Genetic and Hemostatic Risk Factors for Stroke

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Contents

Chapter 1

Introduction

Introduction

Cardiovascular disorders are the main causes of death worldwide, with stroke accounting for 9-10% of all deaths[1,2]. Moreover, stroke is the most frequent cause of disability in the western world[3]. In the Netherlands alone, over 39,000 persons are admitted to hospitals with stroke each year[4].
There are two main types of stroke; ischemic stroke (occurring in about 80% of cases) and hemorrhagic stroke (20%). Hemorrhagic strokes can be further subdivided into intracerebral hemorrhage (15%) and subarachnoid hemorrhage (5%).
In ischemic stroke, an artery or arteriole is blocked by thrombosis or an embolus, impeding blood flow to a part of the brain and causing tissue damage in the supplied territory. Intracerebral hemorrhage, on the other hand, results from rupture of a blood vessel, leading to the formation of a hematoma in the brain parenchyma. Although ischemic stroke and intracerebral hemorrhage are in a way opposite, they share several risk factors such as coagulation abnormalities, atherosclerosis and cardiac disorders.

It is known that individuals with a positive family history for cardiovascular diseases have a higher risk of stroke. This may be explained by shared environmental risk factors and socioeconomic status, similar dietary habits (e.g. high cholesterol intake), but most obviously by common genetic material. The observation of increased risk of cerebrovascular disease in first degree relatives of stroke patients has fostered the notion of a genetic component of stroke. Until a few years ago, knowledge of the genetic background of stroke risk was limited, and mostly derived from small, family-based linkage studies[5]. Since the beginning of this millennium, however, advances in genomics have skyrocketed, paving the way for very large genetic studies[6]. New genetic risk factors are discovered continuously, rapidly increasing our knowledge on the genetics of complex diseases. Also in stroke, these studies are being undertaken to gain more insight in the role of genetic factors in stroke etiology.

Genes are the blueprint of life, but their actual effects are mediated by their products: proteins. Therefore, it seems only logical to study not only genes but also gene products, as these are truly affecting the risk of stroke. In ischemic stroke, an important final common pathway is clot formation.
Blood contains many proteins dedicated to maintaining hemostasis, both procoagulant and anticoagulant. All proteins act in a strictly coordinated cascade, which is self-regulating. The final step in the coagulation cascade is the conversion of fibrinogen to fibrin, which forms the actual clot. Many studies have shown an association between levels of fibrinogen and risk of stroke[7,8]. In the last several years, interest has shifted to an isoform of fibrinogen, fibrinogen γ', which seems to be associated with cardiovascular disease. Its properties are still unclear, however, just like its role in stroke[9-13].

The aim of this thesis is to investigate new risk factors for stroke and transient ischemic attacks, with a focus on genetic and hemostatic factors. To this end, we not only used stroke as main outcome event, but also studied intermediate endpoints such as atherosclerosis and levels of hemostatic proteins. All studies described in this thesis are conducted in the Erasmus Stroke Study, a large hospital-based cohort

of patients with stroke or transient ischemic attack, set up to find new risk factors for cerebrovascular disorders.

In chapter 2, I discuss unbiased, large-scale genetic studies, aimed at uncovering new genetic risk factors for stroke; first the findings from a large gene discovery study in search of new genetic risk factors for ischemic stroke (chapter 2.1), and next the association between phenotypes and risk alleles found in the previous study (chapter 2.2). Next, I describe the results of our multicenter genomewide association study in patients with intracerebral hemorrhage (chapter 2.3). Chapter 3 focuses on the genetic influence of specific genes on hemostatic and inflammatory risk factors. Over the past years several very large genetic studies have uncovered the genetic influence on metabolic traits such as lipids and glucose levels[14-19]. However, the influence of these genes on cerebrovascular disease is still unclear. Therefore, I studied associations between genes for metabolic traits, such as lipids and glucose metabolism, and atherosclerosis in patients with cerebral ischemia (chapter 3.1). C-reactive protein is an acute phase protein and thereby a marker for tissue damage. However, it is still undetermined whether it also has a causal role in increasing brain damage after stroke by enhancing the inflammatory reaction in the infarct area[20]. We therefore studied the genetic influence on levels of C-reactive protein in patients with ischemic stroke (chapter 3.2). The next chapter addresses fibrinogen γ', specifically the influence of the fibrinogen γ gene on levels of fibrinogen γ' in stroke patients (chapter 3.3). Next, we explore the level of fibrinogen γ' and its role in ischemic stroke (chapter 4.1) and intracerebral hemorrhage (chapter 4.2). Recent studies have suggested a role for von Willebrand factor in ischemic stroke[21]. However, conflicting results were found for associations between genetic variations in the von Willebrand factor gene, which influence levels, and risk of ischemic stroke[22,23]. Therefore, we studied the extent of atherosclerosis with regard to levels of von Willebrand antigen (chapter 4.3). In the final chapter, I summarize our findings and try to put them in a broader perspective (chapter 5). Also, I discuss methodological issues of our studies and give recommendations for future research.

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Chapter 2

Genomewide association studies in stroke

Chapter 2.1

Genomewide Association Studies of Stroke

Abstract

Background

The genes underlying the risk of stroke in the general population remain undetermined.

Methods

We carried out an analysis of genomewide association data generated from four large cohorts composing the Cohorts for Heart and Aging Research in Genomic Epidemiology consortium, including 19,602 white persons (mean [+/-SD] age, 63+/-8 years) in whom 1544 incident strokes (1164 ischemic strokes) developed over an average follow-up of 11 years. We tested the markers most strongly associated with stroke in a replication cohort of 2430 black persons with 215 incident strokes (191 ischemic strokes), another cohort of 574 black persons with 85 incident strokes (68 ischemic strokes), and 652 Dutch persons with ischemic stroke and 3613 unaffected persons.

Results

Two intergenic single-nucleotide polymorphisms on chromosome 12p13 and within 11 kb of the gene NINJ2 were associated with stroke ($P<5x10(-8)$). NINJ2 encodes an adhesion molecule expressed in glia and shows increased expression after nerve injury. Direct genotyping showed that rs12425791 was associated with an increased risk of total (i.e., all types) and ischemic stroke, with hazard ratios of 1.30 (95% confidence interval [CI], 1.19 to 1.42) and 1.33 (95% CI, 1.21 to 1.47), respectively, yielding population attributable risks of 11% and 12% in the discovery cohorts. Corresponding hazard ratios were 1.35 (95% CI, 1.01 to 1.79; P=0.04) and 1.42 (95% CI, 1.06 to 1.91; P=0.02) in the large cohort of black persons and 1.17 (95% CI, 1.01 to 1.37; P=0.03) and 1.19 (95% CI, 1.01 to 1.41; P=0.04) in the Dutch sample; the results of an underpowered analysis of the smaller black cohort were nonsignificant.

Conclusions

A genetic locus on chromosome 12p13 is associated with an increased risk of stroke.

Introduction

Stroke is a major disabling disease and neurologic cause of death[1]. Family studies have shown that there is a genetic component in the risk of stroke[2-4], but the genes underlying this risk remain unknown, especially in the general population. Candidate gene studies and other classical approached have had inconsistent results so far[5].

Common variants in complex diseases, such as diabetes[6] and coronary heart disease[7,8], can be discovered using genomewide association studies. There are two previous genomewide association studies of stroke, but they used a case-control design which make them vulnerable to survival and selection bias, which is less in prospective cohort studies[9,10]. In this study we combined data from four prospective cohort studies: the Atherosclerosis Risk in Communities (ARIC) cohort[11], the Cardiovascular Health Study cohort[12], the Framingham Heart Study cohort[13,14], and the Rotterdam Study cohort[15]. All consist of predominantly white participant. The cohorts are part of the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE)[16], together generating a discovery sample of 19,602 participants. Additionaly we included three replication samples: a prospective cohort of 2430 black participants from the ARIC study, a cohort of 574 black participants in the Cardiovascular Health Study, and a case-control sample of 4265 Caucasian Dutch persons.

Methods

Study design and samples

The ARIC study is a prospective population-based study of four US communities, for the purpose of this study only self-reported whites were included. Only individuals who were free of stroke or TIA at baseline were included. After quality control, including sex mismatches, identity by state and incident subarachnoid hemorrhage, 7686 persons were included in the analyses. Genotyping was performed using the Affymetrix Genome-Wide Human SNP Array 6.0.

The CHS is a population-based study including participants from four centers in the US. Only self-identified blacks were excluded from the discovery cohort. Genotyping was performed using the Illumina 370CNV Duo BeadChip system, and after quality control 2,022 persons free from stroke at baseline were included.

