affect ion transport through the actin cytoskeleton and modulation of the NA^+-K^+ pump activity.[7,8] Carriers of the 460Trp allele (variant allele) of the α-adducin gene (*ADD1)*, located on chromosome 4p16.3, show a higher NA^+-K^+ pump activity and therefore higher rates of renal tubular sodium reabsorption compared with homozygous carriers of the Gly460 allele (wild type allele).[9,10] The Gly460Trp polymorphism has been associated with blood pressure levels and the risk of hypertension in many, but not all populations.[11]

Furthermore, this polymorphism has been associated with salt-sensitivity,[12] which reportedly is a risk factor for cardiovascular events.[13] Indeed, carriers of the 460Trp allele were found to have an increased risk of cardiovascular disease, although findings have not been consistent.[11,14] So far, no association was found between *ADD1* and ischaemic stroke.[15,16] We studied the Gly460Trp polymorphism in relation to atherosclerosis, myocardial infarction and cerebrovascular disease. Also, we studied the interaction between hypertension and the Gly460Trp polymorphism with respect to all above-mentioned outcomes.

METHODS

Study populations

The Rotterdam Study is an ongoing prospective population-based cohort study on chronic and disabling diseases in the elderly. Baseline examinations were done between 1990 and 1993. A total of 7983 subjects (age ≥ 55 years) participated in this study. In 6471 (81.1%) participants, the Gly460Trp polymorphism was successfully genotyped.

The Rotterdam Scan Study was designed to study the aetiology and natural history of age-related brain changes in the elderly. Baseline examinations, which included brain MRI scanning, were performed in 1995 and 1996 in 1077 participants (aged 60 to 90 years).[17] In 1018 (94.5%) participants, the Gly460Trp polymorphism was successfully genotyped. The medical ethics committee of Erasmus Medical Centre, Rotterdam, approved both studies and all participants gave written informed consent and permission to retrieve information from treating physicians.

Measurements

Body mass index (BMI) was calculated from height and weight. Blood pressure was measured twice using a random-zero sphygmomanometer. The average of two measurements was used for analyses. Hypertension was defined as a systolic blood pressure of 160 mmHg or higher or a diastolic blood pressure of 100mmHg or higher (grade 2 and 3 of the 1999 WHO criteria)[18] or use of blood pressure lowering medication. Information on smoking habits was obtained during a home interview.

We collected non-fasting blood samples from all participants. We defined diabetes mellitus as a random glucose level ≥ 11.1 mmol/l or use of oral anti-diabetics or insulin. Total serum cholesterol and HDL-cholesterol were determined by means of an automated enzymatic method.

Measurements of atherosclerosis

Intima-media thickness of the common arotid artery (CCA IMT) was assessed by duplex scan ultrasonography over an average distance of 10 mm.[19] We used the average of the measurements of three still images of both the left and right arteries. CCA IMT was determined as the mean of the mean IMT of near and far wall measurements of both the left and right arteries. These measurements were done in 5643 participants. For 5083 participants, genotypic information was available.

Stroke and myocardial infarction

Stroke and MI were assessed as part of the Rotterdam Study. A prevalent stroke or MI was determined during the baseline interview and verified by checking medical records. Incident stroke and MI were determined by continuously monitoring subjects for major events. Research physicians reviewed information on all possible strokes and transient ischaemic attacks (TIA) with an experienced stroke neurologist (P.J.K.) to verify all diagnoses. Subarachnoid haemorrhages and retinal strokes were excluded. A stroke was classified ischaemic when a patient had typical symptoms and a CT or MRI, that was made within 4 weeks after the stroke occurred, ruled out other diagnoses, or when indirect evidence (deficit limited to 1 limb or completely resolved within 72 hours or atrial fibrillation in absence of anticoagulants) pointed to an ischaemic nature of the stroke. A stroke was classified haemorrhagic when a relevant haemorrhage was shown on CT or MRI scan, or the subject lost consciousness permanently or died within hours after the onset of focal signs. If a stroke did not match any of these criteria, it was classified unspecified. During follow-up, 637 first ever strokes occurred. Genotype data was available for 498 of these individuals.

Two research physicians independently coded all reported cardiovascular events according to the *International Classification of Diseases, 10th Edition* (ICD-10).[20] Incident MI was defined as the occurrence of a fatal or non-fatal MI (ICD-10 code I21) after the baseline examination. Follow-up started at baseline and lasted until January 1st 2002 for stroke and until January 1st 2003 for MI. Of all participants 2.6% were lost to follow-up. For these subjects, the follow-up time was computed until the last date of contact. We ascertained 371 incident MI cases. For 272 cases, genotypic data was available.

Silent brain infarcts and white matter lesions

Presence of silent brain infarcts (SBI) was assessed in the Rotterdam Scan Study. Infarcts were defined as focal hyperintensities on T2-weighted images, 3 to 20 mm in size. SBI were defined as evidence for one or more infarcts on MRI, without a history of a (corresponding) stroke or TIA.[21] We observed SBI in 217 participants. Genotypic data was available for 119 cases.

White matter lesions (WML) were scored present if visible as hyperintense on proton-density and T2-weighted images, without prominent hypointensity on T1-weighted scans and, according to their location, as periventricular or subcortical.[22] Periventricular WML were rated semi-quantitatively (range 0-9). A total volume of subcortical WML was approximated based on number and size of lesions (volume range 0-29.5 ml).

Genotyping

Genotyping was performed using TaqMan allelic discrimination Assays-By-Design (Applied Biosystems, Foster City, CA).[23] Forward primer sequence was 5'-GAGAAGACAAGATGGCT-GAACTCT-3' and reverse primer sequence 5'-GTCTTCGACTTGGGACTGCTT-3'. The minor groove binding probes were 5'-VIC-CATTCTGCCCTTCCTC-NFQ-3' and 5'-FAM-ATTCTGCCATTCCTC-NFQ-3'. We used the reverse strand design. The assays utilized 5 nanograms of genomic DNA and 5 micro liter reaction volumes. The amplification and extension protocol was as follows: an initial activation step of 10 minutes at 95 °C preceded 40 cycles of denaturation at 95 °C for 15 seconds and annealing and extension at 50 °C for 60 seconds. Allele-specific fluorescence was then analysed on an ABI Prism 7900HT Sequence Detection System with SDS v 2.1 (Applied Biosystems, Foster City, CA). Based on the analysis of blind duplicates, there was a 98% concordance in genotyping.

Statistical analyses

Hardy-Weinberg equilibrium proportions were tested using GENEPOP-package.[24] Baseline characteristics were compared using univariate ANOVA or χ^2 statistics. Univariate analyses of variance were used to assess the relation between the Gly460Trp polymorphism and CCA IMT

and WML. Cox proportional hazards regression analysis was used to assess the association between the Gly460Trp polymorphism and stroke and MI. For the analyses on incident stroke, we excluded prevalent strokes, for the analyses on incident MI, we excluded prevalent MI, at baseline from the analyses. We performed a binary logistic regression analysis to study the relation between the Gly460Trp polymorphism and SBI. All analyses were adjusted for age and sex and additionally for hypertension, BMI, total cholesterol, diabetes mellitus and smoking and performed in SPSS version 11.0.

RESULTS

Genotype frequencies were in Hardy-Weinberg equilibrium in both study populations. Table 1 shows the baseline characteristics stratified by *ADD1* genotype. No significant differences were observed between the genotype groups with the exception of smoking. Within the Rotterdam Study, there were significantly more smokers among the variant carriers, compared with wild type homozygotes. After adjusting for age and sex, no association between the Gly460Trp polymorphism and blood pressure or hypertension was found in either of the two study populations (not shown).

Table 1. General characteristics stratified by *ADD1* genotype

	***ADD1* Gly460Trp**	
	Wild type homozygotes (GG)	**Variant carrier (GT/TT)**
Rotterdam Study		
Number of subjects (%)	4018 (62.1)	2453 (37.9)
Age, y	69.6 ± 9.3	69.3 ± 8.9
Sex, % men	40.0	41.1
BMI (kg/m^2)	26.3 ± 3.7	26.3 ± 3.8
Current smoking, %	21.8	24.1*
Hypertension, %	34.3	32.8
Total Cholesterol (mmol/)	6.6 ± 1.2	6.6 ± 1.2
Diabetes Mellitus, %	10.3	9.6
Rotterdam Scan Study		
Number of subjects	649 (63.8)	369 (36.2)
Age, y	72.2 ± 7.5	72.1 ± 7.2
Sex, % men	50.7	45.3
BMI (kg/m^2)	26.7 ± 3.6	26.7 ± 3.7
Current smoking, %	18.3	15.0
Hypertension, %	51.2	53.1
Total Cholesterol (mmol/)	5.9 ± 1.0	5.9 ± 1.0
Diabetes Mellitus, %	7.4	5.7

All values are percentages or means ± SD, *p<0.05 compared with wild type homozygotes.

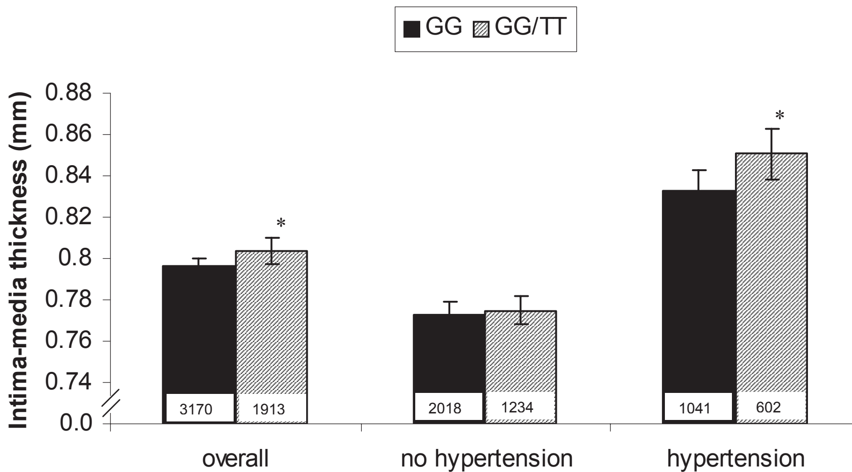

Figure 1. Association between the *ADD1* polymorphism and common carotid intima-media thickness in overall, non-hypertensive and hypertensive subjects
*p<0.05 compared with GG genotype

Figure 1 shows that overall, adjusted for age and sex, variant carriers had an increase in mean CCA IMT (0.80 mm) compared with wild type homozygotes (0.79 mm, p=0.04). This finding did not remain significant after additional adjustment for smoking and other cardiovascular risk factors. Within hypertensive subjects, mean CCA IMT was 0.85 mm in variant carriers, compared with 0.83 mm in wild type homozygotes (p=0.03). After additional adjustment for smoking and other cardiovascular risk factors, this finding remained significant. No significant differences were observed within normotensive subjects. The interaction term hypertension**ADD1* was not a significant predictor of CCA IMT (p=0.07) in the model including age, sex, hypertension and *ADD1*.

We classified 291 stroke cases as ischaemic stroke, 47 as haemorrhagic stroke and 160 as unspecified stroke. Table 2 shows the hazard ratio (HR) for incident stroke and MI, by genotype adjusted for age and sex. Variant carriers were found to have a an increased risk of any stroke (HR 1.22, 95% CI: 1.02-1.45), ischaemic stroke (HR 1.29, 95% CI: 1.02-1.63) and MI (HR 1.33, 95%

Table 2. The Rotterdam Study: Risk of incident stroke and myocardial infarction in relation to the *ADD1* polymorphism

***ADD1* Gly460Trp**		**GG**	**GT/TT**
	n	**HR (95% CI)**	**HR (95% CI)**
Any incident stroke	498	1 (ref)	1.22 (1.02-1.45)*
Incident ischaemic stroke	291	1 (ref)	1.29 (1.02-1.63)*
Incident haemorrhagic stroke	47	1 (ref)	1.07 (0.59-1.92)
Incident MI	272	1 (ref)	1.33 (1.05-1.69)*

All hazard ratios are adjusted for age and sex. For incident stroke, all prevalent strokes were excluded, for incident MI, all prevalent MI's were excluded, n=absolute number of cases, MI=myocardial infarction, HR=hazard ratio
*p<0.05 compared with the GG genotype

Table 3. Rotterdam Scan Study: Association of the *ADD1* polymorphism with SBI and white matter lesions

ADD1 Gly460Trp		SBI		Periventricular WML		Deep subcortical WML
	n	OR (95 % CI)	n	Mean ± SE	n	Mean ± SE
GG	119	1 (ref)	649	2.36 ± 0.08	647	1.24 ± 0.10
GT/TT	85	1.36 (0.98-1.88)	369	2.37 ± 0.10	367	1.45 ± 0.14

All odds ratios and means are adjusted for age and sex, n=absolute number of cases, SBI=silent brain infarction, OR=odds ratio, SE=standard error, WML=white matter lesions

Table 4. The Rotterdam Study: Interaction between hypertension and the ADD1 polymorphism in relation to incident stroke and myocardial infarction

Hypertension, ADD1	Any stroke		Ischaemic stroke		Haemorrhagic stroke		Myocardial infarction	
	n	HR (95% CI)	n	HR (95% CI)	n	HR (95% CI)	n	HR (95% CI)
HT-, GG	154	1 (ref)	91	1 (ref)	15	1 (ref)	90	1 (ref)
HT-, GT/TT	94	1.02 (0.79-1.31)	57	1.03 (0.74-1.43)	7	0.77 (0.31-1.88)	67	1.21 (0.88-1.66)
HT+, GG	130	1.46 (1.15-1.84)*	69	1.42 (1.03-1.94)*	13	1.59 (0.75-3.36)	56	1.33 (0.95-1.86)
HT+, GT/TT	104	2.18 (1.70-2.79)**	62	2.32 (1.68-3.21)**	11	2.48 (1.13-5.42)*	42	1.81 (1.26-2.62)*
p for interaction		0.04		0.05		0.3		0.6

All hazard ratios are adjusted for age and sex, HT- = hypertension absent, HT+ = hypertension present, HR=hazard ratio
*p<0.05, **p<0.001 compared with the reference group HT-, GG

CI: 1.05-1.69), compared with wild type homozygotes. Findings remained significant after additional adjustment for smoking and other cardiovascular risk factors. No significant association was found between the Gly460Trp polymorphism and haemorrhagic stroke.

Table 3 shows that for variant carriers, after adjusting for age and sex, we observed an increased risk for the prevalence of SBI (OR 1.36, 95% CI: 0.98-1.88). There was no difference in mean periventricular WML grade between variant carriers and wild type homozygotes (Table 3). There was an increase in mean subcortical WML volume for variant carriers (1.45 ml ± 0.14) compared with wild type homozygotes (1.24 ml ± 0.10), but this difference was not significant (p=0.22).

Within the Rotterdam study, we investigated the interaction between hypertension and the Gly460Trp polymorphism in relation to atherosclerosis (see figure 1), stroke and MI. Table 4 shows the interaction between hypertension and the Gly460Trp polymorphism in relation to stroke and MI. We found an increased risk for variant carriers with hypertension for any stroke (HR 2.18, 95% CI: 1.70-2.79), ischaemic stroke (HR 2.32, 95% CI: 1.68-3.21), haemorrhagic stroke (HR 2.48, 95% CI: 1.13-5.42) and MI (HR 1.81, 95% CI: 1.26-2.62), compared with wild type homozygotes without hypertension, adjusted for age and sex. Adjusting for smoking and other cardiovascular risk factors did not alter these results. The interaction term hypertension**ADD1* was a significant predictor of any stroke (p=0.04) in the model including age, sex, hypertension and *ADD1* and in the full model including age, sex, hypertension, *ADD1*, BMI, total cholesterol, diabetes mellitus and smoking (p=0.04). This interaction term was also significant for

ischaemic stroke in the model including age, sex, *ADD1* and hypertension (p=0.05), but only borderline in the full model (p=0.06). No interaction was found between hypertension and SBI or WML.

DISCUSSION

In the Rotterdam Study, we found an increased mean CCA IMT and a higher risk for any stroke, ischaemic stroke and MI for carriers of the 460Trp allele. Consistent with these findings, we found in the Rotterdam Scan Study that variant carriers had an increased risk of SBI and an increase in mean subcortical WML volume, but these findings were not significant. We found a significant interaction between the Gly460Trp polymorphism and hypertension in relation to any and ischaemic stroke. To our best knowledge, this is the first study to find an association between the Gly460Trp polymorphism and CCA IMT, ischaemic stroke and MI.

The strengths of our study are the size of our two study populations and the fact that we were able to study the effect of the *ADD1* in relation with both clinical stroke and SBI and white matter lesions. SBI and WML have previously been found to be risk factors for stroke.[21,25]

An early marker of ischaemic stroke, CCA IMT[26] was increased in variant carriers in this study, especially in hypertensive subjects. Also, we observed significant associations between the Gly460Trp polymorphism and any and ischaemic stroke and MI. Hypertension has found to be a strong risk factor for all of these outcomes.[1-3,13,27] Also, it has been reported that variant carriers have an increased left ventricular mass[28] and are at increased risk of coronary heart disease.[14] Recently, it was found that hypertension and stroke, as well as hypertension and MI coaggregate strongly within families.[5,6] This suggests that overlapping genetic factors influence susceptibility to hypertension, stroke and MI.

We did not observe an association between the Gly460Trp polymorphism and haemorrhagic stroke. This may be due to small numbers. Also, this may be the result of survival bias. This is especially important as case fatality is higher in patients with haemorrhagic stroke compared with ischaemic stroke patients.[29,30] Especially in haemorrhagic stroke patients with high blood pressure on admission, prognosis was found to be poor.[31]

Consistent with the association between stroke and SBI and WML,[21,25] and the association between the Gly460Trp polymorphism and stroke in our study, we observed an association between the Gly460Trp polymorphism and SBI and subcortical WML. The lack of statistical significance may have been due to lack of power.

We found a significant interaction between the Gly460Trp polymorphism and hypertension with respect to any and ischaemic stroke, suggesting that hypertension is an effect modifier in the development of these diseases. This does not mean that the effect of *ADD1* on these outcomes may be solely attributed to an effect on blood pressure. We did not observe a relationship between the Gly460Trp polymorphism and blood pressure or hypertension. Also,

when adjusting for hypertension or blood pressure, the associations between the Gly460Trp polymorphism and IMT, stroke and MI remained significant, suggesting that hypertension and blood pressure are not part of the intermediate pathway. A potential pathway could be salt sensitivity, which was found to be a risk factor for cardiovascular events, independent of blood pressure[13].

It has previously been reported that there is an interaction between the 460Trp allele and diuretic therapy.[15] Hypertensive subjects carrying the variant allele and treated with diuretics were at lower risk of stroke and MI compared with other antihypertensive therapies. We found that in hypertensive subjects carrying the variant allele of the Gly460Trp polymorphism, the risk of stroke was significantly increased. Therefore, it is of great importance to optimize treatment of hypertension for these patients. When selecting anti-hypertensive medication, the genetic profile of a patient may need to be taken into account in the future.

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Chapter 4.3

Polymorphisms of the Renin-Angiotensin System are associated with blood pressure, atherosclerosis and cerebral white matter pathology

Objective – The renin-angiotensin system is involved in the development of hypertension, atherosclerosis and cardiovascular disease. We studied the association between the M235T polymorphism of the angiotensinogen gene (*AGT*) and the C573T polymorphism of the angiotensin II type 1 receptor (*AT1R*) and blood pressure, carotid atherosclerosis and cerebrovascular disease.
Methods – We genotyped over 6000 subjects of the Rotterdam Study and over 1000 subjects of the Rotterdam Scan Study. We used logistic regression and univariate analyses, adjusting for age and sex, with for *AGT*, the MM, and for *AT1R*, the TT genotype as reference.
Results - We found that *AGT*-235T increased systolic (p for trend=0.03) and diastolic blood pressure (p for trend=0.04). The prevalence of carotid plaques was 1.25 fold increased (95% CI: 1.02-1.52) in *AGT*-TT carriers. There was a significant increase in mean volume deep sub cortical white matter lesions (WML) for *AGT*-TT carriers (1.78 ml versus 1.09 ml in the reference group, p=0.008). A significant interaction was found between *AGT* and *AT1R*, further increasing the effect on periventricular and subtotal WML (p for interaction=0.02).
Conclusions – We found an association between *AGT* and blood pressure, atherosclerosis and WML. Also, we found synergistic effects between *AGT* and *AT1R* on the development of WML. These findings raise the question whether the RAS may be a therapeutical target for the prevention of cerebral white matter pathology.

INTRODUCTION

The renin-angiotensin system (RAS) regulates blood pressure, cardiovascular homeostasis and vascular tone.[1] Polymorphisms in genes that encode for the proteins of the RAS are candidate genes for hypertension, cardiovascular and cerebrovascular diseases. The angiotensin (*AGT*) and the angiotensin II type I receptor (*AT1R*) genes are two key players in the AGT protein metabolism.

The *AGT*-M235T polymorphism encodes the substitution of methionine by threonine at residue 235 of the AGT protein, increasing plasma AGT levels in 235T homozygotes.[2] A haplotype at the *AGT* promoter, which was in complete linkage disequilibrium with the M235T polymorphism, was found to increase transcriptional activity in astrocytes.[3] *AGT* has been associated with hypertension, carotid atherosclerosis, cardiovascular and cerebrovascular disease, although findings have been inconsistent.[4-7] *AGT* has been associated consistently with cerebral small vessel disease.[8-10]

The *AT1R* gene has been associated with hypertension, cardiovascular and cerebrovascular disease.[11-15] The *AT1R*-C573T polymorphism, which is in linkage disequilibrium with the frequently studied A1166C polymorphism,[14,15] has been associated with blood pressure and vascular complications in hypertensive patients.[16,17]

Hypertension, atheroclerosis and cerebrovascular diseases are all complex diseases. For these traits, a network of interactions between genetic factors can be supposed.[18] Both the *AGT* and the *AT1R* gene products are part of the RAS. So far, an interaction has not been reported. We studied the *AGT*-M235T and the *AGT*-C573T polymorphisms in relation to blood pressure, carotid atherosclerosis and small- and large-vessel cerebral pathology. Also, we studied the interaction between *AGT* and *AT1R* with respect to all above-mentioned outcomes.

METHODS

Study populations

The Rotterdam Study is an ongoing prospective population-based cohort study on chronic and disabling diseases in the elderly.[19] Baseline examinations were done between 1990 and 1993. A total of 7983 subjects (age ≥ 55 years) participated in this study. In 6,444 (80.7%) and 6,367 (79.8%) participants, the M235T polymorphism of *AGT* and the C573T polymorphism of *AT1R*, respectively, were successfully genotyped. No DNA was available for 1455 subjects and there was a genotyping failure in 84 (*AGT*) and 161 (*AT1R*) subjects.

The Rotterdam Scan Study was designed to study the aetiology and natural history of age-related brain changes in the elderly, using a similar protocol (baseline between 1995). A total of 1,077 non-demented elderly persons (age ≥ 60 years) participated in this study. In

1995/1996, subjects aged between 60 and 90 years were selected randomly in strata of age (5 years) and sex, from the Zoetermeer Study[20] and the Rotterdam Study. In 1,048 (97.3%) participants, the M235T polymorphism of *AGT* and in 1011 (93.9%) participants, the C573T polymorphism of *AT1R,* were successfully genotyped. There was a genotyping failure in 29 (*AGT*) and 66 (*AT1R*) subjects. The medical ethics committee of the Erasmus Medical Centre, Rotterdam, approved both studies and all participants gave written informed consent and permission to retrieve information from treating physicians.

Measurements

Height and weight were measured and body mass index (BMI in kg/m^2) was calculated. Blood pressure was based on the average of two measurements with a random-zero sphygmomanometer. Hypertension was defined as a systolic blood pressure (SBP) ≥ 140 mmHg and/or a diastolic blood pressure (DBP) ≥ 90 mmHg and/or use of anti-hypertensives. For 1184 (14.8%), blood pressure measurements were not available. Information on smoking habits was obtained.

We collected non-fasting blood samples from all participants. We defined diabetes mellitus as a random glucose level ≥ 11.1 mmol/l and/or use of oral anti-diabetics or insulin. Total serum cholesterol and HDL-cholesterol were determined using an automated enzymatic method.[21]

Measurements of atherosclerosis and stroke

As part of the Rotterdam study, the total number of plaques was assessed by duplex scan ultrasonography.[22] Plaques were defined as focal widening of the vessel wall with protrusion into the lumen. The total plaque score reflected the total number of sites with plaques ranging from 0-6 (left and right sided, common carotid arteries, bifurcation, and internal carotid arteries). This score was dichotomised (0,1 or 2 versus >2). For 2372 (29.7%) participants the number of plaques could not be assessed.

Incident stroke was also assessed as part of the Rotterdam Study. A prevalent stroke was determined during the baseline interview. Research physicians reviewed information on all possible strokes with an experienced stroke neurologist to verify all diagnoses. Subarachnoid haemorrhages and retinal strokes were excluded. A stroke was classified ischaemic when a patient had typical symptoms and a CT or MRI ruled out other diagnoses, or when indirect evidence (deficit limited to 1 limb or completely resolved within 72 hours or atrial fibrillation in absence of anticoagulants) pointed to an ischaemic nature of the stroke. A stroke was classified haemorrhagic when a relevant haemorrhage was shown on CT or MRI scan, or the subject lost consciousness permanently or died within hours after the onset of focal signs.

Cerebral infarcts and white matter lesions

As part of the Rotterdam Scan Study, we obtained axial T1, T2 and proton-density MRI scans of the brain. Infarcts were defined as focal hyperintensities on T2-weighted images, 3 to 20 mm in size. Silent brain infarctions (SBI) were defined as evidence for infarcts on MRI, without a history of a (corresponding) stroke or TIA.[23]

White matter lesions (WML) were scored as hyperintense on proton-density and T2-weighted images, without prominent hypointensity on T1-weighted scans and, according to their location, as periventricular or sub cortical.[24] Periventricular WML were rated semi-quantitatively (range 0-9). A total volume of sub cortical WML was approximated based on number and size of lesions (volume range 0-29.5 ml). In 4 participants (0.004%), sub cortical WML could not be measured, due to the quality of the MRI scans.

Genotyping

Genotyping of the *AGT* M235T polymorphism and the *AT1R* C573T polymorphism was performed using TaqMan allelic discrimination Assays-By-Design (Applied Biosystems, Foster City, CA). Based on the analysis of blind duplicates (326 control pairs), there was a 99.4 % concordance in genotyping *AGT* and 100 % concordance in genotyping *AT1R*. The 2 discordant pairs were set to missing.

Statistical analyses

Hardy-Weinberg equilibrium proportions of the M235T and the C573T polymorphisms were tested using the GENEPOP-package. Baseline characteristics were compared using univariate ANOVA or χ^2 statistics. Analyses of variance was used to assess the relation between *AGT* and *AT1R* and SBP and DBP, as well as WML. In order to obtain a normal distribution of WML, we used the natural logarithm transformation. Cox proportional hazards regression analysis was used to assess the relative risk of stroke. For this analysis, we excluded prevalent stroke. We used logistic regression to assess the odds ratio for carotid artery plaques and SBI for *AGT* and *AT1R* (SPSS version 11.0). All analyses were adjusted for age and sex (model 1) and additionally adjusted for SBP, DBP, BMI, total cholesterol, diabetes mellitus and smoking (model 2). The analyses on blood pressure levels were also adjusted use of anti-hypertensives. A p-value <0.05 was considered statistically significant.

RESULTS

Genotype frequencies were in Hardy-Weinberg-Equilibrium for both study populations. Table 1 shows the baseline characteristics stratified by *AGT* and *AT1R* genotype. No significant differences were observed between genotype groups and baseline characteristics.

In Table 2 we show that for *AGT*, SBP (p for trend=0.03) and DBP (p for trend=0.04) increased with the number of *AGT*-235T alleles. In the fully adjusted model, including anti-hypertensive medication use, these findings remained significant. Also, a (borderline) significant increase

Table 1. Baseline characteristics stratified by *AGT* and *AT1R* genotype

***AGT* M235T**	**MM**	**MT**	**TT**
Rotterdam Study (n)	2341 (36.3)	3084 (47.9)	1019 (15.8)
Age, y	69.4 ± 9.1	69.7 ± 9.2	69.0 ± 9.1
Sex, % men	39.6	41.0	40.7
BMI (kg/m^2)	26.4 ± 3.8	26.3 ± 3.7	26.2 ± 3.6
Current smoking, %	21.9	23.1	23.2
Total Cholesterol (mmol/)	6.6 ± 1.2	6.6 ± 1.3	6.6 ± 1.2
Diabetes Mellitus, %	10.1	10.2	8.9
Rotterdam Scan Study (n)	386 (36.8)	503 (48.0)	159 (15.1)
Age, y	72.2 ± 7.3	72.2 ± 7.4	72.4 ± 7.6
Sex, % men	46.4	50.3	42.8
BMI (kg/m^2)	26.7 ± 3.6	26.7 ± 3.6	26.5 ± 3.6
Current smoking, %	18.1	16.1	17.7
Total Cholesterol (mmol/)	5.9 ± 1.1	5.9 ± 1.0	5.9 ± 1.1
Diabetes Mellitus, %	5.2	8.3	7.5
***AT1R* C573T**	**TT**	**CT**	**CC**
Rotterdam Study (n)	1484 (23.3)	3126 (49.1)	1755 (27.6)
Age, y	69.5 ± 9.3	69.4 ± 8.9	69.5 ± 9.4
Sex, % men	38.8	41.3	40.6
BMI (kg/m^2)	26.2 ± 3.7	26.3 ± 3.7	26.4 ± 3.8
Current smoking, %	21.6	23.9	21.4
Total Cholesterol (mmol/)	6.6 ± 1.2	6.6 ± 1.2	6.6 ± 1.2
Diabetes Mellitus, %	9.4	10.0	10.8
Rotterdam Scan Study (n)	219 (21.7)	504 (49.9)	288 (28.5)
Age, y	72.6 ± 7.5	72.1 ± 7.1	71.8 ± 7.2
Sex, % men	46.1	49.8	48.3
BMI (kg/m^2)	26.7 ± 4.2	26.8 ± 3.6	26.4 ± 3.3
Current smoking, %	17.4	15.7	19.9
Total Cholesterol (mmol/)	5.9 ± 1.0	5.9 ± 1.0	5.9 ± 1.1
Diabetes Mellitus, %	5.5	7.3	7.3

All values are percentages or means ± SD

Table 2. The Rotterdam Study: Association between the M235T and the C573T polymorphism with SBP and DBP and hypertension