The FHS is a community-based cohort study that comprises three generations of inhabitants of Framingham (US); since the third generation is still young only the first and second generations are used in this study, almost exclusively comprised of whites. Genotyping was performed using the Affymetrix GeneChip Human Mapping 500K Array Set and 50K Human Gene Focused Panel, after quality control 4,131 participants remained for this study.

The Rotterdam Study is a population-based study from the Netherlands, almost entirely made up of Caucasians. All participants stroke-free at baseline were included in the discovery cohort. Genotyping was performed using the Illumina Infinium II HumanHap550chip v3.0 array; after quality control 5,763 participants were included for this study.

Each study had an institutional review board that approved the separate studies. All participants gave written informed consent. Stroke-free participants were followed until occurrence of incident stroke. Baseline characteristics of all participants are shown in table 1.

Stroke definition and classification

Stroke was defined as a focal neurological deficit of presumed vascular origin, lasting at least 24 hours or until death if this occurred within 24 hours. Based on clinical and imaging criteria, strokes were classified as ischemic, hemorrhagic or unspecified. Ischemic strokes were subclassified into atherothrombotic and cardioembolic ischemic stroke. All strokes except subarachnoid hemorrhages were included.

Statistical analysis

Individual studies

We used Cox-proportional hazards models to evaluate time until first stroke. Patients were excluded at death or at the last time they were known to be stroke-free. For the ischemic stroke analyses, persons were also excluded if they had another type if stroke. Every study fit an additive genetic model, association the gene dose (0 to 2) to the outcome (total or ischemic stroke). Primary analyses were age- and sex adjusted. The ARIC study and CHS additionally adjusted for study site, FHS also adjusted for familial structure. Also, we adjusted for hypertension, diabetes, current smoking, and atrial fibrillation. We also determined the association between two SNPs reaching genomewide significance and an atherothrombotic stroke subtype. There were no indications for population stratification in any of the populations.

Combined analysis of studies

We performed a meta-analysis from the separate study results using invariance-weighted analysis. The analysis was restricted to autosomal SNPs that were present in all studies. A priori a significance threshold of $4x10^{-8}$ was decided, which corresponds to a p-value of 0.05 with Bonferroni correction for 1 million independent tests. SNPs with p-values $<5x10^{-8}$ but $>1x10^{-5}$ were considered highly suggestive associations, SNPs with p values $>1x10^{-5}$ and $<1x10^{-4}$ were considered moderately suggestive for association.

Replication

There were three replication cohorts. The first comprised a group of black participants from the ARIC study, who were stroke-free at baseline and were followed until occurrence of stroke (N=2430). The second cohort included 574 blacks from CHS. The third cohort was a Dutch Caucasian case-control sample of 652 stroke cases and age-matched controls. In all replication cohorts the two top SNPs, rs11833579 and rs12425791 were genotyped.

Results

During a mean follow-up of 11 years, 1544 incident strokes were observed (1164 ischemic strokes) among 19,602 persons (table 1). In figures 1A en 1B results of the genomewide association study for total and ischemic stroke are shown. For loci with p-values <1x10-5, considered highly suggestive, hazard ratios and population attributable risks for the minor allele are shown in table 2.

Table 1. Characteristics of the study population in the discovery cohorts for analysis of incident total stroke and incident ischemic stroke

Variable	ARIC (N=7686)	CHS (N=2022)	FHS (N=4131)	Rotterdam (N=5763)
Female sex (%)	53	55	55	59
Mean follow-up (yr)	15	11	6	10
Mean age (yr)				
At DNA draw	54±6	73±6	66±12	69±9
At incident stroke	66±7	81±6	80±10	80±8
Strokes (no.)				
Prevalent	12	0	135	170
Incident total	312	459	156	617
Incident ischemic	277	389	131	367
Incident atherothrombotic	243	264	82	296
Cardiovascular risk factors at baseline				
Systolic blood pressure (mmHg)	118±17	138±22	131±20	139±22
Diastolic blood pressure (mmHg)	72±10	71±11	74±10	74±12
Hypertension (%)	27	61	52	61
Diabetes mellitus (%)	6	14	12	10
Current smoker (%)	25	11	14	23
Prevalent cardiovascular disease other than stroke (%)	5	0	16	10

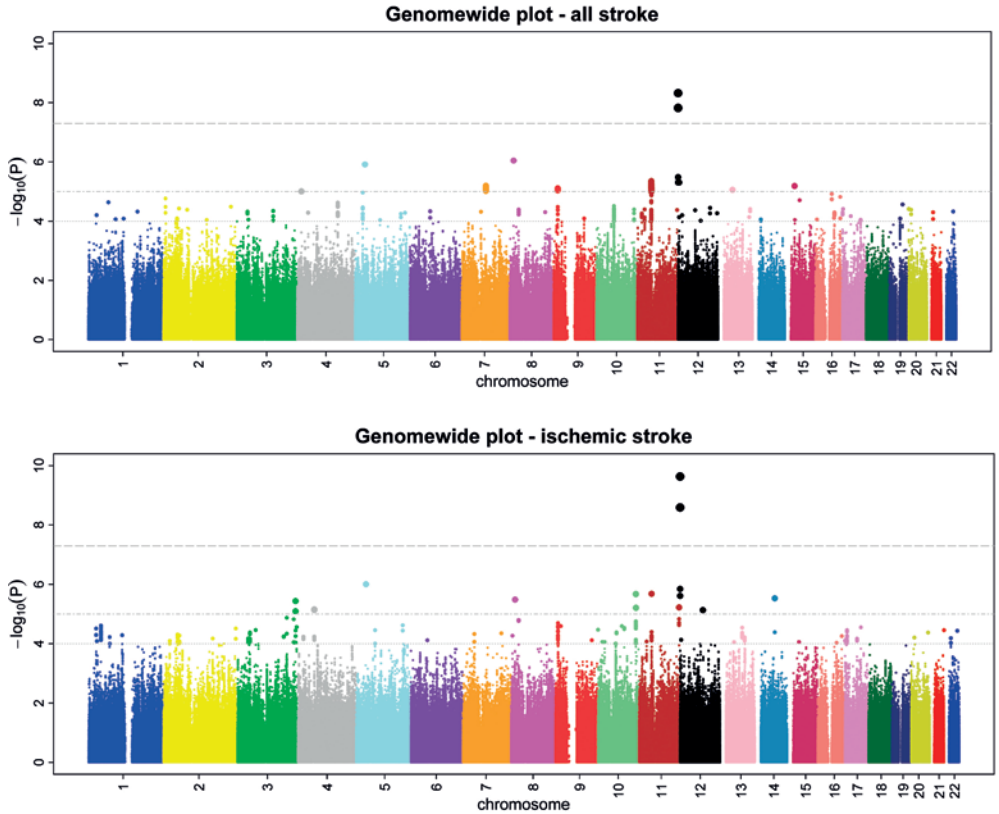
Plus-minus values are means ± SD.

Two SNPs (rs11833579 and rs12425791), located on chromosome 12p13, passed our predefined significance threshold for total and ischemic stroke. Despite a smaller number than the total number of strokes, the hazard ratios were larger and the p-values smaller in ischemic stroke. Results from our analyses suggested that the hazard ratio[HR] for total stroke was increased by 1.31-1.32 (95% confidence interval [CI] 1.19-1.44), for ischemic stroke by 1.39-1.41 (95% CI 1.27-1.56). The population attributable risks were 11-13% for total stroke and 14-17% for ischemic stroke. We found no associations between either of the SNPs and nonischemic stroke (HR 1.13, 95%CI 0.94-1.36, p=0.20 for each SNP). For both SNPs, risk estimates were similar across all four studies.

We observed stronger associations between the two top SNPs and atherothrombotic ischemic stroke, than with all ischemic strokes together. For rs11833579, hazard ratios for total stroke, ischemic stroke and atherothrombotic ischemic stroke were 1.26 (95% CI 1.16-1.37), 1.33 (95% CI 1.21-1.47) and 1.35 (95% CI

1.21-1.50), respectively. For rs12425791 the respective hazard ratios were 1.30 (95% CI 1.19-1.42), 1.33 (95% CI 1.21-1.47) and 1.37 (95% CI 1.23-1.54) (figure 2).

Figure 1. Results of tests for association between stroke and each SNP in the genomewide association study.