***AGT* M235T**		**SBP (mmHg) Mean ± SE**			**DBP (mmHg) Mean ± SE**			**Hypertension OR (95% CI)**	
	n	**Model 1**	**Model 2**	**n**	**Model 1**	**Model 2**	**n**	**Model 1**	**Model 2**
MM	2198	138.2 ± 0.5	138.2 ± 0.5	2197	73.4 ± 0.2	73.4 ± 0.2	2206	1.0 (reference)	1.0 (reference)
MT	2910	139.5 ± 0.4*	139.4 ± 0.4*	2910	73.7 ± 0.2	73.7 ± 0.2	2927	1.13 (1.01-1.27)*	1.15 (1.02-1.30)*
TT	955	139.6 ± 0.7	139.6 ± 0.7	955	74.2 ± 0.4*	74.2 ± 0.4*	964	1.17 (1.00-1.36)	1.20 (1.02-1.41)*
P for trend		0.03	0.03		0.04	0.04			
***AT1R* C573T**									
TT	1397	139.4 ± 0.6	139.6 ± 0.6	1396	73.7 ± 0.3	73.8 ± 0.3	1409	1.0 (reference)	1.0 (reference)
CT	2942	139.3 ± 0.4	139.2 ± 0.4	2942	73.9 ± 0.2	73.9 ± 0.2	2952	1.04 (0.91-1.18)	1.02 (0.89-1.17)
CC	1657	138.6 ± 0.5	138.4 ± 0.5	1657	73.4 ± 0.3	73.4 ± 0.3	1669	0.95 (0.82-1.10)	0.92 (0.79-1.07)
P for trend		0.3	0.1		0.4	0.3			

All means and ORs are adjusted in model 1 for: age and sex, in model 2 for: age, sex, body mass index, total cholesterol, diabetes mellitus, smoking (and use of anti-hypertensive medication in SBP and DBP analyses), n=absolute number of subjects, SBP=systolic blood pressure, DBP=diastolic blood pressure, OR=odds ratio, SE=standard error, *$p<0.05$ compared with the MM genotype

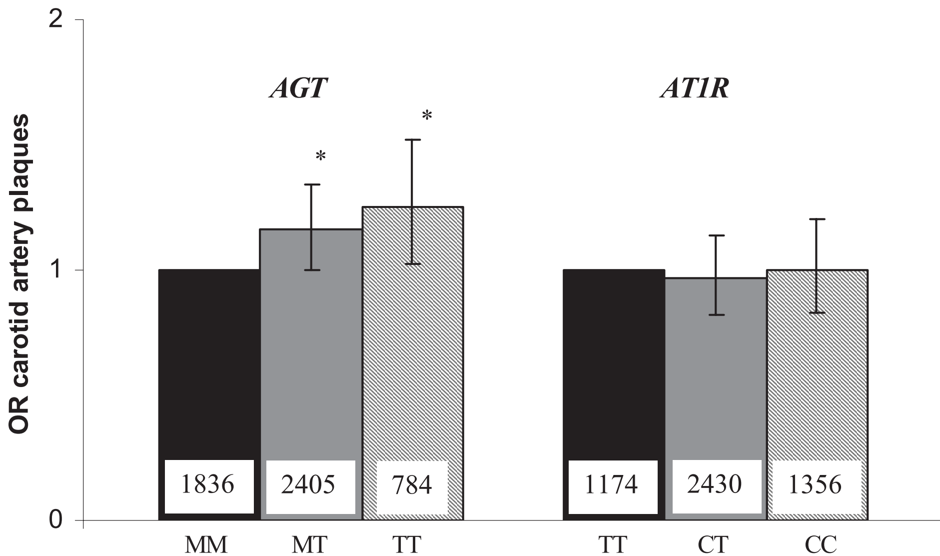

Figure 1. The Rotterdam Study: Association between the AGT and AT1R genotypes and carotid artery plaques. From a total plaque score of 0-12, plaques were scored low if the total plaquescore was 0, 1 or 2, and high if the total plaque score was greater than 2. *p<0.05 compared with TT genotype, adjusted for age and sex. OR=odds ratio.

in prevalence of hypertension was found. *AT1R* was not associated with blood pressure or hypertension.

Figure 1 shows that carriers of the *AGT*-235T allele had an increased risk of plaques. The OR for the *AGT*-MT genotype was 1.16 (95% CI: 1.00-1.34, p=0.05), and for the TT genotype 1.25 (95% CI: 1.02-1.52, p=0.03). In the fully adjusted model, the OR for the *AGT*-MT genotype was 1.17 (95% CI: 1.01-1.37, p=0.04), and for the TT genotype 1.27 (95% CI: 1.03-1.57, p=0.03). We did not find an association between *AT1R* and carotid artery plaques.

Periventricular WML were present in 219 (20.3%), deep sub cortical WML in 84 (7.8%) participants. No association was found between *AGT* or *AT1R* and periventricular WML. Participants with the TT genotype of *AGT*, had an increased volume of deep sub cortical WML (1.78 ml versus 1.09 ml for the reference group, p=0.008; figure 2). Participants with the CT genotype of the *AT1R* genotype also showed an increased volume (1.45 ml versus 0.99 ml for the reference group, p=0.03). Findings remained significant in the fully adjusted model (*AGT*-TT 1.81 ml versus 1.08 ml for the reference group, p=0.004 and *AT1R*-CT 1.39 ml versus 0.98 ml for the reference group, p=0.05).

We observed 217 (20.2%) participants with a SBI (Rotterdam Scan Study) and 637 (8.0%) with incident stroke (Rotterdam Study). The prevalence of SBI was increased in *AGT*-TT carriers (OR=1.44 (95% CI: 0.89-2.33, p=0.14), and *AT1R*-CC carriers (OR=1.43 (95% CI: 0.89-2.30, p=0.14), model 2), however, findings were not significant. No significant association was found between *AGT* or *AT1R* and overall stroke (*AGT*-TT HR=0.99 (95% CI: 0.75-1.30), *AT1R*-CC

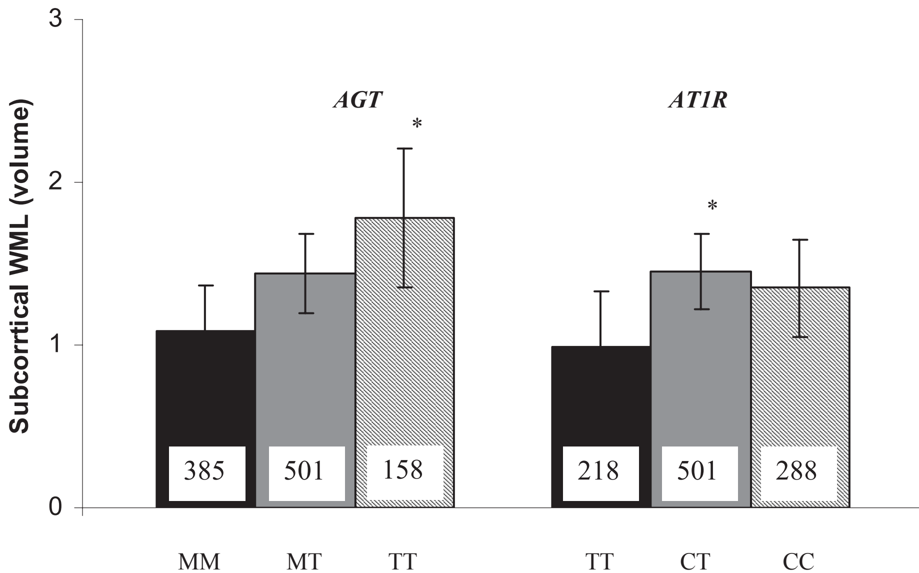

Figure 2. The Rotterdam Scan Study: Association of the AGT and the AT1R genotypes with subcortical white matter lesions (WML)
*p<0.01 compared with the MM genotype for AGT and the TT genotype for AT1R, adjusted for age and sex

HR=1.19 (95% CI: 0.91-1.56), model 2), ischaemic stroke (*AGT*-TT HR =1.05 (95% CI: 0.73-1.50), *AT1R*-CC HR=1.34 (95% CI: 0.94-1.92), model 2), or haemorrhagic stroke (*AGT*-TT HR=1.19 (95% CI: 0.50-2.80), *AT1R*-CC HR=1.14 (95% CI: 0.52-2.52), model 2).

Finally, we studied the interaction between *AGT* and *AT1R*. Within the Rotterdam Study, SBP and DBP were highest in participants carrying both the TT genotype of *AGT* and the CC genotype of *AT1R* (n=234) compared with the reference group of participants with MM genotype of *AGT* and the CC genotype of *AT1R* (n=593) (model 1: SBP 141.9 mmHg versus 137.3 mmHg, p=0.007, model 2: SBP 141.7 mmHg versus 137.2 mmHg, p=0.007 and model 1: DBP 74.8 mmHg versus 72.7 mmHg, p=0.02 and model 2: DBP 75.0 mmHg versus 72.6 mmHg, p=0.005).

For periventricular WML, we found the highest degree in participants with both the TT genotype of *AGT* and the CC genotype of *AT1R* (model 1: 3.06 (n=37), versus 2.06, (n=105), in the reference group, p=0.008, model 2: 2.93 versus 2.09, p=0.03), as well as for deep sub cortical WML (model 1: 2.60 ml (n=37), versus 0.93 ml (n=105) in the reference group, p=0.001, model 2: 2.67 ml versus 0.98 ml, p=0.001). The p for interaction was significant for both periventricular (p=0.02, both models) and deep sub cortical WML (p=0.02, both models). No interaction was found for carotid artery plaques or SBI.

DISCUSSION

Within the Rotterdam Study, we found that mean systolic and diastolic blood pressure levels increased with the number of T alleles of the M235T polymorphism of *AGT*, as did the risk for the prevalence of carotid artery plaques. Within the Rotterdam Scan Study, we found that subjects with the TT genotype of *AGT* and the CT genotype of *AT1R*, had an increase in mean volume deep sub cortical WML. A significant interaction between *AGT* and *AT1R* was found for periventricular and deep sub cortical WML.

We believe the strength of our study lies in the size of our study populations and the follow-up of patients over time. Also, we were able to study the effect of *AGT* and *AT1R* in two study populations, which made it possible to study both clinical and sub clinical stroke (SBI), as well as white matter lesions of the brain and atherosclerosis. Results on plaques and incident stroke were obtained from the Rotterdam Study, while results on SBI and WML were obtained from the Rotterdam Scan Study. None of these outcomes were available in both cohorts. Participants of the Rotterdam Scan Study were randomly selected from the Rotterdam Study and the Zoetermeer Study in strata of age (>60 years) and sex. Participants of the Rotterdam Study and the Rotterdam Scan Study therefore party overlap. Both cohorts consist of elderly participants, living in the Netherlands, who are participants of two large prospective population-based studies, and are therefore comparable cohorts.

For genotyping measurements, as well as measurements of blood pressure and plaques, there was missing data. We did not find differences between participants with and without a genotype with regard to demographic or cardiovascular characteristics. Participants with missing data on blood pressure and plaques, were significantly older and more often males. As there were no genotype differences between these participants, this will most likely not have biased our results.

Carriers of the 235T allele of the *AGT* gene have reported to have increased plasma angiotensinogen levels,[2] hypertension and atherosclerosis.[5,6] In line with these findings, we found an increase in SBP and DBP in *AGT*-235T allele carriers in the Rotterdam Study and a (borderline) significant increased risk of hypertension. Also, we observed a significant association between *AGT* and carotid artery plaques. Previously, an association between *AGT*-M235T and carotid intima-media thickness (IMT) was reported.[5,25] Even though we found an association between *AGT*-M235T and SBP and DBP, the observed association with carotid artery plaques may not be solely attributable to the effect on blood pressure. After adjusting for blood pressure, the association between the M235T polymorphism and carotid artery plaque remained significant, suggesting that blood pressure levels may not be part of the intermediate pathway.

Within the Rotterdam Scan Study, we found that the risk of SBI was increased in *AGT*-TT and *AT1R*-CC carriers, although not statistically significant. *AGT* was significantly associated with deep sub cortical WML. We did not find an association with periventricular WML. We used

two different scales to define periventricular and deep sub cortical WML, categorical and volumetric, respectively. As the scale to define sub cortical WML was quantitative and the scale to define periventricular WML semi-quantitatively, the power to detect an effect is most likely higher for sub cortical WML. This may explain why a significant association was only observed for sub cortical WML in this study.

So far, three studies found an association between *AGT* and small-vessel disease and periventricular hyperintensity grade.[8-10] This finding has been explained by an increase in plasma AGT levels, which may lead to increased formation of angiotensin II, which has several pro-atherogenic effects,[26] and may also explain the effect we found on carotid atherosclerosis. However, the lack of a convincing association with SBI or stroke, does not support this pathway. Another mechanism explaining our findings and these of others, may be related to the fact that an independent renin-angiotensin system exists in the brain, which might amplify cerebrovascular pathology, in particular WML.[27] Also, a haplotype in at the *AGT*-promoter has been found to increase transcriptional activity in astrocytes.[3]

As plaques and WML are precursors of stroke,[28,29] they are less heterogeneous compared with (sub) clinical stroke. This may also explain why we did not find a significant association for stroke and SBI, not even after sub typing stroke in order to increase homogeneity. As genes involved in so-called complex diseases, such as stroke, usually have small effects, they may be difficult to detect, even in large study populations. Studying intermediate phenotypes increases homogeneity as they focus on a specific pathohysiological pathway.

An interaction was observed between *AT1R* and *AGT*. Cross talk between genes is plausible as both the *AGT* and the *AT1R* gene products are part of the RAS. This is the first study addressing the interaction between *AGT* and *AT1R*. In complex traits, such as WML, one may expect joint effects of multiple genes. Other genes of the RAS may also be of interest in relation to small vessel pathology.

There is increasing interest in the association between cognitive decline and depression, and severe WML.[30,31] The consistent association between *AGT* and WML, and the interaction with *AT1R* reported here, raises the question whether the RAS may be a therapeutical target for the prevention of cerebral small vessel pathology. Previously, subjects with hypertension and the DD genotype of the insertion/deletion polymorphisms of the angiotensin converting enzyme (ACE) gene, also part of the RAS, showed a relative resistance to ACE-inhibitor therapy and therefore an increase in cardiovascular mortality.[32] In addition, several other studies found that this polymorphism may influence antihypertensive response, particularly when using ACE inhibitors.[33] Also, *AGT* was found to be an independent predictor of blood pressure response to ACE inhibititors[34] and a protective association between ACE inhibitor use and nonfatal stroke was found among 235T allele carriers of *AGT*.[4]

In conclusion, we found that the *AGT*-235T allele was associated with increased blood pressure levels and carotid artery plaques. With respect to WML, we found evidence for interaction between *AGT* and *AT1R*. As no significant evidence was found for an association with SBI or

stroke, the effect of *AGT* and *AT1R* may be specific for small vessel pathology, perhaps related to blood pressure early in life.

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Chapter 5

Genome-wide association study

Chapter 5.1

A genome-wide association study on blood pressure in a genetically isolated population

Objective – The aaetiology of hypertension is partly genetic. Many studies have performed genome-wide linkage analysis and identified regions linked to blood pressure or hypertension. We have performed a genome-wide association study on systolic (SBP) and diastolic (DBP) blood pressure in a genetically isolated population.
Methods – From the Erasmus Rucphen Family (ERF) study, 200 partcipants were selected. We genotyped 262,000 single nucleotide polymorphisms (SNPs) using the Affymetrix GeneChip Mapping 500K. Genome-wide association analyses were performed using GenABEL. We performed both single SNP and haplotypic analyses. P-values were estimated using the permutation test.
Results – We found 228 SNPs associated with SBP and 266 SNPs with DBP (nominal p-value ≤0.001). Seven SNPs, all intergenic, located on chromosomes 18q22.3, 19p12 and 1p21.1, were associated with blood pressure with a p-value ≤ 10^{-5}. A SNP located in the gene *NR3C2* was associated with DBP (p=0.0009). This gene was earlier associated with early-onset hypertension. None of our findings reached genome-wide empirical significance. When performing haplotypic analyses, the haplotype rs1781860-rs7250372-rs2210744, on chromosome 19, showed the strongest association ($p<10^{-6}$ with SBP).
Conclusions - This is a first step to genome-wide association analysis of blood pressure. We identified several SNPs associated with blood pressure in regions earlier identified in linkage studies. Genes of interest in these regions are *C18orf55, CYB5A, CNDP1, SLC35A3* and *CNN3*. As our study was performed in a small series of individuals, we are currently replicating our findings.

INTRODUCTION

Hypertension is a common determinant of coronary artery disease and stroke, accounting for more than 12 million deaths annually worldwide (httpp://www.who.int/en). It has been recognised for long that the disease is for a large part of genetic origin.[1-5] For subjects with an affected first degree relative, the relative risk of hypertension is 2 to 5 fold increased, depending on the age of onset in the affected relative. Between 30% and 40% of the blood pressure variation is estimated to be genetically determined.[1-5]

Many studies aimed to identify genes contributing to common essential hypertension. At present, over 20 genome linkage scans have been reported in the literature. Most scans have reported nominal or suggestive evidence for linkage.[6-28] A number of regions have been found to be linked to hypertensions in more than one study, suggesting these regions do incorporate loci involved in blood regulation. This includes the regions on chromosome 1q, 2p, 3p, 6q, 7q, 11q, 12q, 15,q, 16q, 18q, and 19p.[28]

All genome-wide scans to date have been based on linkage in families. Most population-based studies using association analysis have targeted specific candidate genes, which were selected on the basis of their involvement in the pathophysiology of the disease. Hypertension is a complex disease in which besides multiple genes, environmental factors are involved as well. The risk factors that have been consistently related to hypertension include age, sex, body mass index, diabetes, dyslipidemia, smoking and alcohol intake. A recent development in research of the genetics of complex diseases, is genome-wide association studies, using arrays with dense maps of single nucleotide polymorphisms (SNPs). So far hypertension or blood pressure have not been studied.[29-32] In this study, we present our findings on a genome-wide association study on systolic (SBP) and diastolic blood pressure (DBP) in a genetically isolated population in the Netherlands.

METHODS

Setting

All participants are Dutch inhabitants of a genetically 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 program aims to identify genetic risk factors in the development of complex disorders.[33] The study was approved by the Medical Ethics Committee of Erasmus Medical Centre Rotterdam. Written informed consent was obtained from all participants.

Participants

The isolate was founded by less than 400 individuals around the middle of the 18th century. Minimal inward migration and considerable population growth characterised this population. About 20,000 inhabitants are now scattered over eight adjacent villages. Church and the municipality records were used to reconstruct genealogical information and is currently available in the form of a large pedigree-database including over 63,000 individuals. Twenty couples that had at least 6 children baptised in the community church between 1850-1900 were identified with the help of genealogical records. All living descendants of these couples and their spouses were invited to participate in the ERF study.

For the current study, 200 individuals were selected from the ERF study. This selection was based on height. We selected 50 men ≤ 172cm, 50 men ≥ 185cm, 50 women ≤ 158cm and 50 women ≥ 172cm. According to the principle of Mendelian randomisation, selecting samples based on height should not affect blood pressure. Indeed blood pressure did not differ significantly ($p=0.85$ for SBP and $p=0.10$ for DBP) between those who are short (SBP=131.2 mmHg and DBP=78.4 mmHg) and those who are tall (SBP=131.4 mmHg and DBP=76.2 mmHg). Subjects were all distantly related (≥ 5 generations) or unrelated. Three individuals were excluded as they were close relatives of other study participants. In total 197 individuals were selected.

Data collection

Participants of the ERF study were invited for a series of clinical examinations at our research centre, located within the community. For the current study blood pressure measurements were used. Blood pressure was measured twice in the sitting position at the right upper arm using an automated device (OMRON 711, automatic IS). Height and weight were measured with the participant dressed in light under clothing and body mass index (BMI) was calculated (kg/m^2).

Genotyping

We genotyped all participants using the Affymetrix GeneChip Mapping 500K. This array set is comprised of two Nsp arrays, each capable of genotyping approximately 262,000 SNPs. About 250 ng of genomic DNA was digested with two restriction enzymes and processed according to the Affymetrix protocol. All SNPs on the X-chromosome were excluded from our analyses, as were all monomorphic markers. In total, 229,779 SNPs were considered for further analyses.

Statistical analyses

Baseline characteristics were estimated using SPSS 11.0.1. An exact test of Hardy-Weinberg equilibrium was performed using GenABEL package 1.1-4.[34,35] Genome-wide association analyses were also done using GenABEL. For every SNP, a score test was performed, as implemented in the qtscore procedure, on SBP and DBP, adjusting for age, sex and BMI. From this, we obtained nominal p-values with 1df. To adjust for multiple testing, 500 permutations were repeated in order to estimate empirical genome-wide significance, using the 'emp.qtscore' function. To compare blood pressure levels between genotypes of selected SNPs, we performed univariate analyses of variance, adjusting for age, sex and BMI. For the most significant SNPs, 500 kb surrounding regions were analysed in further detail. We conducted haplotype analyses using a 3-SNP sliding window using GenABEL interface to the 'haplo.stats' library.[36] For each possible haplotype, the association with SBP and DBP was assessed, adjusting for age, sex and BMI. We ran 20,000 simulations in order to obtain p-values for the global test of significance. The simulated p-value for the global score statistic is the number of times the global score statistic, estimated using permutaed data, exceeds the observed, divided by the total number of simulations. The association with blood pressure was also investigated for haplotypes formed by all possible pairs of SNPs in the selected region (2D scan). We ran 20,000 simulations in order to obtain p-values for the global test of significance. Next, linkage disequilibrium (LD) was estimated between all SNP pairs in the regional analyses, using Lewontin's D' as implemented in the "genetics" library. Heatmaps representing LD data and the data from the 2D scan were produced. We searched the Ensembl Project (www.ensembl.org) and the National Centre for Biotechnology Information (NCBI) RefSeq and UniGene Human Sequence Collection databases (www.ncbi.nlm.nih.gov) for known genes surrounding or containing the SNPs associated with blood pressure in this study.

RESULTS

General characteristics of our study population are presented in Table 1. The mean age was 31.2 years. Mean SBP was 130.7 mmHg and DBP 77.2 mmHg.

Table 1. General characteristics

Total Number	**197**
Age – years	31.2 ± 6.5
Sex - % male	49.2
Height – cm	172.0 ± 12.1
BMI – kg/m^2	25.5 ± 4.6
SBP – mmHg	130.7 ± 14.1
DBP – mmHg	77.2 ± 9.7

All values are means ± SD or percentages

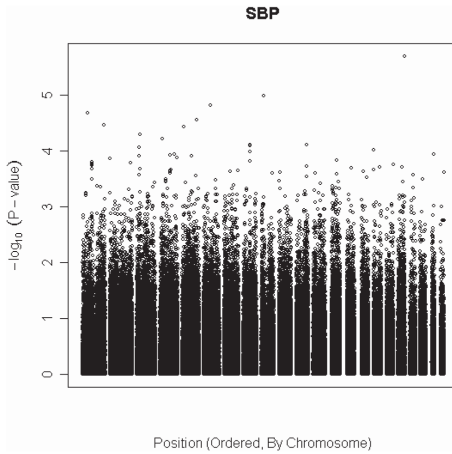

Figure 1a. Genome-wide association for SBP. Nominal p-values adjusted for age, sex and BMI.

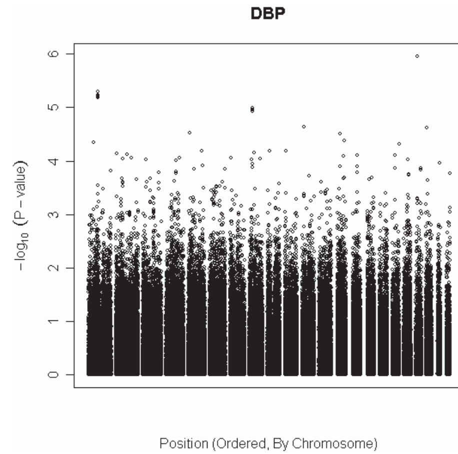

Figure 1b. Genome-wide association for DBP. Nominal p-values adjusted for age, sex and BMI.

The results of the genome-wide association analyses are displayed in Figure 1a (SBP) and 1b (DBP). In these figures, $-\log_{10}$ p-values per SNP are plotted, adjusted for age, sex and BMI. On the x-axis, the physical position of the SNP in the genome is displayed. A large number of SNPs was associated with SBP (n=228) or DBP (n=266) with a p-value $\leq 10^{-3}$ (= $-\log_{10}$(p-value) ≥ 3).

SNPs associated with blood pressure with a nominal p-value $\leq 10^{-4}$ (1df) are presented in Table 2a (SBP) and 2b (DBP). Genotype frequencies of the SNPs rs726815, rs2210744,

Table 2a. Single nucleotide polymorphisms significantly ($p<10^{-4}$) associated with SBP

SNP	rsnumber	Nominal p-value (1df) x 10^5	Chromosome	Position	Gene	Flanking genes
SNP_A-2152540	rs12958471	0.2	18	69635902		*NETO1, FBXO15*
SNP_A-1879827	rs1838430	1.1	9	27492077	*MOBKL2B*	
SNP_A-4226863	rs9442895	1.5	6	73913789	*KCNQ5*	
SNP_A-2020876	rs726815	2.1	1	55502882		*USP24, FLJ45337*
SNP_A-4226078	rs11738617	2.9	5	158813854		*IL12B, ADRA1B*
SNP_A-1989071	rs10495356	3.5	1	231337373		*IRF2BP2, TOMM20*
SNP_A-2255170	rs6554739	3.8	5	12699312		*CTNND2, DNAH5*
SNP_A-4237336	rs4478120	5.3	3	31081143		*GADL1, STT3B*
SNP_A-1976455	rs1352802	6.2	4	23412617		*GBA3, PPARGC1A*
SNP_A-1993704	rs831741	7.9	8	70311811		*C8orf34, SULF1*
SNP_A-2093307	rs4447204	8.0	11	112099134		*C11orf34, NCAM1*
SNP_A-4213005	rs1809311	8.3	8	70314533		*C8orf34, SULF1*
SNP_A-1972092	rs1909520	8.9	3	25038705		*THRB, RARB*
SNP_A-2062593	rs587388	9.8	16	5458795		*NPM1P3, A2BP1*

Table 2b. Single nucleotide polymorphisms significantly ($p<10^{-4}$) associated with DBP

SNP	rsnumber	Nominal p-value (1df) x 10^5	Chromosome	Position	Gene	Flanking genes
SNP_A-1959826	rs2210744	0.1	19	21205922		*ZNF431, ZNF493*
SNP_A-4219788	rs1887914	0.5	1	104411993		*AMY1C, PRMT6*
SNP_A-1955725	rs1330400	0.6	1	104349547		*AMY1C, PRMT6*
SNP_A-4224836	rs7515655	0.6	1	104422124		*AMY1C, PRMT6*
SNP_A-2095756	rs12747997	0.6	1	104423146		*AMY1C, PRMT6*
SNP_A-4237981	rs12078274	0.7	1	104438986		*AMY1C, PRMT6*
SNP_A-4212913	rs10503962	1.0	8	34443391		*FUT10, UNC5D*
SNP_A-2258370	rs16882469	1.1	8	34561686		*FUT10, UNC5D*
SNP_A-1876224	rs4289807	1.2	8	34266892		*FUT10, UNC5D*
SNP_A-4214114	rs4465365	2.3	11	24541909	*LUZP2*	
SNP_A-1967732	rs4813932	2.4	20	10284912		*SNP25, MKKS*
SNP_A-2255170	rs6554739	3.0	5	12699312		*CTNND2, DNAH5*
SNP_A-2203765	rs9525514	3.1	13	40963578		*NARG1L, KIAA0564*
SNP_A-4227306	rs729218	4.3	13	87784586		*SLITRK5, GPC5*
SNP_A-2131845	rs6670302	4.5	1	56511281		*FLJ45337, PPAP2B*
SNP_A-2185820	rs8078438	4.9	17	76591190		*CHMP6, BAIAP2*
SNP_A-4204602	rs2225699	6.5	10	8832293		*GATA3, CUGBP2*
SNP_A-1879827	rs1838430	6.6	9	27492077	*MOBKL2B*	
SNP_A-2179071	rs10055809	6.6	5	143746439	*KCTD16*	
SNP_A-2274965	rs270794	7.1	8	56012658		*RP1, XKR4*
SNP_A-4229271	rs10929808	7.3	2	12519594		*LPIN1, TRIB2*
SNP_A-2128231	rs17009670	7.7	2	123770088		*TSN, CNTNAP5*
SNP_A-2274627	rs1358174	7.8	17	36011285		*CCR7, SMARCE1*
SNP_A-1795529	rs6573780	8.0	14	67094811	*PLEKHH1*	
SNP_A-2155964	rs2497410	8.2	13	84746002		*SLITRK1, SLITRK6*
SNP_A-4212175	rs10237920	8.6	7	9018814		*NXPH1, NDUFA4*
SNP_A-2237528	rs11099678	8.7	4	149410455	*NR3C2*	
SNP_A-2207013	rs12478446	8.7	2	167020447		*SCN9A, SCN7A*
SNP_A-1963855	rs10193916	9.0	2	76670743		*C2orf3, LRRTM4*
SNP_A-4233048	rs7838203	9.2	8	116123468		*CSMD3, TRPS1*
SNP_A-1811561	rs16953549	9.4	18	47360544		*RKHD2, DCC*
SNP_A-1802283	rs7826247	9.4	8	23454180		*ENTPD4, SLC25A37*
SNP_A-4231895	rs17034609	9.5	3	36228796		*ARPP21, STAC*
SNP_A-1893445	rs2597874	9.7	4	112894273		*PITX2, C4orf16*

rs6573780 and rs17034609, were out of HWE. The tables also show the chromosome, the position on the chromosome, and the gene in which the SNP is located. None of our adjusted analyses reached genome-wide empirical significance. The lowest empirical p-value was 0.24 for SNP rs12958471 on chromosome 18, for SBP, and 0.27 for SNP rs2210744 on chromosome 19, for DBP. For DBP, it is remarkable that within a 90 kb region on chromosome 1, we found 5 SNPs associated with DBP with nominal p-values ranging from 0.5×10^{-5} to 0.7×10^{-5}. In a 300 kb region on chromosome 8, we found 3 SNPs associated with DBP (p-values $\leq 1.2 \times 10^{-5}$). For SBP, we found two intragenic SNPs, one on chromosome 9 (rs1838430) positioned in the *MOBKL2B* gene and one on chromosome 6 (rs9442895) positioned in the *KCNQ5* gene. The latter gene is of interest as it belongs to the potassium voltage gated channel subfamily. For DBP we found 5 intragenic SNP. These SNPs are located on chromosome 11 (rs4465365) in the *LUZP2* gene, on chromosome 9 (rs1838430) in the *MOBKL2B* gene, on chromosome 5 (rs10055809) in the *KCTD16* gene, on chromosome 14 (rs6573780) in the *PLEKHH1* gene and on chromosome 4 (rs11099678) in the gene *NR3C2*. Of particular interest are the genes *KCTD16* and *NR3C2*. *KCTD16* is a potassium channel tetramerisation domain containing gene. *NR3C2* encodes for both the mineralocorticoid receptor, such as aldosterone, and the glucocorticoid receptor, such as corticosterone or cortisol.