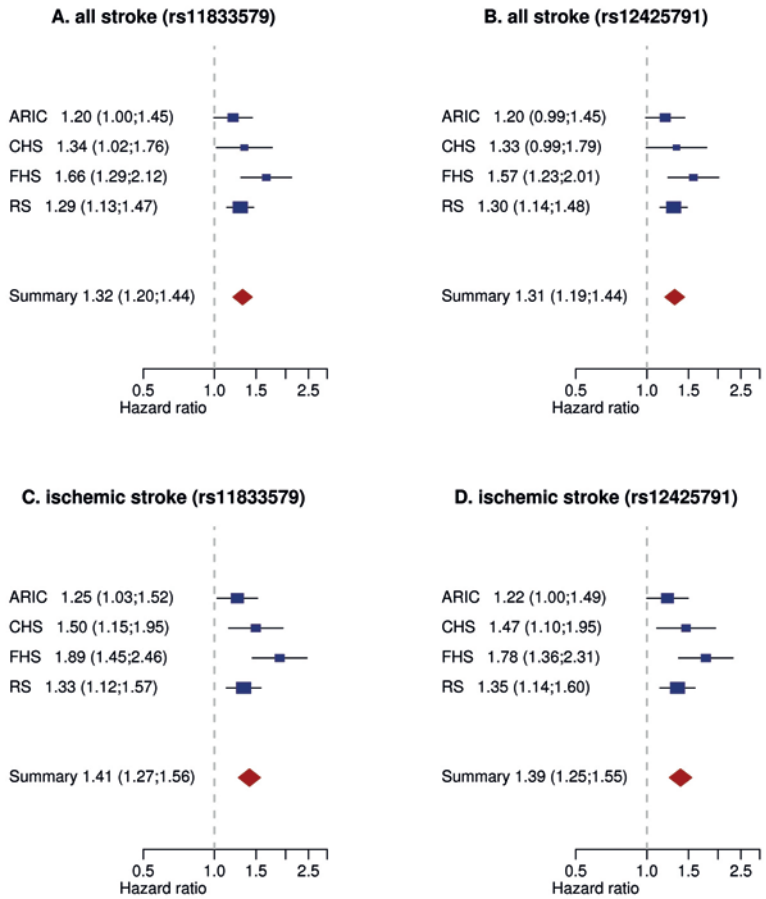


P values are shown in Manhattan plots, relative to their genomic position, for all stroke (fig 1A) and for ischemic stroke (fig 1B). For each chromosome, SNPs are plotted from left to right starting at the p-terminal end. The dashed line indicates the chosen threshold for genomewide significance, $P=5x10^{-8}$; the middle line indicates the threshold for $p=10^{-5}$; the dotted line indicates the threshold for $p=10^{-4}$.

We observed stronger associations between the two top SNPs and atherothrombotic ischemic stroke, than with all ischemic strokes together. For rs11833579, hazard ratios for total stroke, ischemic stroke and atherothrombotic ischemic stroke were 1.26 (95% CI 1.16-1.37), 1.33 (95% CI 1.21-1.47) and 1.35 (95% CI 1.21-1.50), respectively. For rs12425791 the respective hazard ratios were 1.30 (95% CI 1.19-1.42), 1.33 (95% CI 1.21-1.47) and 1.37 (95% CI 1.23-1.54) (figure 2).

Both SNPs are in proximity to NINJ2, coding for the ninjurin2 protein. Based on HapMap CEU data, both SNPs are in linkage disequilibrium with one another (r^2=0.73), and with SNPs in the 5' untranslated region of the NINJ2 gene. Figure 3 shows SNPs and genes within a 200 kb range of the two SNPs, alongside recombination rates. Two nearby SNPs also showed a strong, though nonsignificant, association with total and ischemic stroke.

Figure 2. Forest plots showing associations between SNPs and stroke



Associations between rs11833579 and rs12425791 and total stroke and ischemic stroke, based on directly genotyped data. Individual cohort studies (blue boxes) are plotted against individual hazard ratios. Red diamonds indicate the overall hazard ratios. The size of the blue box is inversely proportional to the variance. Horizontal lines indicate 95% confidence intervals. The dashed vertical line shows the value for no effect (hazard ratio = 1.0).

Table 2. Most significant associations between SNPs and stroke phenotype

SNP	SNP function	Minor Allele	Chromosome: position	Hazard Ratio (95% CI)	P value	PAR	Closest gene*		Second closest gene*		Additional SNPs at $P<10^{-5}$†
							Name	Distance	Name	Distance	
Total stroke											
rs11833579	Upstream	A	12:645460	1.32 (1.20-1.44)	$4.8x10^{-9}$	0.13	*NINJ2*	2.4	*WNK1*	87.0	3
rs12425791	Intergenic	A	12:653745	1.31 (1.19-1.44)	$1.5x10^{-8}$	0.11	*NINJ2*	10.7	*WNK1*	78.7	3
rs713536	Intergenic	T	8:12943177	1.23 (1.13-1.33)	$9.1x10^{-7}$	0.18	*C8orf79*	11.5	*DLC1*	42.1	
rs4867131	Intergenic	A	5:33047743	1.41 (1.23-1.62)	$1.2x10^{-6}$	0.09	*C5orf23*	220.1	*NPR3*	224.7	
rs10734548	Intronic	T	11:46744149	1.23 (1.13-1.35)	$4.4x10^{-6}$	0.10	*CKAP5*	WG	*F2*	26.5	49
rs11609145	Intergenic	G	12:3885559	1.31 (1.17-1.48)	$4.9x10^{-6}$	0.12	*PARP11*	32.7	*EFCAB4B*	153.0	
rs3211928	Intronic	G	7:79942371	0.83 (0.77-0.90)	$6.4x10^{-6}$	0.21	*CD36*	WG	*SEMA3C*	74.1	14
rs877087	Intronic	T	15:31661567	1.22 (1.12-1.33)	$6.5x10^{-6}$	0.17	*RYR3*	WG	*AVEN*	284.1	
rs7853368	Intergenic	G	9:13447920	1.20 (1.11-1.29)	$7.8x10^{-6}$	0.14	*MPDZ*	207.5	*NFIB*	623.9	1
rs4151467	Intronic	C	13:47817924	1.44 (1.23-1.69)	$8.7x10^{-6}$	0.05	*RB1*	WG	*P2RY5*	65.2	
rs6449093	Intergenic	G	4:14638765	1.32 (1.27-1.49)	$9.8x10^{-6}$	0.06	*CPEB2*	43.0	*C1QTNF7*	379.1	

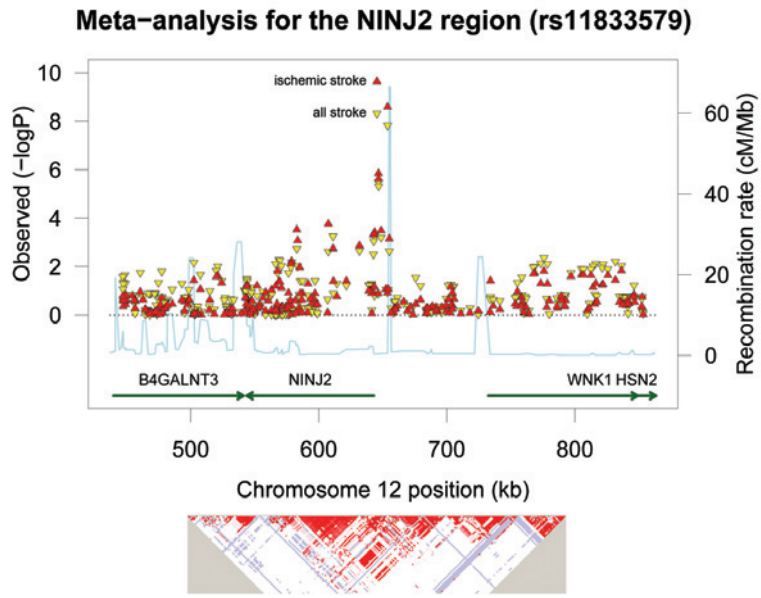
Table 2. (Continued)