Table 3a. Systolic blood pressure values per genotype for single nucleotide polymorphisms with $p<10^{-5}$

SNP	N	Allele1_Allele1 (1)	N	Allele1_Allele2 (2)	N	Allele2_Allele2 (3)
rs12958471	176	130.6 ± 0.8	5	156.2 ± 4.8		
rs2210744			86	128.3 ± 1.2	101	133.8 ± 1.1
rs1887914			17	140.2 ± 2.6	172	130.3 ± 0.8
rs1330400			20	138.7 ± 2.4	161	130.0 ± 0.9
rs7515655	171	130.3 ± 0.8	17	140.2 ± 2.6		
rs12747997			17	140.3 ± 2.6	170	130.5 ± 0.8
rs12078274	171	130.3 ± 0.8	17	140.2 ± 2.6		

All values are means (mmHg) ± SD and adjusted for age, sex and BMI, N=total number

Table 3b. Diastolic blood pressure values per genotype for single nucleotide polymorphisms with $p<10^{-5}$

SNP	N	Allele1_Allele1 (1)	N	Allele1_Allele2 (2)	N	Allele2_Allele2 (3)
rs12958471	176	76.8 ± 0.7	5	90.5 ± 4.1		
rs2210744			86	73.9 ± 0.9	101	80.0 ± 0.9
rs1887914			17	85.2 ± 2.1	172	76.3 ± 0.7
rs1330400			20	84.7 ± 2.0	161	76.2 ± 0.7
rs7515655	171	76.3 ± 0.7	17	85.2 ± 2.1		
rs12747997			17	85.3 ± 2.1	170	76.4 ± 0.7
rs12078274	171	76.3 ± 0.7	17	85.2 ± 2.1		

All values are means (mmHg) ± SD and adjusted for age, sex and BMI, N=total number

We estimated the effect of the SNPs passing a nominal threshold p-value ≤ 10^{-5} on SBP and DBP along with the 3 intragenic SNPs, adjusting for age, sex and BMI (Table 3a and b). Differences in SBP levels between genotypes ranged from 5.5 to 25.6 mmHg, in DBP levels from 6.1 to 13.7 mmHg. These differences were all significant (p ≤ 0.001).

Figures 2a-d show heatmaps of the regions surrounding the SNPs with a p-value <10^{-5}, as well as for the 3 SNPs located in the 300 kb region on chromosome 8. The results of the 2D scan are presented in the upper left triangle, the LD results are presented in the lower right tri-

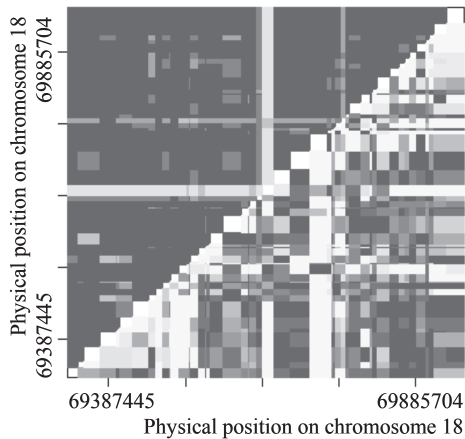

Figure 2a. Heatmap for 500 kb region containing 53 SNPs with index SNP rs12958471, chromosome 18. Results from the 2D scan are presented in the upper left triangle, LD is presented in the lower right triangle.

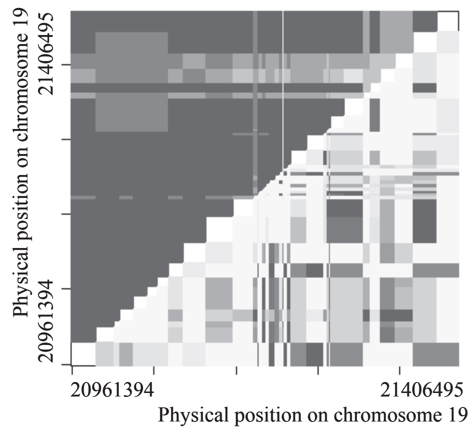

Figure 2b. Heatmap for 500 kb region containing 40 SNPs with index SNP rs2210744, chromosome 19. Results from the 2D scan are presented n the upper left triangle, LD is presented in the lower right triangle.

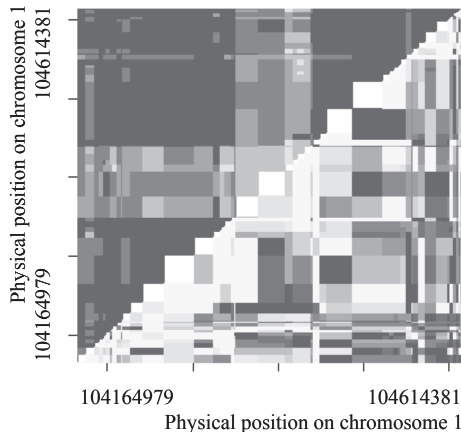

Figure 2c. Heatmap for 500 kb region containing 53 SNPs with index SNPs rs1887914, rs1330400, rs7515655, rs12747997 and rs12078274, chromosome 1. Results from the 2D scan are presented in the upper left triangle, LD is presented in the lower right triangle.

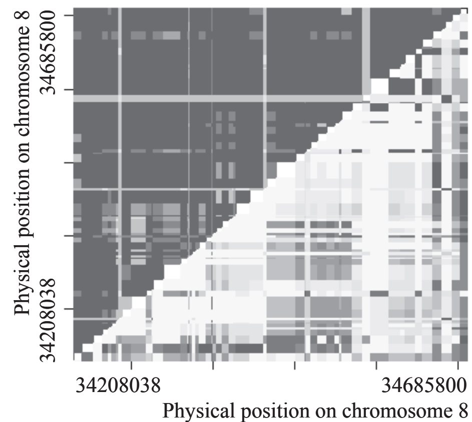

Figure 2d. Heatmap for 500 kb region containing 68 SNPs with index SNPs rs10503962, rs16882469 and rs4289807, chromosome 8. Results from the 2D scan are presented in the upper left triangle, LD is presented in the lower triangle.

Table 4. Haplotypes containing single nucleotide polymorphisms with $p<10^{-5}$

Haplotype	Chromosome	Global p-value SBP	Global p-value DBP
rs1991621 / rs12455621 / **rs12958471**	18	0.00005	0.03
rs12455621 / **rs12958471** / rs8086918	18	0.00005	0.03
rs12958471 / rs8086918 / rs2872	18	0.0007	0.06
rs7250372 / rs2884599 / **rs2210744**	19	0.00005	0.003
rs2884599 / **rs2210744** / rs1645806	19	0.00005	0.003
rs2210744 / rs1645806 / rs669038	19	0.00005	0.004
rs1999228 / rs118007 / **rs1330400**	1	0.004	0.007
rs11800756 / **rs1330400** / rs12116936	1	0.03	0.06
rs1330400 / rs12116936 / rs10494007	1	0.008	1.0
rs12116936 / rs10494007 / **rs1887914**	1	0.009	0.07
rs1494007 / **rs1887914** / **rs7515655**	1	0.002	0.03
rs1887914 / **rs7515655** / **rs1274799**	1	0.0005	0.001
rs7515655 / **rs12747997** / **rs12078274**	1	0.0003	0.002
rs12747997 / **rs12078274** / rs12407548	1	0.001	0.002
rs12078274 / rs12407548 / rs11185388	1	0.007	0.01
rs4289807 / rs4237087 / **rs4289807**	8	0.0002	0.00005
rs4237087 / **rs4289807 /** rs2953932	8	0.004	0.0003
rs4289807 / rs2953932 / rs2719293	8	0.005	0.0004
rs1445197 / rs13261718 / **rs4212913**	8	0.04	0.35
rs13261718 / **rs4212913** / rs7816886	8	0.003	0.0004
rs4212913 / rs7816886 / rs7833514	8	0.006	0.001
rs16882460 / rs7357540 / **rs16882469**	8	0.03	0.003
rs7357540 / **rs16882469** / rs7819030	8	0.02	0.004
rs16882469 / rs7819030 / rs4480120	8	0.15	0.03

All analyses are adjusted for age, sex and BMI. The index SNPs are printed in bold.

angle. The shade is brighter when the association for a SNP pair with blood pressure is higher, as well as when LD between two SNPs is higher. For all 4 regions, the association with blood pressure was strongest for SNP pairs containing an index SNP (p-value = $5x10^{-5}$).

The SNPs with a nominal p-value ≤ 10^{-5}, and the 3 SNPs in the region on chromosome 8, were also used to construct haplotypes with a 3-SNP sliding window. Haplotypes were created from all SNPs genotyped in an area of 250kb up and downstream of these SNPs. These data are shown in Table 4. For SBP, the most significant haplotypes were located on chromosome 18 and 19 ($p=5x10^{-5}$). For DBP, the most significant haplotype was located on chromosome 8 ($p=5x10^{-5}$).

DISCUSSION

We have performed a genome-wide association analysis of blood pressure in a genetically isolated population in the Netherlands. We found that 228 SNPs were associated with SBP and 266 SNPs with DBP with a p-value ≤ 10^{-3}. As the series are small, many associations are false positives. Seven SNPs, located on chromosome 18, 19 and 1, were associated with blood pressure with a p-value ≤ 10^{-5}. All of these SNPs were intergenic. One intragenic SNP was of particular interest as it was located in the gene NR3C2, encoding for both the mineralocorticoid receptor and the glucocorticoid receptor. When adjusting for multiple testing by repeating 500 permutations, none of our findings reached genome-wide empirical significance. The regional analyses showed that for SBP the most significant haplotypes were located on chromosomes 18 and 19, and for DBP on chromosome 8 (p-value = $5x10^{-5}$). SNP pairs formed by one of the index SNPs and one of the SNPs in a 500 kb surrounding region, showed strong association with blood pressure (p-value = $5x10^{-5}$). To our knowledge, this is the first genome-wide association study on blood pressure.

For complex diseases, resulting from genes with common variants and small effects, association has been found to be more powerful than linkage. As hypertension is a complex disease, our study may have more power to identify such genes compared with previously performed linkage analyses. Another approach to study the genetics of blood pressure and hypertension is the candidate gene approach, in which genes are studied that have been selected on the basis of their function. An advantage of our study is that genome-wide association, as well as linkage, does not require a previous hypothesis and both analyses may be used to explore the full genome for new genes.

Our study was performed in a genetically isolated population. Individuals selected from such a population are likely to share a more common genetic and environmental background,[37] and linkage disequilibrium (LD) is detectable over greater distance, compared with the general population.[38] Therefore, identifying genes may be easier in such isolated populations. The main problem is that our analyses were performed on a small number of participants. This might explain why we did not find genome-wide significant associations with blood pressure.

So far, no genome-wide association studies have been performed using blood pressure traits as an outcome. In contrast, many genome-wide linkage scans for SBP and DBP have been conducted. Some of these studies have reported evidence for linkage in regions of the genome in which we found SNPs that were associated with blood pressure in this study. The strongest association with SBP was found for SNP rs12958471. This SNP is located on chromosome 18q22.3. Previous studies have reported linkage of hypertension to chromosome 18q21,[21] of postural systolic blood pressure changes to chromosome 18q21[39] and of autosomal dominant orthostatic hypotensive disorder to 18q21-22.[40] For DBP, SNP rs2210744 showed the strongest association. This SNP is located on chromosome 19p12. This region was also

identified in previous linkage studies. Four studies have found linkage of blood pressure to chromosome 19p12 and 19p13[12,18,20,41] and 1 study found linkage of hypertension to chromosome 19p13.[27] Genotype frequencies of this SNP were out of HWE. Next, we found association between DBP and 5 SNPs on chromosome 1p21.1. Three studies have reported to find linkage of blood pressure to chromosomes 1p13, 1p21, 1p22 and 1p31 for blood pressure.[12,42,43] Last, we found association between DBP and 3 SNPs on chromosome 8p12. No previous studies have reported linkage of hypertension or blood pressure to this chromosome.

The 7 SNPs that showed the strongest association with blood pressure in this study, were all intergenic. We searched the Ensembl website to identify surrounding genes (www.ensembl.org). For chromosome 18q22.3, there were several genes in the surrounding area (<1Mb). One is the gene *C18orf55*, which encodes for TIM21-like protein, which is a mitochondrial precursor. It may participate in the translocation of transit peptide-containing proteins across the mitochondrial inner membrane. Another gene is *CYB5A* encoding cytochrome b5. This is a membrane bound hemoprotein functioning as an electron carrier for several membrane bound oxygenases. Defects in *CYB5A* are known to cause type IV hereditary methemoglobinemia.[44] It was proposed that the rate-limiting step in microsomal cytochrome P450-mediated hydroxylation reactions was modified through the involvement of the transfer of electrons through cytochrome b5. In turn, cytochrome P450 is involved in salt-sensitivity and has been associated with blood pressure regulation.[45] Last, the gene *CNDP1* was close to the position our SNP. It encodes for carnosine dipeptidase 1 and was found to be involved in homocarnosinosis.[46] Also, diabetic patients with a variant allele (Mannheim allele) of *CNDP1* were found to be less susceptible for nephropathy.[47]

In the region of chromosome 1p21.1, the genes *SLC35A3* and *CNN3* (4-10cM from our SNP) were earlier proposed as candidate genes for blood pressure.[42] *SLC35A3* encodes for a UDP-N-acetylglucosamine transporter in the Golgi apparatus, while *CNN3* encodes for calponin-3, which is implicated in the regulation and modulation of smooth muscle contraction. There were no genes in the surrounding region of chromosome 19p12 and 8p12 that may explain the association we found with blood pressure. So far, no studies have reported an association between the above-mentioned genes and blood pressure or hypertension.

We found evidence for association with blood pressure for several intragenic SNPs. Of particular interest are the genes KCNQ5 and KCTD16, as they are part of potassium channel subfamilies. Even more interesting may be the gene NR3C2, which encodes for both the mineralocorticoid receptor, such as aldosterone, and the glucocorticoid receptor, such as corticosterone or cortisol. The receptor binds to mineralocorticoid response elements and transactivates target genes. The effect of mineralocorticoids is to increase ion and water transport and thus raise extracellular fluid volume and blood pressure and lower potassium levels. This gene has been associated with autosomal dominant pseudohypoaldosteronism type I[48] and early onset hypertension with severe exacerbation in pregnancy.[49] This disease is character-

ized by the onset of severe hypertension before the age of 20, and by suppression of aldosterone secretion.

We have presented the results from a genome-wide association analysis of blood pressure. We were able to detect 7 SNPs on chromosomes 18q22.3, 19p12 and 1p21.1 that were most significantly associated wih blood pressure. Haplotypes containing these SNPs also showed strong association with blood pressure. Genes of interest in the surrounding regions of these SNPs were *C18orf55, CYB5A, CNDP1*, *SLC35A3* and *CNN3*. We also showed association between several intragenic genes and blood pressure, of which the gene *NR3C2* was of particular interest as this gene was earlier associated with early-onset hypertension. Currently, we are replicating our findings.

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Chapter 6

Discussion

6.1 General discussion

Stroke is a complex disease and in many cases is resulting from many environmental and genetic risk factors as well as a complex network of gene-gene and gene-environment interactions[1]. Dissecting the genetics of stroke has proven to be difficult. Loci involved in the pathogenesis of stroke most likely confer a small relative risk, and may influence subtypes of stroke, intermediate phenotypes or risk factors, or have a direct effect on stroke.[2] Many studies have attempted to unravel the genetic aetiology of stroke, however, findings have been inconsistent.[3]

In the introduction of this thesis, I have discussed several options to reduce the complexity of identifying genotype-phenotype correlations for complex diseases, like studying intermediate phenotypes and homogeneous populations. In this chapter I will discuss our main findings, address the problems of studying complex diseases and discuss the future prospectives of stroke genomics.

GENETIC SUSCEPTIBILITY OF STROKE

There is substantial evidence for an inherited risk of stroke. Several studies have described that a positive family history of stroke is a risk factor for stroke occurrence.[4,5] Also animal studies have shown evidence for a genetic susceptibility of stroke.[6,7] An obstacle in studying the genetics of stroke is that patients are often distantly related, in which case extensive genealogic information is necessary. Further, the age of onset of stroke is high and other affected relatives, especially parents, have often deceased. Family history studies rely on information and memory of relatives, which often leads to inadequate information. In **chapter 3.1** we used extensive genealogic data instead of family history. We have found that stroke patients were significantly closer related compared with controls derived from the same genetically isolated population. Also, the isolate allowed us to study the effect of inbreeding on stroke risk, which we found to be associated with stroke in this study.

REDUCING THE COMPLEXITY OF STROKE

Intermediate phenotypes – heritability studies

Many intermediate phenotypes can be treated as quantitative traits and they potentially yield more statistical power for a given sample size. They represent an intermediate stage of the disease and they are more homogeneous phenotypes and therefore, their aetiology may be

less genetically diverse. These traits can be obtained from population-based samples and overcome the problem of subclinical disease (i.e. in a case control study, the control may be at risk to develop the disease, but is yet in the subclinical stage). These advantages have led to an increase in interest in studying intermediate phenotypes of complex diseases.

In this thesis, we have studied blood pressure, carotid atherosclerosis and cerebral white matter lesions (WML), which can be considered quantitative intermediate phenotypes of stroke.[8-12] Hypertension is the most important risk factor for stroke[13] and hypertension and stroke have found to co-aggregate strongly within families, suggesting overlapping genetic factors.[14-16] A key issue is that these intermediate phenotypes are heritable. For carotid atherosclerosis, data was available for individuals derived from our genetically isolated population. We found that the heritability of IMT was 41% and for plaques 28%, and therefore these intermediate phenotypes may be of interest in the identification of stroke genes (**chapter 2.1**). Heritability estimates for systolic and diastolic blood pressure were 34% and 37%, respectively (**chapter 2.2**).

Intermediate phenotypes – gene variants

In **chapter 4.2** we studied the α-adducin gene (*ADD1*) and found that IMT was significantly higher in carriers of the 460Trp allele (variant allele) of the Gly460Trp polymorphism (compared with the homozygous Gly460 allele carriers). A significantly increased risk of stroke and myocardial infarction (MI) was observed for variant carriers in this study, as well as an interaction between *ADD1* and hypertension with respect to ischaemic stroke. Previously, variant carriers showed a higher NA^+-K^+ pump activity and therefore higher rates of renal tubular sodium reabsorption, which affects blood pressure.[17,18] Furthermore, *ADD1* has been associated with salt-sensitivity, a risk factor for cardiovascular events.[19,20] For two other genes, angiotensinogen (*AGT*) and angiotensin II type 1 receptor (*AT1R*), we could not observe an increased risk of stroke. However, we were able to detect an association between *AGT* and blood pressure, carotid plaques and WML (**chapter 4.3**).

Also, we observed an interaction between *AGT* and *AT1R* and WML. The RAAS regulates blood pressure, cardiovascular homeostasis and vascular tone.[21] Renin, secreted by the kidney, converts angiotensinogen (AGT) into angiotensin I, which in turn is converted into angiotensin II by angiotensin-converting enzyme (ACE). The effect of angiotensin II, the key peptide in the RAAS, is regulated through the angiotensin II type 1 and 2 receptors. Polymorphisms in genes that encode for the proteins of the RAAS are candidate genes for hypertension, cardiovascular and cerebrovascular diseases. Also, angiotensin II was found to have several proatherogenic effects, explaining the effect we found on carotid atherosclerosis.[22]

In **chapter 3.2** we present a study on the gene encoding the 5-lipoxygenase activating protein (*ALOX5AP*). This gene was identified by deCODE in the isolated Icelandic population in a linkage analysis on MI families. In the same population, they found an increased risk of

stroke for *ALOX5AP*.[23] We found an association between *ALOX5AP* and ischaemic stroke, but also blood pressure and body mass index (BMI), which is another well known risk factor for stroke.[24,25] ALOX5AP encodes the 5-lipoxygenase (5-LO) activating protein. This protein plays a role in inflammation, as it is involved in the synthesis of leukotrienen, which are proinflammatory lipid mediators.[26-28]

Homogeneous populations

Individuals derived from genetically isolated populations show significantly less genetic diversity compared with individuals from outbred populations and linkage disequilibrium (LD) is detectable over greater distance in young isolates, compared with the general population. Other advantages of using genetic isolates in gene identification are the environmental homogeneity and known genealogy and founders.[29]

In this thesis, we have performed two candidate gene studies in the isolated population (**chapter 3.1 and 3.2**). We studied two genes associated with stroke that have been identified by a genome wide search within multiple families derived from the isolated population of Iceland. The *PDE4D* gene, on chromosome 5q12, plays a role in cAMP regulation.[30] In our genetically isolated population, SNP 45 and SNP 39 of *PDE4D* were significantly associated with small-vessel ischaemic stroke, in inbred individuals. A recent meta-analysis reported a significant association between ischaemic stroke and SNP 87, SNP 83 and SNP 41.[31] The *ALOX5AP* gene, on chromosome 13q12-13, was described in a previous paragraph. The SG13S32 polymorphism of *ALOX5AP* was significantly associated with blood pressure, BMI and ischaemic stroke. The TGC haplotype was also significantly associated with ischaemic stroke in our isolated population.

Subtyping stroke

Stroke is a heterogeneous disease, which can be divided into two main subtypes: ischaemic stroke and haemorrhagic stroke. Ischaemic stroke itself is again a heterogeneous disease and can be grouped into different subtypes, all with a partly different and overlapping pathogenesis. These diverse pathways are very likely to be under different genetic influences. Therefore, it has been proposed to study subtypes of ischaemic stroke, instead of overall ischaemic stroke. For this purpose, classification systems were developed, like the Trial of Org 10172 in Acute Stroke Treatment (TOAST),[32] which divides ischaemic stroke into 5 different pathophysiological categories (large-artery atherosclerosis, small-artery atherosclerosis, cardioembolism, stroke of other aetiology and stroke of undetermined aetiology). Family history studies have reflected the aetiologic origin of subtypes of ischaemic stroke, as stroke due to small- and large-artery atherosclerosis was found to be heritable, while cardio-embolic stroke was not.[14] Another subgroup of stroke concerns the early-onset stroke, as it is known that ischaemic

stroke in the young is different from that in the elderly.[33] Furthermore, a positive family history of stroke was a stronger risk factor for early-onset than for late-onset ischaemic stroke patients, suggesting a higher genetic susceptibility for early-onset stroke.[14,34,35]

In line with these findings, **chapter 3.1** shows that the degree of relationship was higher for large-vessel stroke, compared with small-vessel stroke and highest for early-onset stroke. Furthermore, we found that the gene encoding phosphodiesterase 4D (*PDE4D*) was only associated with small-vessel stroke and not with large-vessel stroke. This gene was identified by deCODE and was associated with combined cardiogenic and carotid stroke in the Icelandic population.[30] PDE4D degrades second messenger cAMP which plays a central role in signal transduction.[36] PDE4D is expressed in vascular smooth muscle cells, [37] but also, low levels of cAMP increase proliferation of endothelial cells.[38] Therefore, PDE4D may be involved in both atherosclerosis, and thus carotid stroke, as well as small-vessel stroke resulting from endothelial proliferation. Other studies have found an association between *PDE4D* and cardioembolic stroke or large-vessel stroke, but findings have been inconsistent.[39-41]

Also for *ADD1* we found evidence for subtype specific effects as variant carriers had an increased risk of ischaemic stroke, but not of haemorrhagic stroke (**chapter 4.2**). For genes of the RAAS and the IGF-I gene, we did not find an association with stroke overall, or subtypes of stroke. *ALOX5AP* was found to be associated with ischaemic stroke, but was not studied in ischaemic stroke subtypes.

APPROACHES

There are two principle approaches to identify disease genes: linkage analysis and association studies. So far, most association studies have focused on candidate genes. A challenge in association studies is the selection of candidate genes. These genes are chosen, as they are known to encode for proteins involved in the pathogenesis of the disease. In this thesis, two candidate genes, *PDE4D* and *ALOX5AP*, were identified in a genome-wide linkage analysis in multiple affected families with stroke and MI.[23,30] Other candidate genes were selected based on their known involvement in blood pressure regulation and atherosclerosis (*ADD1*, *AGT* and *AT1R*),[42-46] or their role in the development of atherosclerosis, infarct volume and neurological outcome (*IGF-I*).[47,48]

Instead of limiting ourselves to candidate genes, we can also target the complete genome in a genome-wide association study, which does not require a previous hypothesis. In **chapter 5.1** we have presented a genome-wide association study on blood pressure in the isolate genotyping over 262,000 SNPs in 200 individuals. So far, no genome-wide association studies have been published on blood pressure. In contrast, many studies have performed linkage analysis, but findings were often not replicated.[49,50] We found 228 SNPs that were significantly associated with SBP and 266 SNPs with DBP ($p<0.001$). For 7 SNPs, the p-value was <0.0001.

Haplotypes containing one of the 7 SNPs also showed strong association with blood pressure. The 7 SNPs were located on chromosomes 18q22.3, 19p12 and 1p21.1. All of the regions were identified previously in linkage studies.[51-61] Several genes were present in the surrounding area of the 7 SNPs. None of these genes so far have been associated with blood pressure. Some have been proposed as candidate genes for blood pressure, amongst which was the *CNN3* gene, which was involved in the regulation of smooth muscle contraction.

6.2 The impact of genetic testing on stroke

Genetics has made remarkable progress in unraveling the aetiology of common, multifactorial diseases. These so-called complex diseases, such as stroke, diabetes mellitus or Alzheimer's disease, appear to be due to interactions of multiple genes and environmental factors. The improved understanding of the genetic basis of these conditions has fuelled great expectations about future clinical applications of genetic testing.[62-67] An example is a recent editorial in the New England Journal of Medicine, in which it was stated that 'we have recently entered a transition period in which specific genetic knowledge is becoming critical to the delivery of effective health care for everyone'.[66] Further, the editors wrote that 'the individual patient's genome will help determine the optimal approach to care, whether it is preventive, diagnostic, or therapeutic.[66] Moreover, numerous genetic association studies are published that claim that the application of the findings will ultimately improve patient care.

However, it is yet unclear to what extend genetic testing could eventually be useful in the clinical care of common disorders, risk assessment, diagnosis, therapy, secondary prevention and prognosis. We aimed to quantify the theoretically possible impact of genes-to-be-discovered on clinical practice of complex diseases. As an illustration, we modeled common ischaemic stroke.

Genes are involved in ischaemic stroke in several ways. First, the aetiology of ischaemic stroke is partly genetic.[4,30] Genetic risk factors could thus be used in risk assessment. Furthermore, as genetic variation may be related to the response to drugs ,[63,68] genetic testing might identify patients with optimal response to secondary prevention. Third, cerebral ischaemia is a potent inducer of gene expression[69,70] and several of these genes have different variants. It is therefore likely that genetic variation partly explains the outcome after ischaemic stroke, and that genetic testing could be used to predict the prognosis. Therapy was not discussed, as thrombolysis should be given in the first hours after a stroke, which does not give the clinician enough time for genetic testing. Further, we did not evaluate diagnostic models, as it is unlikely that a complex disorder like ischaemic stroke will once be fully diagnosed with genetic testing.

We modeled hypothetical genotypes (combinations of alleles) with a reasonable frequency (5 % or higher) to mimic the clinical setting where patients present from the general population, assuming common variants underlying a common trait. It is unlikely that a series of common ischaemic strokes include rare genetic variants in which stroke is inherited in a classical Mendelian pattern (for example cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy (CADASIL)).[4,71] As all risk genotypes of presumed susceptibility genes for ischaemic stroke were found to have a frequency of 0.05 or higher,[4,71] we used these frequencies in our models. We further modeled relatively weak effects, as all presumed susceptibility genotypes for ischaemic stroke seem to be associated with a relative risk below 2.0.[4,71] One genotype was considered at a time. This hypothetical gene was defined with index genotype BB, the AB genotype and the reference genotype AA. The genotype and allele frequencies were assumed to be in Hardy-Weinberg. This assumption indicates that the population is large and stable, and not subject to inbreeding, selective survival or selective mating.[72]

For counseling, we modeled the risk of ischaemic stroke for various frequencies (0.05-0.30) and relative risks (0.50-3.00) of the BB genotype. For this purpose, we computed the incidence rate (IR) of ischaemic stroke for a person-aged 55 years or over who carries this BB genotype. We defined genotypic relative risks (GRR) as follows: the risk for subjects with the AB genotype is γ times greater than risk for subjects with the AA genotype. We assume a multiplicative model, so that the GRR for BB if γ^2.[73] The incidence rate (IR) in the reference group with AA was adapted in every computation to keep the IR in the general population at 9.4 per 1000 person-years (py).[74]

We further evaluated the interaction of a hypothetical genotype with particular treatment in the secondary prevention of ischaemic stroke. We defined that persons with genotype BB, benefit more from a certain type of secondary prevention than people with the AB or the AA genotype. The annual risk of a recurrent stroke without treatment was set at 0.05,[75] and at 0.0435 (0.05 times 0.87) in case of treatment with low dose aspirin.[76] Genotype specific treatment in people with the AA genotype was defined to have the same effect as this standard treatment. We defined the annual risk of a recurrent stroke for the BB group to be the risk in the AA group times the BB associated relative risk with genotype specific treatment. For the AB genotype, this was defined as the risk in the AA group times the square root of the BB associated relative risk (multiplicative model). We then computed the number needed to screen for a possible secondary prevention program for different frequencies (0.05-0.30) and relative risks (0.17-0.90) associated with BB. In this case, the number needed to screen is the number of people one needs to genotype, to prevent one ischaemic stroke from happening when genotype specific treatment could be given instead of standard treatment. It gives an estimate of the feasibility of a screening program, and is calculated as the reciprocal of the absolute risk reduction of a recurrent stroke as a result of screening and treatment.