SNP	SNP function	Minor Allele	Chromosome: position	Hazard Ratio (95% CI)	P value	PAR	Closest gene*		Second closest gene*		Additional SNPs at $P<10^{-5}$†
							Name	Distance	Name	Distance	
Ischemic stroke											
rs11833579	Upstream	A	12:645460	1.41 (1.27-1.56)	$2.3x10^{-10}$	0.17	*NINJ2*	2.4	*WNK1*	87.0	3
rs12425791	Intergenic	A	12:653745	1.39 (1.25-1.54)	$2.6x10^{-9}$	0.14	*NINJ2*	10.7	*WNK1*	78.7	3
rs4867131	Intergenic	A	5:33047743	1.49 (1.27-1.75)	$9.9x10^{-7}$	0.10	*C5orf23*	220.1	*NPR3*	224.7	
rs10837576	Intergenic	A	11:41103075	0.78 (0.70-0.85)	$2.1x10^{-6}$	0.27	*LRRC4C*	830.8	*API5*	218.7	
rs10794579	Downstream	T	10:124676646	1.24 (1.13-1.35)	$2.1x10^{-6}$	0.18	*C10orf88*	3.7	*FAM24A*	14.0	3
rs2318308	Intergenic	A	14:65460852	0.75 (0.66-0.85)	$3.0x10^{-6}$	0.29	*FUT8*	181.1	*GPHN*	583.0	
rs713536	Intergenic	T	8:12943177	1.26 (1.14-1.38)	$3.3x10^{-6}$	0.20	*C8orf79*	11.5	*DLC1*	42.0	
rs17429019	Intergenic	G	3:190727847	1.49 (1.26-1.76)	$3.6x10^{-6}$	0.05	*TP63*	104.1	*TPRG1*	203.8	1
rs12786704	Intronic	G	11:131684538	1.36 (1.19-1.55)	$6.0x10^{-6}$	0.10	*HNT*	WG	*OPCML*	105.5	
rs6820391	Intronic	A	4:54255624	1.24 (1.13-1.36)	$6.9x10^{-6}$	0.12	*LNX1*	WG	*FIP1L1*	88.8	2
rs11615969	Intergenic	C	12:75229404	1.53 (1.27-1.84)	$7.4x10^{-6}$	0.09	*BBS10*	11.3	*OSBPL8*	18.6	

P values, hazard ratios, and 95% CIs are based on a fixed-effects (inverse-variance weighted) analysis.
MAF: minor allele frequency, PAR: population attributable risk, WG: within gene.
* The closest gene and second closest gene show the two genes located closest to each SNP and the distance of the associated SNP from the 5' end of the gene in kilobase pairs.

† Number of additional SNPs at the same locus, within 250 kb of the specified SNP, that were also associated with the phenotype with a P value of less than 10^{-5}.

Figure 3. Associations in the region centered on rs11833579 and containing NINJ2.



All SNPs are plotted with their P values against their genomic position. P values for ischemic stroke and all stroke are shown. The blue line indicates expected recombination rates. Green arrows indicate gene annotations.

The next closest gene to both SNPs is WNK1, coding for lysine-deficient protein kinase 1. The SNPs and WNK1 are separated by a recombination hotspot. In several studies, WNK1 has been associated with blood pressure levels and hypertension[29, 30]. Adjustment of the analyses for systolic blood pressure, hypertension and other cardiovascular risk factors had negligible effects on the observed associations.

In the replication cohort of the ARIC study, 215 persons had an incident stroke over a follow-up period of 15 years (191 ischemic strokes, of which 153 atherothrombotic ischemic stroke). In this cohort we replicated the association between rs12425791 and stroke (total stroke: HR 1.35, 95% CI 1.01-1.79, p=0.04; ischemic stroke: HR 1.42, 95% CI 1.06-1.92, p=0.02; atherothrombotic stroke: HR 1.41, 95% CI 1.01-1.95, p=0.04). For rs11833579 we did not replicate our findings, possibly due to low linkage disequilibrium (r^2=0.35) between the SNPs in this cohort.

Tests of association in the CHS replication cohort included only 68 incident ischemic strokes, and yielded no evidence of replication. This cohort had 21% power to detect a 30% risk increase at an alpha level of 0.05.

We observed replication of the association between rs12425791 and stroke in the Dutch case-control sample (total stroke: odds ratio [OR] 1.17, 95% CI 1.01-1.37, p=0.03; ischemic stroke: OR 1.19, 95% CI 1.01-1.41, p=0.04; atherothrombotic stroke: OR 1.29, 95% CI 1.08-1.54, p=0.005). Also, we replicated the association of rs11833579 with atherothrombotic stroke (OR 1.19, 95% CI 1.00-1.40, p=0.05).

When the discovery and replication cohorts of white participants were combined, we found an overall hazard ratio for the risk of ischemic stroke of 1.29 (95% CI 1.19-1.41, $p=1.1x10^{-9}$). This analysis also yielded highly suggestive associations with total and ischemic stroke in 4 SNPs, with total stroke only in 71 SNPs and with ischemic stroke only in 13 SNPs (table 2).

Discussion

In this analysis of four large population-based cohorts, two SNPs on chromosome 12p13 were consistently associated with total stroke, ischemic stroke and an atherothrombotic subtype of ischemic stroke. These findings were replicated in two independent samples: a cohort of black persons from the ARIC study and in Dutch whites. We did not observe replication in a smaller cohort with North American black persons.

Originally, we had considered total stroke our primary outcome. Stroke is a heterogeneous phenotype, and when studying subgroups we found no associations between rs11833579 and rs12425791 and nonischemic stroke. On the contrary, the associations we had found for total stroke proved to be stronger in ischemic stroke, and atherothrombotic stroke in particular. The estimated hazard ratios for stroke were similar in all four cohorts and in the black replication sample.

Ninjurin2 is a transmembrane protein of the ninjurin family. It is a cell-cell adhesion molecule and interacts with matrix metalloproteinases[17]. It has been shown to be upregulated in Schwann cells and dorsal root ganglia in case of nerve injury, and promotes neurite extension, leading to nerve regeneration[18]. It has also been shown to be expressed in radial glia, in low levels[18]. Possibly, the expression of ninjurin2 determines the response and tolerance of the brain to ischemic injuries.

Wnk1 was shown to be expressed in developing aorta and brain vasculature, and the central nervous system, in mice[19]. It is a regulator of transmembrane transport of sodium, potassium and chloride ions[20,21]. Mutations in Wnk1 have been related to familial hyperkalemic hypertension[20], elevated ambulatory blood pressure[22] and severity of essential hypertension[23]. Hypertension is a known strong risk factor for stroke[24], but adjustment for systolic blood pressure and hypertension did not attenuate the association between the two found SNPs and stroke, suggesting that the found genetic associations are not explained by blood pressure regulation.

A recent genomewide association study in stroke did not show an association in the 12p13 region[10]. However, the genotyping platform used did not cover either rs11833579 nor rs12425791, and has no SNP in linkage disequilibrium ($r^2 \geq 0.4$)with these SNPs.

Our study has several limitations. The SNPs found are likely to not be the causal variants, but are probably in linkage disequilibrium with the true causal variants. Further studies of this genomic region with denser genotyping will be required. Also, the number of strokes was too small to study associations between genetic variations and phenotypes underlying subtypes of atherothrombotic stroke, such as lacunar or cortical ischemic stroke. Finally, our power to detect small effects in rare variants was limited; nevertheless, our study has identified a previously unknown association of a common genetic variant with stroke.

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Chapter 2.2

Association of two SNPs from genomewide association studies with clinical phenotypes of cerebral ischemia

Abstract

Background Recent genomewide association studies have revealed an association between two single nucleotide polymorphisms (SNPs), rs11833579 and rs12425791, and ischemic stroke.

Aims To gain more insight in pathophysiological mechanisms underlying the found associations by exploring relationships between these SNPs and clinical and radiological characteristics of patients with cerebral ischemia.

Methods Our cohort consisted of 660 Caucasian patients with cerebral ischemia; from all patients detailed clinical and radiological data were available. Etiologic subtype of cerebral ischemia was determined according to the TOAST classification and an alternative classification. We studied associations between risk alleles and etiologic subtype, duration of ischemia, occurrence of multiple events, functional outcome and findings on CT-angiography (CTA), by means of logistic regression analysis.

Results The risk allele of rs11833579 was associated with an atherothrombotic etiology of cerebral ischemia, but not with other etiologic subtypes. Risk alleles of both SNPs were related to events of shorter duration (<24 hours), the risk allele of rs11833579 with occurrence of multiple events. There was no association between either SNP and clinical outcome. Both SNPs were associated with presence of stenotic calcifications and stenosis >30% in a symptomatic artery on CTA.

Conclusions This is the first study to show an association of rs11833579 with multiple episodes of cerebral ischemia of atherothrombotic origin, and of rs11833579 and rs12425791 with short duration of ischemia. Also, we found an association of both SNPs with atherosclerotic lesions in the extracranial vessels on CT-angiography. Together this suggests a relationship between the two SNPs and large artery pathology.

Introduction

Cerebral ischemia is a complex multifactorial disorder, in which genetic factors are thought to play a role[1]. Thus far results of genetic studies, using approaches such as linkage analysis and candidate gene analysis, have been disappointing. Since 2007 genomewide association studies (GWAS) have dominated genetic research of complex disorders, as it is a cost-effective method to study the entire genome of large numbers of patients. These large-scale studies generate new etiological hypotheses for multifactorial disorders, including ischemic stroke[2-5].