Prognosis was modeled similarly as risk (outlined above). The cumulative incidence (CI) of handicap (Rankin score[77] 3, 4 or 5) or death one year after a stroke was set at 51.0 % overall.[78]

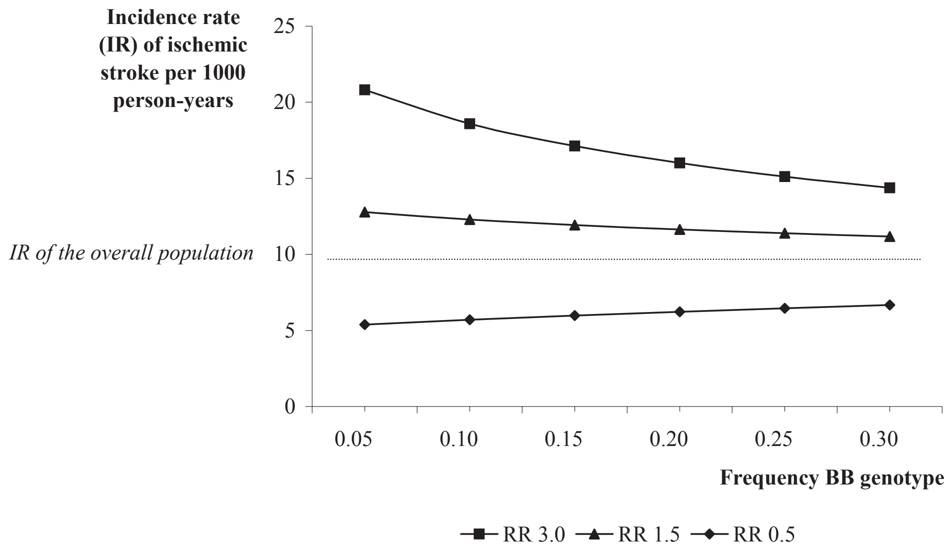

Figure 1. Incidence rate of ischaemic stroke for people aged 55 years or older for different frequencies and effects of the BB genotype (as compared with the AA genotype). The *incidence rate of the overall population* is represented by the dotted line (9.4/1000 person-years).

Figure 1 shows the IR of ischaemic stroke for different frequencies and relative risks of the BB genotype. It demonstrates that the IR among BB carriers levels off with increasing frequency of BB. In other words, the more frequent the index genotype, the less informative genotyping will be, as the risk in persons with the index genotype will approach the overall population risk. It is also shown that when the relative risk approaches one (effect becomes weaker), the IR in BB carriers resembles the overall IR. Considering genotype BB, with a relative risk of 1.2 (which is realistic for a complex disease) and a frequency of 10 %, the increase in IR of ischaemic stroke from the baseline risk, for subjects with the BB genotype would be 13 % (from 9.4 to 10.6 per 1000 py). Considering a protective genotype BB with a relative risk of 0.85, would give an IR of 8.4 per 1000 py (11 % lower than baseline). As is shown in figure 1, increasing the frequency of the BB genotype to 20 or 30 %, does not change the IR substantially for genotypes with these moderate effects.

Figure 2 shows the number needed to screen as a function of the strength of the pharmacogenetic interaction and frequency of the BB genotype. For relatively infrequent genotypes it is shown that a large number of persons need to be screened. This is most striking when the interaction of the BB genotype with treatment is limited (relative risk approaches one). As the risk reducing effect increases or the frequency of BB goes up, the number needed to screen goes down. For instance, to apply pharmacogenetic secondary prevention in patients with a history of ischaemic stroke, a particular genotype with a frequency of 10 % and a relative risk of 0.67, 204 subjects would have to be screened. Following the Hardy-Weinberg equilibrium, 20 (10 %) of them are expected to carry BB, 90 (44 %) AB and 94 (46 %) AA. This means that we need to prescribe genotype specific treatment to 110 persons with the AB or BB genotype

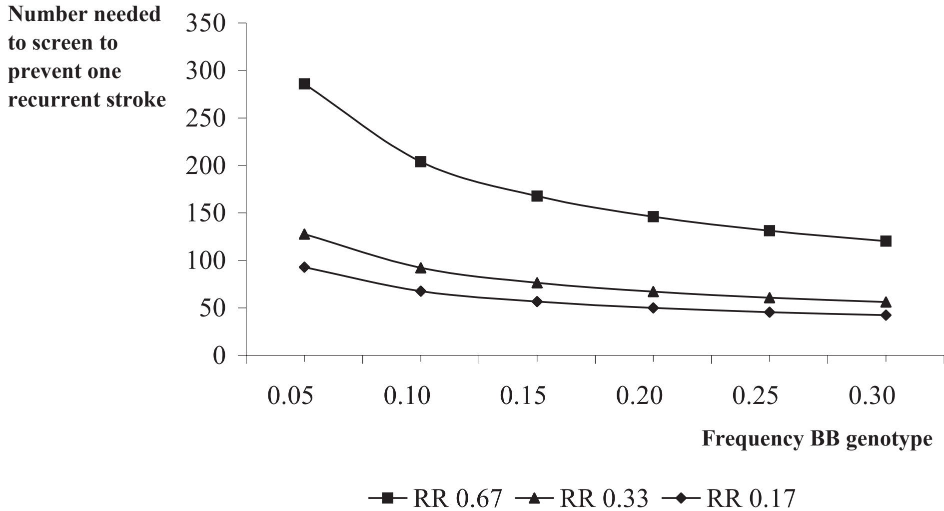

Figure 2. The number needed to screen to prevent one recurrent stroke when genotype specific therapy would be given compared with low dose aspirin, as a function of the frequency and effect of the BB genotype (compared to AA).

for one year, to prevent one recurrent stroke compared with low dose aspirin. With stronger means for secondary prevention (and therefore lower baseline risk of recurrence) the number needed to screen will be higher. For example, when standard treatment reduces the risk of a recurrent stroke with 33 %, 266 persons would have to be screened of which 144 need to be treated with genotype specific treatment, to prevent one recurrent stroke compared with this standard treatment, given the same other assumptions. If we assume a more moderate effect of the BB genotype, a relative risk of 0.90 and the same frequency of 10 %, the NNS would increase to 721. Of this, 389 people would carry the B allele and should therefore be treated with genotype specific treatment. The NNS also depends on the frequency of the BB genotype, even if the effects are only moderate. For instance, if we assume that the frequency of the BB genotype is 20 %, the NNS lowers to 511.

Figure 3 shows how the one-year risk of handicap (Rankin score 3, 4 or 5) or death after a stroke varies with different frequencies and effects of the BB genotype. For a detrimental genotype (i.e. a genetic risk factor for impaired recovery after stroke), the risk of poor outcome increases when the BB genotype becomes less frequent. The risk of handicap or death further increases when the relative risk of the BB genotype increases. We calculated the one-year risk of handicap or death for subjects with the BB genotype assuming a relative risk of 1.2 and a frequency of 10 % and found a CI of 0.57 (12 % increase compared with baseline risk of 0.51). Considering a genotype that improves the outcome after death with a relative risk of 0.85, the CI of death or handicap after a stroke would be 0.45. As for the effect on risk of stroke, increasing the genotype frequency does not alter these results substantially (see figure 3).

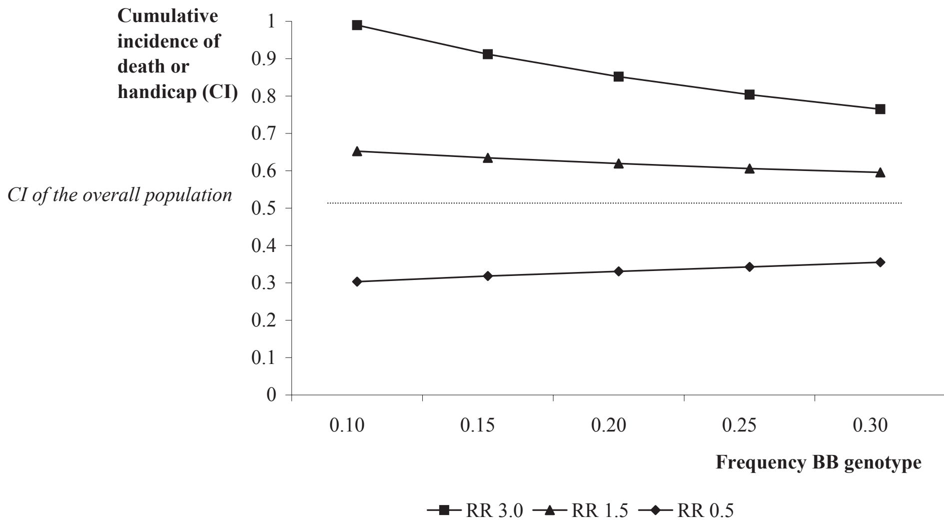

Figure 3. One-year cumulative incidence of handicap (Rankin score 3,4 or 5) or death after a stroke for different frequencies and effects of the BB genotype (as compared to the AA genotype). The *cumulative incidence of the overall population* is represented by the dotted line and is the one-year cumulative incidence of handicap or death for the overall stroke population (0.51).

We showed the theoretically possible impact of genetic testing on ischaemic stroke, an example of a complex disease. We found that genetic testing will be most promising for secondary prevention and to a lesser extent for risk assessment and prognosis. Although common ischaemic stroke was chosen as an example, the conclusions are applicable to other complex diseases and refer to a variety of clinical problems. A limitation of this approach is that it is based on several assumptions.

Given all reported breakthroughs in the lay press, one may expect a major increase in requests for risk assessment from unaffected individuals about their risk of a complex disorder. Since genetic risk factors for common diseases found so far are relatively frequent and have weak effects,[4] it is unlikely that they will substantially alter risk assessment of a complex disorder. Using simulation models, we showed that common genetic risk factors add little in the prediction of future risk of a complex disease for an individual from the general population, when the relative risk is lower than 3. In the example above, considering genotype BB with a relative risk of 1.2 and a frequency of 10 %, the increase in IR of ischaemic stroke for subjects with the BB genotype would only be 13 %. Classical risk factors like hypertension have been shown to increase the IR of stroke with 97 %.[79] However, in subjects with a high risk, testing for such genes may have higher predictive value. Further, single-gene defects are usually strong and rare and can therefore cause an important increase in lifetime risk. Such risk factors usually operate in families or genetically isolated populations. Screening for these risk factors will thus be more efficient in such populations than in the general population.

We did not model diagnosis, as it is unlikely that a diagnosis of a complex disease will ever be made by genotyping alone. Even in case of a major mutation, the use for diagnosis is al-

ways limited since a carrier might not have developed the phenotype *yet*. Still, genetic testing may be important to detect subtypes of a complex disease, when the overall diagnosis (e.g. 'ischaemic stroke') has been made. An example for stroke is CADASIL, which differs in many respects from common stroke syndromes. Given the low proportion of familial stroke patients, it is however not to be expected that large numbers of common stroke patients will once be diagnosed with an underlying single gene defect.

Numerous medication effects are assumed to be genetically regulated.[63,68] Variation in genes may result in differences in the occurrence of beneficial effects and side effects between different users of a particular drug. We evaluated the possible impact of a hypothetical gene on secondary prevention and showed that genotype specific treatment can be feasible. In our calculations, the number needed to screen for pharmacogenetic secondary prevention (204 to 266) is lower than the number needed to screen for breast cancer by mammography (490).[80] Only if we assume really small effects of the BB genotype (relative risk of 0.90), the NNS exceeds the NNS for breast cancer (721). However, we defined a relatively low frequency of the BB genotype of 10 %. If we defined a genotype frequency of 20 %, the NNS would already lower to 511. We assumed that the particular genotype has a low prevalence and limited effects. This assumption may however be incorrect, as an evolutionary decrease in frequency of such a genotype is less likely as compared with an unfavorable genetic risk factor. In other words, we can not exclude that genes-to-be-discovered involved in the effects of treatment, may be more frequent and/or more potent with therefore lower numbers needed to screen.

Genetic testing may also play a role in estimating the prognosis of a patient with a complex disorder. For ischaemic stroke however, few of these genes are known. The detection of new polymorphic genes may be difficult, as expression of these genes is conditional on the occurrence of ischaemia. Animal models are needed, followed by research on variation in corresponding human genes, and investigations on the functional consequences of these variations. In addition to these difficulties, we showed that a genetic prognostic factor needs to be rare and strong in order to be useful in the assessment of prognosis. As calculated previously, for subjects with genotype BB (assuming a relative risk of 1.2 and a frequency of 10 %), there was increase in risk of handicap or death after a stroke of 12 %. This is less than was found know prognostic factors like severity of stroke, stroke subtype, heart failure and history of diabetes.[81,82]

We used a multiplicative model. We chose this model, since it is customary when modeling complex diseases.[73] We considered the impact of one gene, as most candidate gene studies focus on the effect of single genes. Nearly all genes found so far have only small effects on complex diseases like stroke. This model gives insight in the contribution of single genes using a realistic model for complex diseases. However, it has been debated if for complex diseases, genetic screenings should involve multiple risk-associated markers.[83,84] Basically, the same considerations hold for multiple genes, with a certain combined frequency and effect. It is to be expected that a combination of genes will have a stronger effect, but the frequency

of this combination of genes, will be lower compared with a single gene. Janssens et al.[85] have shown that the discriminative ability of the multiplex genetic test increased by the addition of more genes, however, they found that the performance for use as a screening instrument was rather inefficient. Yang et al.[85] responded that the use of multiple genes would identify only a few high-risk subjects per million. This will therefore only be useful for predicting disease in individual patients. In our model, using multiple genes for screening will thus be useful for risk assessment and prognosis (for individual patients), but not for secondary prevention.

As for using a multiple-marker model, taking into consideration gene-gene and gene-environment interactions, will improve the estimation of the effect of genetic screening on complex diseases. However, there is not much known about frequencies and effects of these interactions. We expect however, that the effect of gene-gene or gene-environment interactions will be stronger than using single genes, but that the frequency of these combinations will be lower. As described above, this will be beneficial for risk assessment and prognosis, however, it will probably not be very useful for secondary prevention.[85]

Each individual differs from another genetically, even monozygotic twins. Yet, when we enter the clinic, we are treated almost the same. In the future, we will be able to use the genetic profile of a patient to provide better individually tailored clinical care. Secondary prevention and therapy seem to be the most fruitful areas. It is theoretically unlikely that genetic findings will substantially alter the assessment of risk or prognosis of a complex disorder, except for Mendelian types of disease.

We have focussed the above analyses on single genes and found that the most promising area for application of genetic testing would be in secondary prevention. As genes involved in stroke, as in other comlex diseases, are often common with small effects, risk assessment using one gene is of limited value. Genetic profiling, testing at multiple loci, might be useful in the future. The usefulness of genetic profiling has been proposed to be evaluated by the area under the receiver-operating characteristic curve (AUC).[86] The AUC can be calculated from the heritability and the prevalence (lifetime risk) of a disease. An AUC ~ 0.80 has been proposed to be useful for identifying individuals at risk of disease (screening). To diagnose a disease before the onset of symptoms, a test with an AUC > 0.99 would be required. We calculated the AUC for stroke, assuming a heritability of 20% to 40%. The AUC varied from 0.80 to 0.90 depending on the lifetime risk (15% to 20%[87]). From this, we can conclude that genetic screening for stroke may be feasible, once we have identified all genes, but diagnosing stroke using genetic testing will not be. One problem may be that genes with very small effects may be difficult to identify, even using modern technologies.