A recent large GWAS identified 2 SNPs, rs11833579 and rs12425791, to be significantly associated with ischemic stroke, in particular non-cardioembolic ischemic stroke[4]. This finding could not be replicated in the International Stroke Genetics Consortium and Wellcome Trust Case-Control Consortium 2[6], but a recent independent large case-control study from Japan did replicate the association between rs12425791 and atherothrombotic stroke[7].

The mechanisms by which the two SNPs influence the risk of stroke, are as yet unclear.

AIMS

We aimed our study to provide more insight in the pathophysiological mechanisms underlying the observed associations, by exploring the relationship between the two identified SNPs and clinical and radiological phenotypes of patients with cerebral ischemia in the Erasmus Stroke Study, a large hospital-based patient registry with detailed clinical information.

Methods

Study population

The Erasmus Stroke Study (ESS) is an ongoing prospective registry of all patients with transient ischemic attack (TIA) or stroke treated at Erasmus MC University Medical Center Rotterdam since December 2005. As specified below, highly detailed clinical data including extensive ancillary investigations to determine the most likely etiology, blood samples and DNA are collected from all patients. A standardized fasting blood drawing is performed to detect disorders in glucose and lipid metabolism. An ECG is made on admission, followed by a more elaborate cardiac workup including Holter registration and transoesophageal echocardiography when indicated. Functional outcome was assessed by means of the modified Rankin Scale at discharge and dichotomized as favorable (=<2) or unfavorable (>2). Informed consent was obtained from all participants in the ESS. The study was approved by the local Medical Ethics Committee (Erasmus MC, Rotterdam, the Netherlands).

For the current study we included all patients with cerebral ischemia (TIA or ischemic stroke) enrolled between December 2005 and February 2009.

Imaging

In all patients a plain brain CT scan was routinely performed, followed if possible by CT-angiography (CTA) from the ascending aortic arch to the intracranial vessels according to a standard protocol.

CT-angiography was scored for presence of calcifications and plaques, and severity of stenosis according to the North American Symptomatic Carotid Endarterectomy Trial (NASCET)[8] criteria, per vessel segment. Based on CT-angiography patients were categorized into one of the following groups: a) no signs of atherosclerosis, b) presence of small, non-stenotic calcifications without other signs of atherosclerosis, c) presence of plaques without signs of calcifications, d) presence of stenotic calcifications without signs of plaques, or e) presence of both plaques and calcifications. Stenosis of a symptomatic vessel was defined as a stenosis in an ipsilateral artery (common or internal carotid artery, vertebral artery, or basilar artery) related to clinical symptoms.

Genotyping

From all patients, DNA was isolated from EDTA plasma and stored at -80°C until use. The SNPs genotyped were rs11833579 and rs12425791. Genotyping was performed with the Sequenom platform using a multiplex with other SNPs not used for this study (Sequenom Inc., San Diego, USA). Both SNPs were in Hardy Weinberg equilibrium. The minor allele was regarded as the risk allele; for both SNPs this was the A allele.

Etiologic subtype of cerebral ischemia

Based on all available information, etiologic subtype of ischemic stroke was determined according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification[9]. This classification was used in the original GWAS, but was not designed for, and may not be well suited for studies on genetic associations. The TOAST classification categorizes patients with co-existing causes as having an undetermined cause, thereby ignoring valuable information[10]. We therefore also categorized patients according to the recent Perform Trial classification[11].

In accordance with that classification, patients can have coexisting causes. Lacunar stroke was defined as the presence of a small deep infarct on imaging <15 mm in the territory corresponding to symptoms, in a patient with a compatible clinical syndrome. Atherothrombotic cerebral ischemia comprised the categories definite and likely atherothrombotic cerebral ischemia, but not the category lacunar stroke. Definite atherothrombotic cerebral ischemia was defined as the presence of an ipsilateral extra- or intracranial stenosis >50% or mobile thrombus in the aortic arch. Likely atherothrombotic cerebral ischemia was defined as the presence of visible atherosclerotic plaques or calcifications in the carotid, vertebral or intracranial arteries, or the presence of two or more risk factors (current smoking, hypertension, hypercholesterolemia, diabetes mellitus). Cardioembolic cerebral ischemia was defined as presence of prosthetic valve, history of atrial fibrillation or atrial flutter, cardiac left-sided thrombus, recent myocardial infarction, sick sinus syndrome, dilated cardiomyopathy, endocarditis or atrial myxoma. Other causes of cerebral ischemia were arterial dissection, vasculitis, essential thrombocytemia and other rare causes. Patients who did not meet criteria for any subtype were categorized as cerebral ischemia of unknown cause.

Statistical analysis

Associations between the presence of risk alleles and etiologic subtypes, symptom duration (categorized as short-lasting (<24 hours) or long lasting (>=24 hours), multiplicity of events (occurrence of one or more cerebral ischemic events during the year before the inclusion event) and clinical outcome were tested using logistic regression adjusted for age and sex, and given as odds ratios with 95% confidence interval (CI) per increase in one copy of the risk allele. Associations between the presence of risk alleles and CT-angiography findings were evaluated by means of logistic regression adjusted for age, sex, hypertension, hypercholesterolemia, diabetes mellitus and current smoking, and presented as odds ratios with 95% CI.

Hypertension, hypercholesterolemia and diabetes were defined as use of respectively antihypertensives, cholesterol lowering drugs, and anti-diabetics before the event.

All analyses were performed with the risk alleles both as a continuous and dominant model.

Because we studied only two SNPs, which are in relatively high linkage disequilibrium (r^2 = 0.87 in our genotyping data) and the clinical variables are not independent, Bonferroni correction was not performed as this method was considered too conservative[12].

Statistical analyses were performed using SPSS 15.0 for Windows (SPSS Inc., Chicago, USA).

Results

From December 2005 until February 2009, 1187 patients were enrolled in the ESS; 944 of them had cerebral ischemia. In 135 patients DNA was not available and in 20 patients genotyping was unsuccessful for both SNPs, leaving 789 patients in whom one or both SNPs were genotyped. We successfully genotyped rs11833579 in 774 patients (649 Caucasians), and rs12425791 in 763 patients (636 Caucasians). As genotype distribution between ethnic groups was different (data not shown) and Caucasians constituted the largest group, we analyzed only Caucasians (N=660).

Baseline characteristics of patients including genotype distributions are shown in table 1.

The risk alleles of both SNPs were not associated with any etiologic subtype according to the TOAST classification (table 2). However, on the basis of the Perform classification, the risk allele of rs11833579 was associated with an atherothrombotic cause of cerebral ischemia. The risk allele of rs12425791 was also associated with an atherothrombotic subtype, with an effect size similar to that of rs11833579, but this association was not significant.

The risk allele of rs11833579 was significantly associated with the occurrence of multiple cerebral ischemic events in the past year, whereas no such association was found for rs12425791.

Carriers of one or more risk alleles of both SNPs more often had transient ischemic events (lasting <24 hours) than longer lasting symptoms. There was no association between both SNPs and functional outcome.

Table 1. Baseline characteristics of the study population (N=660)

Age (years)		64 (55-74)	
Male		350 (53)	
Etiologic subtype of cerebral ischemia		TOAST classification	Perform Trial classification*
Lacunar / small vessel occlusion		102 (16)	44 (7)
Atherothrombotic / large artery disease		110 (17)	393 (60)
Cardioembolic		96 (15)	82 (12)
Other		37 (6)	49 (7)
Undetermined		315 (47)	160 (24)
Multiple events		148 (22)	
Transient Ischemic Attack		296 (45)	
Unfavourable functional outcome†		90 (14)	
rs11833579 genotypes	GG	374 (58)	
	GA	235 (36)	
	AA	40 (6)	
rs12425791 genotypes	GG	421 (66)	
	GA	188 (30)	
	AA	27 (4)	

Presented are N(%), except for age where median (IQR) is shown
*Numbers add up to more than 100% because 68 patients (10%) had coexisting causes
†Defined as modified Rankin Scale score >2

Table 2. Associations between presence of risk alleles and clinical characteristics of cerebral ischemia. OR (95%CI) per risk allele, adjusted for age and sex.

		rs11833579	**rs12425791**
Etiologic subgroup*	Large artery atherosclerosis	1.12 (0.80-1.57)	1.05 (0.73-1.51)
	Small vessel occlusion	0.88 (0.62-1.26)	0.88 (0.60-1.30)
	Cardioembolism	0.80 (0.55-1.16)	0.83 (0.55-1.25)
	Other determined etiology	1.12 (0.65-1.94)	0.99 (0.53-1.83)
	Undetermined etiology	1.08 (0.84-1.40)	1.14 (0.86-1.50)
Etiologic subgroup†	Definite or likely atherothrombotic	1.31 (1.01-1.72)	1.32 (0.98-1.76)
	Lacunar	0.85 (0.50-1.45)	0.87 (0.49-1.56)
	Cardioembolic	0.82 (0.54-1.24)	0.79 (0.50-1.25)
	Other causes	1.03 (0.63-1.69)	0.97 (0.56-1.66)
	Unknown cause	0.87 (0.64-1.19)	0.91 (0.65-1.27)
Multiple events		1.44 (1.07-1.93)	1.21 (0.88-1.66)
Symptoms <24 hours vs >=24 hours		1.41 (1.09-1.83)	1.34 (1.01-1.78)
Unfavourable functional outcome (mRS >2)		0.93 (0.63-1.35)	1.02 (0.68-1.53)

*According to TOAST classification[9]
†According to Perform Trial classification[11]

CTA was available in 585 patients; the main reasons why it was not performed in 75 patients were poor renal function, or very severe stroke with likely fatal outcome.