Stroke is a major cause of morbidity and mortality and with increasing life expectancy, the incidence of stroke will tend to increase. Preventing stroke will thus have a major impact in public health. Unravelling the genetics of stroke can play a crucial role by increasing our

knowledge about the aetiology, prognosis and treatment of stroke. With this thesis I have attempted to make a contribution to this knowledge.

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Chapter 7

Summary / Samenvatting

Summary

Stroke is a leading cause of death and disability in the Western world. It is a complex disease resulting from environmental factors and genetic factors, as well as gene-gene and gene-environment interactions. Many studies have attempted to unravel the genetic aetiology of stroke, but results have been inconsistent. Most have used the candidate gene approach, but genome-wide linkage analyses have also been performed. Recently, results of genome-wide association studies have been reported, however, this approach has not yet been used to study the genetics of stroke. Stroke is a heterogeneous disease, which can be subtyped into ischaemic stroke (80%) and haemorrhagic stroke (20%), with different underlying pathways. In order to reduce the complexity of stroke, intermediate phenotypes have been studied, such as hypertension, carotid atherosclerosis and cerebral white matter lesions. Another approach has been to study stroke in a genetically isolated population.

In Chapter 1, an introduction is given on the genetic epidemiology of stroke. An overview of genes associated with stroke so far, is presented in this chapter.

Chapter 2 describes the results of two heritability studies, performed in the Erasmus Rucphen Family study (ERF). This is a family-based study conducted in a genetically isolated population. In Chapter 2.1 we present the heritability estimates of carotid-femoral pulse wave velocity (PWV), carotid intima-media thickness (IMT) and carotid plaques. Heritability estimates were 0.36 for PWV, 0.41 for IMT and 0.28 for plaques. In Chapter 2.2 we show that heritability estimates range from 0.24 to 0.37 for four blood pressure traits. In this study we also found that polymorphisms of the genes *ADD1*, *AGT*, *AT1R* and *GNB3* explained less than 1% of the blood pressure variance. From these studies it becomes clear that genetic factors play an important role in the variation of intermediate phenotypes of stroke.

In Chapter 3, we present the findings of two candidate gene studies performed in the ERF population, from which we selected 91 ischaemic stroke patients. The two candidate genes were the *PDE4D* gene and the *ALOX5AP* gene, both identified in genomic screens on stroke and myocardial infarction (MI) patients in another genetically isolated population, the Icelandic population. Chapter 3.1 first describes that we found familial clustering of ischaemic stroke. Next, we report that the C allele of SNP45 and the T allele of SNP39, significantly increased the risk of small-vessel stroke, in inbred individuals. Results presented in Chapter 3.2 show that allele A of SNP SG13S32, was associated with increased blood pressure, body mass index and increased risk of ischaemic stroke. Our studies thus confirm the results of the genome-wide scans performed in the Icelandic population.

Chapter 4 shows the results of two candidate gene studies, conducted in the Rotterdam study, which is a large population-based cohort study. First, we studied a promoter polymorphism in the *IGF-I* gene in relation to stroke and survival after stroke. Chapter 4.1 shows that non carriers of the 192 bp allele had an increased risk of death after stroke, compared with carriers of this allele. No association with stroke risk was observed. In Chapter 4.2, we present our findings on the Gly460Trp polymorphism of the *ADD1* gene. We found that carriers of the 460Trp allele had increased IMT levels, as well as an increased risk of stroke and MI. Also, we found a significant interaction between this polymorphism and hypertension, for any and ischaemic stroke. Last, we studied the M235T polymorphism of *AGT* and the C573T polymorphism of *AT1R*. Both genes are part of the Renin-Angiotensin System. In chapter 4.3, we show that the 235T allele increased blood pressure, cerebral white matter lesions and the prevalence of carotid plaques. Also, we found an interaction between the polymorphisms of the *AGT* and the *AT1R* genes, with respect to white matter lesions. This chapter underlines the importance of gene-environment and gene-gene interactions when studying the genetics of stroke. Also, it shows that genes can found to be associated with intermediate factors of stroke, while an association with stroke itself, is not always observed.

In Chapter 5.1, we present our findings of a genome-wide association analysis on blood pressure. We found seven SNPs associated with blood pressure with a nominal p-value ≤ 0.00001, all located intergenic. Previous studies have reported linkage of hypertension and blood pressure to the chromosomes in which these SNPs are located. Also, we found seven intragenic SNPs that were associated with blood pressure with a nominal p-value ≤ 0.0001, one of which was in the gene *NR3C2*. This gene was earlier associated with early-onset hypertension. None of our findings reached genome-wide empirical significance.

Finally, in Chapter 6, we provide a general discussion of the results of our studies. In Chapter 6.2 we discuss the clinical impact of genetic testing on stroke. From our simulation studies it becomes clear that the most promising area would be in secondary prevention of stroke. Also, we show that genetic screening for stroke may be feasible, but that diagnosing stroke using genetic testing, will not be feasible.

Samenvatting

Een beroerte is de belangrijkste oorzaak van sterfte en invaliditeit in de Westerse wereld. Het is een 'complexe ziekte', wat inhoudt dat de ziekte het gevolg is van genen, omgevingsfactoren en gen-gen en gen-omgevingsfactor interacties. Veel studies hebben geprobeerd de onderliggende genetische oorzaak van een beroerte te ontrafelen, maar tot dusver zijn de resultaten tegenstrijdig. De meeste studies hebben kandidaat-genen bestudeerd, maar er zijn ook linkage studies gedaan die het hele genoom hebben onderzocht. Een recente ontwikkeling zijn associatie studies waarbij het hele genoom wordt gescreend. Voor een beroerte is dit echter nog niet gedaan. Een beroerte is een erg heterogene ziekte en kan worden onderverdeeld in ischemische (80%) en hemorrhagische (20%) beroerte, met een verschillende pathogenese. Het bestuderen van intermediaire phenotypes, zoals hypertensie, atherosclerose en witte stof afwijkingen in de hersenen, kan een manier zijn om de complexiteit van een beroerte te verminderen. Een andere mogelijkheid is het bestuderen van een beroerte in een genetisch geïsoleerde populatie.

In hoofdstuk 1 wordt de genetische epidemiologie van een beroerte beschreven. Ook wordt in dit hoofdstuk een overzicht gegeven van de genen die tot nu toe zijn geassocieerd met een beroerte.

Hoofdstuk 2 beschrijft twee erfelijkheidsstudies, uitgevoerd binnen het Erasmus Rucphen Familie onderzoek (ERF), een familie onderzoek in een genetisch geïsoleerde populatie in Nederland. In hoofdstuk 2.1 worden de resultaten gepresenteerd van de erfelijkheidsstudies naar pulse-wave velocity (PWV), intima-media dikte van de halsslagader (IMT) en plaques in de halsslagader. Voor PWV was de erfelijkheid 0.36, voor IMT 0.41 en voor plaques 0.28. In hoofdstuk 2.2 laten we zien dat de erfelijkheid van bloeddruk varieert van 0.24 tot 0.37. Binnen dit onderzoek vonden we verder dat variaties binnen de *ADD1*, *AGT*, *AT1R* en *GNB3* genen, minder dan 1% van de variatie in bloeddruk verklaarden. Uit deze studies wordt duidelijk dat genetische factoren een belangrijke rol spelen bij de variaties binnen de intermediaire phenotypes van een beroerte.

In hoofdstuk 3 presenteren we de bevindingen van twee kandidaat-gen studies, die werden uitgevoerd binnen de ERF populatie, waarbinnen we 91 patiënten verzamelden die een ischemische beroerte hadden doorgemaakt. De twee kandidaat genen waren het *PDE4D* en het *ALOX5AP* gen, welke eerder werden geïdentificeerd in een andere genetisch geïsoleerde populatie, IJsland. In hoofdstuk 3.1 beschrijven we eerst dat we clustering vonden van een ischemische beroerte binnen families. Verder wordt beschreven dat binnen bloedverwante deelnemers, het C allel van SNP45 en het T allel van SNP39, het risico verhoogden op het

krijgen van een beroerte met oorprong in de kleine vaten. In hoofdstuk 3.2 vonden we dat zowel bloeddruk als body mass index was verhoogd in dragers van het A allel van SNP SG13S32. Ook verhoogde dit allel het risico op het krijgen van een ischemische beroerte. Deze studies bevestigen de resultaten welke eerder werden gevonden in de IJslandse populatie.

In hoofdstuk 4 laten we de resultaten zien van twee kandidaat-gen studies, die werden uitgevoerd in de Rotterdam studie, een grootschalige populatie studie. Binnen deze studie onderzochten we de relatie tussen een variatie binnen het *IGF-I* gen en een beroerte, als ook de overleving na een beroerte. In hoofdstuk 4.1 vonden we dat deelnemers die geen drager waren van het 192 bp allel, een hogere kans hadden om te overlijden na een beroerte, dan dragers van dit allel. We vonden geen relatie met het risico op het krijgen van een beroerte. In hoofdstuk 4.2 worden de resultaten gepresenteerd van de Gly460Trp variatie in het *ADD1* gen. Wij vonden dat dragers van het 460Trp allel een toegenomen IMT hadden en een verhoogd risico op het krijgen van een beroerte en een hartinfarct. Ook vonden wij dat er een interactie was tussen deze variatie en hypertensie met betrekking op het krijgen van een (ischemische) beroerte. Tenslotte bestudeerden wij de M235T variatie in het *AGT* gen en de C573T variatie in het *AT1R* gen. Beide genen maken deel uit van het Renine-Angiotensine System. In hoofdstuk 4.3 beschrijven we dat dragers van het 235T allel een verhoogde bloeddruk hebben, meer cerebrale witte stof afwijkingen en vaker plaques in de halsslagader. Ook vonden we een interactie tussen de variaties in de *AGT* en *AT1R* genen, met betrekking tot witte stof afwijkingen. Dit hoofdstuk onderschrijft het belang van gen-omgeving en gen-gen interacties bij het onderzoek naar de genetische oorzaak van een beroerte. Ook laat dit hoofdstuk zien dat genen soms niet geassocieerd zijn met het krijgen van een beroerte, maar wel met intermediaire phenotypes.

Hoofdstuk 5 laat de resultaten zien van een associatie studie waarbij het hele genoom werd gescreend. Als uitkomst werd er in dit hoofdstuk naar bloeddruk gekeken. Van zeven SNPs vonden we dat deze geassocieerd waren met bloeddruk (nominale p-waarde $\leq$ 0.00001, allen gelokaliseerd tussen 2 genen in). Eerdere studies lieten linkage zien van hypertensie en bloeddruk aan chromosomen waarbinnen deze SNPs waren gelokaliseerd. Verder vonden wij zeven SNPs die binnen een gen waren gelokaliseerd en die met bloeddruk waren geassocieerd (nominale p-waarde $\leq$ 0.0001). Eén van deze SNPs was gelokaliseerd in het *NR3C2* gen, dat eerder geassocieerd werd met early-onset hypertensie. Geen van onze bevindingen waren significant na correctie voor multiple testing.

Tenslotte bediscussiëren we in hoofdstuk 6 de resultaten van al onze studies. In hoofdstuk 6.2 bespreken we de impact van het testen van genen voor een beroerte in de kliniek. Uit onze simulatie studies komt naar voren dat secundaire preventie het belangrijkste gebied zal zijn waarbinnen genetische testen voor een beroerte een rol zouden kunnen spelen. Ook laten we zien dat het screenen van genen geassocieerd met een beroerte haalbaar zou kunnen zijn, echter, het diagnostiseren van een beroerte met behulp van genetische testen, zal niet haalbaar zijn.

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Het lijkt nog niet zo lang geleden dat ik als geneeskundestudent voor het eerst kennis maakte met de afdeling Epidemiologie & Biostatistiek. Nooit had ik gedacht dat ik 6 jaar later het dankwoord van mijn proefschrift zou schrijven. Toch is het nu zover. De tijd is aangebroken om mijn promotie af te ronden en iedereen te bedanken die de afgelopen jaren heeft bijgedragen aan het tot het tot stand komen ervan, ieder op zijn eigen manier.

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Wat is het verband tussen een beroerte en de ziekte van Creutzfeldt-Jakob? Zelfs ik kan die vraag niet beantwoorden. Toch heb ik uren door Nederland gereden (naar plaatsen waarvan ik niet eens wist dat ze bestonden) om patiënten met deze ziekte te bezoeken. Beste Mark S, wat ben ik blij dat ik het CJD project met jou kon delen. En je weet het hè, als we ooit gaan dementeren als we 65+ zijn…. Ook wil ik je bedanken voor alle gezelligheid, en al het eten dat je me hebt toegestopt als ik weer met een rammelende maag tegenover je zat te werken. Dat ik sinds jou komst elke dag naar de koningin heb moeten kijken, heb ik je hierdoor wel kunnen vergeven. Mark S vulde de grote leegte op die Anna achter liet toen zij de plek tegenover mij verruilde voor een baan in het ziekenhuis. Lieve Anna, wat heb ik je gemist die laatste jaren! Uren hebben we besteed aan het bespreken van onze relatie perikelen. Gelukkig hebben we ook veel kunnen lachen. Bedankt dat je er altijd voor me was. Ik heb meerdere kamergenoten zien komen en gaan. Kristel, ook jij bent een grote steun voor me geweest. Ik heb genoten van de muziek en nog veel gebruik gemaakt van de speakertjes die je voor me achterliet. Dominique, ik hoop dat kleine Bâtise het goed maakt. Annelous, geniet van de nieuwe liefde en zorg goed voor mijn oude werkplekje, het is een van de beste plekken van de afdeling. Mark H, ook jou wil ik bedanken voor de fijne samenwerking. Hoe bevalt het leven tussen de kruikenzeikers? Leonieke, met jou heb ik nooit een kamer gedeeld. Desalniettemin wil ik je toch bedanken voor de gezelligheid. Je was altijd in voor een praatje.

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Woorden
maken heel
Even een andere ruimte
om tot jezelf
te komen

Deze woorden
helen
door te bedekken
wat ooit
kapot is gemaakt

MJ, 12 februari 2007

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van Rijn MJE, van Duijn CM, Slooter AJC. The Impact of genetic testing on complex diseases. Common ischaemic stroke as an example. *Eur J Epidemiol*, 2005;20:383-8.

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About the author

Marie Josee Elisabeth van Rijn was born on December 12, 1978 in Harderwijk. In 1996, she graduated from secondary school at the Eckart college in Eindhoven. In 1997, she backpacked through Australia, New Zealand and Indonesia for one year. In 1998, she started her medical studies at the Erasmus University in Rotterdam. During her studies, she obtained a Master of Science degree in Genetic Epidemiology at the Netherlands Institute of Health Siences, at the Erasmus University. As part of this study program, she studied for four weeks at the Harvard School of Public Health, in Boston, USA. During her medical studies, she worked at the Genetic Epidemiology Unit of the Department of Epidemiology & Biostatistics, where she collected data on stroke patients as part of the research program "Genetic Research in Isolated Populations". After obtaining her bachelor degree of medicine in 2002, she prosponed her clinical training (internships) to continue her research project as a PhD student, working on the studies described in this thesis. In 2005 she started her internships, while she continued working on her thesis. In April 2007 she will obtain her medical degree (cum laude).