After adjustment for age and sex, the risk allele of rs12425791 was associated with presence of a stenosis of more than 30% in a symptomatic artery (table 3). This association was borderline significant when

using a continuous risk allele model; associations were more pronounced using a dominant model. Risk alleles of both SNPs were significantly associated with the occurrence of stenotic calcifications without presence of plaques. The risk allele of rs12425791 is associated with occurrence of calcifications in the carotid bifurcation. Risk alleles of both SNPs were also significantly associated with presence of calcifications in the vertebral arteries. The associations were not attenuated after additional adjustment for hypertension, hypercholesterolemia, diabetes mellitus and current smoking.

Table 3. Associations between presence of risk alleles and findings on CT-Angiography. OR (95% CI) for presence of risk alleles versus no risk alleles (dominant model)

		*Model 1**		*Model 2†*	
		rs11833579	**rs12425791**	**rs11833579**	**rs12425791**
Overall grading	No atherosclerosis	0.89 (0.53-1.47)	0.97 (0.57-1.67)	0.89 (0.52-1.54)	0.99 (0.56-1.76)
	Small calcifications	0.71 (0.40-1.23)	0.71 (0.39-1.30)	0.78 (0.45-1.38)	0.79 (0.43-1.44)
	Plaques only	0.76 (0.44-1.32)	0.79 (0.43-1.44)	0.75 (0.43-1.31)	0.76 (0.41-1.40)
	Stenotic calcifications only	1.86 (1.12-3.09)	1.73 (1.04-2.87)	1.81 (1.06-3.11)	1.76 (1.03-3.02)
	Plaques with calcifications	1.01 (0.67-1.52)	0.92 (0.60-1.41)	1.02 (0.66-1.59)	0.89 (0.56-1.42)
Presence of stenosis >30% in symptomatic artery		1.24 (0.85-1.80)	1.52 (1.03-2.24)	1.31 (0.88-1.95)	1.63 (1.07-2.48)
Presence of calcifications in vertebral artery		1.81 (1.19-2.76)	1.55 (1.01-2.40)	1.80 (1.14-2.85)	1.63 (1.01-2.61)

*Model 1: adjusted for age and sex
†Model 2: adjusted for age, sex, hypertension, hypercholesterolemia, diabetes mellitus, and current smoking

Discussion

In this study of patients with recent cerebral ischemia, we observed an association between presence of the risk alleles of rs11833579 and rs12425791 with a predefined atherothrombotic subtype of cerebral ischemia, although this was significant only for rs11833579. Together with our observation that both SNPs were related to occurrence of ischemic events of short duration and the absence of an association with a lacunar stroke and cardioembolic subtype, this suggests that large artery disease is the most probable pathophysiologic substrate of the SNPs. Further support for a potential role of large vessel disease comes from our finding that risk alleles of the two SNPs were associated with presence of stenotic calcifications without presence of plaques. In particular, risk alleles were significantly associated with presence of calcifications of the vertebral arteries. Also, patients with risk alleles of one SNP had a higher chance of having a symptomatic stenosis of over 30%.

Presence of the risk alleles of rs11833579 and rs12425791 was recently found to be associated with occurrence of ischemic stroke, in particular non-cardioembolic stroke, in a GWAS[4]. A shortcoming of GWAS and other large scale genetic studies is that available clinical information is generally sparse, which hampers further research into etiology and clinical outcomes.
In this study, we have investigated associations between presence of risk alleles of the SNPs and clinical

characteristics of cerebral ischemic in a hospital-based registry with extensive clinical and radiological information. Strengths of our study include availability of highly detailed clinical information from all patients and of standardized CT-angiography of all vascular territories in the vast majority of patients. A weakness of our study is that the sample size is small for a study of genetic associations; however, it was large enough to identify several consistent associations. Another point is that the study population consisted of relatively mild cases of cerebral ischemia with a favorable outcome in most patients, likely to be the consequence of including TIAs as well as strokes. This may have reduced our power to find an association between presence of risk alleles and outcome of cerebral ischemia.
In this study, we have found an association between presence of risk alleles of the studied SNPs and an atherothrombotic subtype according to the Perform Trial classification, but not according to the TOAST classification. This is likely to be the consequence of different criteria for the subtype atherothrombotic stroke. Due to the very strict criteria of the TOAST classification, only a small number of patients was assigned to the large artery atherosclerosis group.

The two SNPs studied are in proximity of two genes on chromosome 12, Ninj2 and Wnk1. NINJ2 codes for Ninjurin-2, a protein involved in repair of peripheral neuronal injury, but it has also been shown to be present in radial glial cells[13]. There is scarce knowledge about the function of Ninjurin-2, a relation with ischemic stroke has not been described. Wnk1 has been associated with blood pressure and essential hypertension[14], which play a role in large vessel disease. It is, however, unlikely that the effect shown is through SNPs in the Wnk1 gene, as there is a recombination hotspot between that gene and the loci of our SNPs.

This is the first study aimed at unraveling the clinical phenotype of two SNPs shown to be related with ischemic stroke in a GWAS. We found that risk alleles of rs11833579 and rs12425791 are likely associated with large artery disease, based both on clinical etiologic subtypes and characteristics of cerebral ischemia, as well as on CT-angiography imaging. If the association with large vessel disease is confirmed in future studies, this may stimulate research into the functionality of the identified SNPs.

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Chapter 2.3

Genomewide association studies in intracerebral hemorrhage

Abstract

Background: The genetic contribution to the etiology of intracerebral hemorrhage (ICH) is largely unknown, except for rare specific mutations. Genomewide association studies provide a powerful tool to investigate common genetic variations involved in ICH. We performed a meta-analysis of genomewide association studies on intracerebral hemorrhage derived from three cohorts.

Methods: We included 153 patients with intracerebral hemorrhage and 12,519 controls, derived from two hospital-based cohorts (Erasmus Stroke Study and Medical University of Graz – ICH study) and the population-based Rotterdam Study. Results of the study-specific genomewide association analyses were meta-analyzed using an inverse variance model.

Results: The CAMTA1 and ATP6V1H loci were found to be associated with intracerebral hemorrhage ($p<5x10^{-8}$). Both genes have not been implicated in traditional risk factors for ICH, nor are they involved in a likely causal pathway. SNPs in the ATP6V1H locus had unidirectional risk estimates in all three cohorts and are therefore most likely to be linked to a functional genetic variant. We did not find an association for risk of ICH with APOE genotypes, as has been suggested previously.

Conclusions: Two previously unsuspected loci are associated with an increased risk of intracerebral hemorrhage in a meta-analysis of genomewide data from three independent cohorts.

Introduction

Intracerebral hemorrhage (ICH) is a devastating subtype of stroke, accounting for approximately 15% of stroke cases[1]. Acute treatments in patients with ICH are often ineffective in limiting brain damage, resulting in high mortality and extensive morbidity in surviving patients[2].

Previous studies have shown that ICH has a substantial genetic basis[4]. However, not many genetic factors affecting the risk of ICH have been identified. Most data on genetics of ICH come from patients with precipitating conditions such as cerebral amyloid angiopathy and rare monogenetic disorders[3]. Moreover, many previous candidate-gene studies on genetic risk factors for ICH have implicated various genes, many of which were either inconclusive or not replicated. Therefore, many other genetic factors contributing to risk of ICH remain unknown.

Genomewide association studies provide a powerful tool to investigate common genetic variants affecting the risk of complex diseases, such as diabetes mellitus or ischemic stroke[5,6]. Given the complex genetic basis of ICH we hypothesized that genomewide association studies would uncover common variants underlying the risk of ICH.

Therefore, we performed genomewide association studies into ICH in three independent cohorts with a meta-analysis of the three studies, aiming to find new, unsuspected genetic variants involved in the etiology of ICH.

Methods

Study population

Patients and controls were derived from two Dutch and one Austrian cohort: the Rotterdam Study (RS), Erasmus Stroke Study (ESS) and the Medical University of Graz ICH study (MUG-ICH). All studies have been described previously[6-8]. Each study included their own controls.

The Rotterdam Study (RS) is a prospective population-based cohort study with 7983 persons aged 55 or older, recruited from the Ommoord district in the city of Rotterdam, the Netherlands. At baseline an interview and physical examinations were performed, as well as blood sampling. We excluded persons with prevalent stroke at baseline. All participants were followed for incident stroke until January 1 2005. Stroke surveillance and stroke subtyping have been described in detail and includes automated linkage of the study database with medical records from general practitioners. We included cases of incident ICH until January 1, 2005 in our analysis[6].

The ESS is an ongoing registry of all patients with neurovascular disorders treated at the Erasmus MC University Medical Center Rotterdam. This registry also includes patients from the St. Elisabeth Hospital and TweeSteden hospital (Tilburg, the Netherlands). In all patients a standardized blood drawing is performed, including blood for DNA isolation. For patients from the ESS, population-based controls were used from the baseline cohort of the Rotterdam Study. Because for this analysis baseline data from RS were used, whereas for the GWAS in RS incident data were used, these two analyses can be considered independent[6].

MUG-ICH is a hospital based cohort of patients with intracerebral hemorrhage, treated at the Medical University of Graz. Controls for this cohort are derived from the Austrian Stroke Prevention Study (ASPS), a population-based cohort of inhabitants of Graz[9].
Virtually all participants of the Rotterdam Study and MUG-ICH are whites; the ESS also includes patients with other ethnicities. To account for genetic differences between ethnicities, only Caucasians were included in this study. All participants gave written informed consent. Institutional ethics committees from each institution approved this study.

Definition of intracerebral hemorrhage

In both hospital-based cohorts (ESS and MUG-ICH), intracerebral hemorrhage was defined as a sudden neurological deficit of presumed vascular origin, with brain imaging (CT scan) showing typical signs of intracerebral hemorrhage concordant with clinical symptoms; patients with subarachnoid hemorrhage were excluded. In RS, stroke was defined as a sudden focal neurological deficit of presumed vascular origin. Stroke was further subclassified as intracerebral hemorrhage based on imaging studies and data from hospital records.

Genotyping

DNA was isolated using standard procedures. In RS genotyping was performed using the Illumina Infinium II HumanHap550chip v3.0 array. In the ESS and MUG-ICH the Illumina Human 610-Quad Chip (Illumina) was used.
We only analyzed genotyped single nucleotide polymorphisms (SNPs) that were genotyped in all three studies, not imputed SNPs.

Statistical analysis

We performed quality control on all data taking into account Hardy Weinberg equilibrium, minor allele frequency, call rate and sex errors. Next, each cohort was analyzed separately, after which a meta-analysis was performed of the three cohorts.
To study associations between the dose of risk alleles (0 to 2) and risk of intracerebral hemorrhage, an additive genetic model was fitted. Analyses were adjusted for age and sex (sex only in MUG-ICH and RS). All studies were screened for population stratification, which was inconsiderable. The separate cohort analyses were performed using the GenABEL R package[10]. After cohort analyses, all data were meta-analyzed using inverse-variance weighting in the METAL program[11].
We used a p-value of $5x10^{-8}$ as the genomewide significance threshold, corresponding to a p-value of 0.05 with Bonferroni adjustment for 1 million independent tests[6].

Results

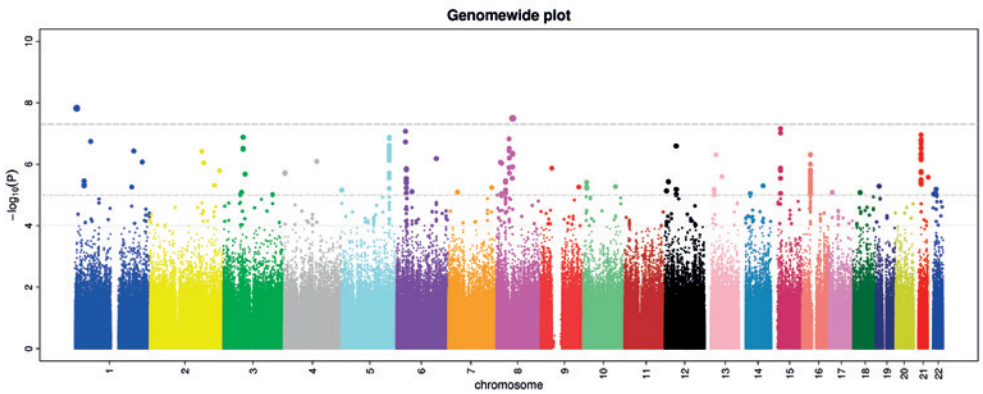
We included a total of 153 patients and 12,519 in our analyses. Baseline characteristics of the study populations are shown in table 1. Figure 1 shows the results for the genomewide association study for intracerebral hemorrhage. Odds ratios of highly suggestive loci, with P values $<1x10^{-5}$, are shown in table 2.

Table 1. Baseline characteristics of the study populations

	ESS		RS		MUG-ICH	
	Patients (N=73)	**Controls (N=5955)**	**Patients (N=55)**	**Controls (N=5708)**	**Patients (N=25)**	**Controls (N=856)**
Female, %	37	59	56	59	44	57
Age, mean (SD)	68.3 (14.4)	69.4(9.1)	*71.6 (8.1)	*69.1 (9.0)	-	65.2 (8.1)

ESS: Erasmus Stroke Study; RS: Rotterdam Study; MUG-ICH: Medical University of Graz ICH study; IQR: interquartile range. * Age at blood drawing

Figure 1. Results of tests for associations between intracerebral hemorrhage and each SNP.



P-values are shown in a Manhattan plot, relative to their genomic position. The top dashed horizontal line represents the threshold for genomewide significance, P=5x10^{-8}.

Three autosomal SNPs in two different loci passed our predefined threshold for genomewide significance ($P < 5x10^{-8}$). The first SNP, rs4737974, is located on chromosome 8q11.2 and was associated with a higher risk of ICH in all three studies, together with several SNPs in the same locus that did not reach genomewide significance. The two other hits, rs2071918 and rs12070592, are located on chromosome 1p36. Both SNPs were associated with higher risk of ICH in the Rotterdam Study, opposed to a lower risk in the ESS and were not genotyped in MUG-ICH.

Table 2. Most significant associations between Single Nucleotide Polymorphisms (SNPs) and intracerebral hemorrhage

SNP	SNP function	Minor allele	Chromosome: Position	Odds ratio (95% CI)	P Value	Closest gene		Additional SNPs at P<10-5
						Name	Distance*	
rs2071918	Intronic	T	1:7073164	0.98 (0.98-0.99)	$1.51x10^{-8}$	CAMTA1	WG	1
rs4737974	Intronic	A	8:54901625	1.03 (1.02-1.04)	$3.21x10^{-8}$	ATP6V1H	WG	3
rs10515775	Intronic	T	5:158523032	0.98 (0.98-0.99)	$1.32x10^{-7}$	RNF145	WG	1
rs202816	Upstream	A	5:157537980	2.79 (1.91-4.10)	$1.41x10^{-7}$	CLINT1	310	4
rs1620525	Intronic	A	8:42984073	0.90 (0.86-0.93)	$1.49x10^{-7}$	HOOK3	WG	3
rs6688451	Downstream	T	1:52858628	1.04 (1.02-1.05)	$1.80x10^{-7}$	GPX7	10	0
rs645722	Intronic	A	12:3226495	1.04 (1.02-1.05)	$2.05x10^{-7}$	TSPAN9	WG	0
rs10876680	Downstream	T	12:37895225	0.94 (0.92-0.96)	$2.55x10^{-7}$	KIF21A	75	1
rs11995771	Downstream	T	8:43545847	0.89 (0.85-0.93)	$3.05x10^{-7}$	POTE8	210	4
rs12629792	Intronic	A	3:66544926	1.09 (1.06-1.13)	$3.05x10^{-7}$	LRIG1	WG	2
rs12469875	Intronic	T	2:173012282	1.03 (1.02-1.05)	$3.81x10^{-7}$	ITGA6	WG	0
rs17056012	Downstream	T	13:37012227	0.98 (0.98-0.99)	$4.92x10^{-7}$	POSTN	20	0
rs3734744	Intronic	T	6:132737709	1.01 (1.01-1.02)	$6.51x10^{-7}$	MOXD1	WG	0
rs1457219	Intronic	T	8:15050696	1.08 (1.05-1.11)	$8.55x10^{-7}$	SGCZ	WG	0
rs12618595	Intronic	A	2:178910166	0.98 (0.97-0.99)	$8.94x10^{-7}$	OSBPL6	WG	0
rs7816853	Upstream	T	8:43243823	0.91 (0.87-0.94)	$1.29x10^{-6}$	POTE8	20	0
rs13415867	Upstream	A	2:231527983	0.96 (0.94-0.97)	$1.61X10^{-6}$	GPR55	30	0
rs8175350	Downstream	A	8:43868883	1.12 (1.07-1.17)	$1.92x10^{-6}$	POTE8	515	1
rs1123121	Downstream	A	3:72779850	0.99 (0.98-0.99)	$2.09x10^{-6}$	SHQ1	94	0
rs17252015	Intronic	T	8:53728728	1.12 (1.07-1.17)	$2.85x10^{-6}$	RB1CC1	WG	0
rs12309339	Intronic	A	12:12552192	0.97 (0.95-0.98)	$3.70x10^{-6}$	DUSP16	WG	0
rs16834529	Downstream	A	1:31748547	0.21 (0.11-0.41)	$4.56x10^{-6}$	SERINC2	66	1
rs2194532	Intronic	A	14:78222164	0.99 (0.98-0.99)	$5.02x10^{-6}$	NRXN3	WG	0
rs2163839	Exonic	A	19:11117982	1.02 (1.01-1.03)	$5.16x10^{-6}$	SPBC24	WG	0
rs12091129	Intronic	T	1:188672954	0.95 (0.93-0.97)	$5.50x10^{-6}$	FAM5C	WG	0
rs913232	Intronic	A	9:126602794	1.01 (1.01-1.01)	$5.52x10^{-6}$	OLFML2A	WG	0
rs6914422	Downstream	T	6:33642858	0.42 (0.28-0.61)	$5.86x10^{-6}$	BAK1	5	0
rs12582802	Intronic	A	12:38271473	1.07 (1.04-1.11)	$6.57x10^{-6}$	ABCD2	WG	0
rs4820748	Downstream	T	22:25990461	2.40 (1.64-3.52)	$6.59x10^{-6}$	MN1	455	0
rs4975533	Upstream	T	5:1041510	1.01 (1.01-1.02)	$6.92x10^{-6}$	NKD2	20	0
rs716970	Intronic	A	6:52363047	0.99 (0.99-0.99)	$7.77x10^{-6}$	PAQR8	WG	0
rs1860790	Exonic	A	7:31822103	1.03 (1.02-1.05)	$8.09x10^{-6}$	PDE1C	WG	0
rs1513751	Intronic	A	17:11275982	1.01 (1.01-1.02)	$8.25x10^{-6}$	FLJ45455	WG	0
rs730105	Upstream	T	14:36144024	0.99 (0.98-0.99)	$9.02x10^{-6}$	NKX2-8	22	0
rs2518827	Intron	A	22:18390730	0.98 (0.98-0.99)	$9.25x10^{-6}$	C22orf25	WG	0
rs1584327	Downstream	T	3:57425175	0.99 (0.98-0.99)	$9.66x10^{-6}$	FLJ44290	33	0
rs2089739	Downstream	A	3:163361407	1.01 (1.01-1.02)	$9.71x10^{-6}$	LOC131149	600	0

CI: confidence interval. *Distance in kilobase pairs. Additional SNPs are SNPs within 250 kb of the specified SNP.

The meta-analysis of the three cohort studies suggest that each copy of the minor allele of rs4737974 increases risk of stroke with an odds ratio of 1.03 (95% CI 1.02-1.04). Each copy of the minor allele of the chromosome 1p36 locus decreases risk of ICH with an odds ratio of 0.98 (95% confidence interval [CI] 0.98-0.99). We did not test for associations in subtypes of intracerebral hemorrhage, as phenotypic subtyping was only available for ESS patients.
The SNP in 8q11.2, rs4737974, is located in an intron of ATP6V1H, encoding "*ATPase, H+ transporting, lysosomal 50/57kDa, V1 subunit H*". The SNPs in the 1p36 locus, rs2071918 and rs12070592, are located in an intron of the CAMTA1 gene, which encodes "*calmodulin binding transcription activator 1*".

Discussion

To the best of our knowledge, this is the first genomewide association study in ICH. We found three SNPs to be associated with risk of intracerebral hemorrhage. Of these, the locus on chromosome 8q11.2 seems most promising in terms of true association with ICH as risk estimates across the cohorts were similar.

Before interpreting the data, some methodological concerns have to be addressed. The number of ICH patients in this study was small, although we combined data from three independent cohort studies. This reflects the difficulty to include patients with ICH, as it is a rare condition when compared with ischemic stroke (up to 15% of stroke cases), has a high mortality in the acute phase and is difficult to ascertain in absence of brain imaging, as in population-based studies. Also, we do not have an independent replication cohort, as they are very scarce. Efforts are ongoing to group multiple cohorts with data on intracerebral hemorrhage in a consortium, but analysis has not started yet. In absence of data from larger consortia, we present these data from three cohorts.

Calmodulin binding transcription activator 1 can bind DNA and activate transcription, and has been implicated as a tumor suppressor gene in neuroblastoma and gliomas, and episodic memory[12-14]. In our cohorts we had limited information on underlying brain tumors so we could not study whether the association of the found SNPs was present only in patients with brain tumors. However, neuroblastoma is a childhood tumor so it would be highly unlikely in our patient population. Gliomas are a rare cause of intracerebral hemorrhage, and thus it is unlikely to cause the observed genomewide significant association with ICH.
ATPase, H+ transporting, lysosomal 50/57kDa, V1 subunit H is a subunit of an ATPase, an enzyme which mediates acidification of intracellular organelles, which is required for several intracellular processes and mitochondrial energy metabolism[15,16]. Very little is known about this enzyme, it has been described in one study describing a role in islet cell dysfunction in the development of diabetes mellitus, found using proteomics[15]. It is unclear whether there may be a role in occurrence of ICH.

Of the two genomewide significant loci, only one had similar risk estimates in all three cohorts, the ATP6V1H gene locus. Therefore, this locus is more likely to be truly associated with risk of intracerebral hemorrhage than the CAMTA1 locus, in which risk estimates were opposite in different cohorts. The SNPs we found are, however, likely not to be the causal variant, but in linkage with the true functional variant. More detailed genotyping of these areas and replication are needed to ascertain a true association with risk of ICH, and find the causal genetic variants.

There has been a large number of publications on candidate gene polymorphisms in ICH, but for most findings there has been no replication[3]. The only robustly replicated finding is the association between APOE genetic variants and risk of ICH, mostly in candidate gene studies[17-20]. A recent genomewide association study embedded in a targeted study found variants in the APOE locus to be significantly associated with lobar ICH, but not with deep ICH[21]. We did not find significant associations with APOE variants; a possible explanation for this discrepancy is that we analyzed all ICH cases, irrespective of localization.
The ATPV61H and CAMTA1 loci have not been implicated in intracerebral hemorrhage previously, most likely due to lack of an obvious disease mechanism and the candidate gene approach of most earlier studies.

In this genomewide association study we have uncovered two loci to be associated with intracerebral hemorrhage, both of which have not been associated with vascular disease previously. Replication studies are needed to validate, and fine-tune our findings.

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Chapter 3

Genetic influences on risk factors for stroke

Chapter 3.1

Associations between recently discovered genetic variations in metabolic traits and arterial stenosis in patients with recent cerebral ischemia

Abstract

Background Recent large genomewide association studies have found many new genes to be associated with metabolic traits including hypertension, lipid levels and diabetes. A next step after gene discovery is to investigate associations between these genes and clinically relevant endpoints. Therefore, we studied the relation between recently discovered genetic variations in metabolic traits and craniocervical artery stenosis, in patients with transient ischemic attack or ischemic stroke.

Methods We included 700 patients with a recent transient ischemic attack or ischemic stroke. In all patients CT-angiography from the aortic arch to the intracranial vessels was performed and scored for degree of stenosis in each artery. Our primary outcome was presence of a stenosis ≥30% in any artery. Genotyping was performed with Metabochip, a targeted gene chip for metabolic traits containing ≈200,000 SNPs.

Results Five loci were found to be strongly associated with presence of stenosis (GLIS3, $p=1.6x10^{-6}$; AGBL2, $p=1.2x10^{-4}$; SBF2, $p=7.9x10^{-5}$; SCAMP5, $p=8.7x10^{-5}$; and MC4R, $p=1.7x10^{-4}$). After adjusting for multiple testing using permutation only the GLIS3 locus remained. For this gene, the risk allele was associated with a 2.2 (95%CI 1.6-3.1)-fold increase of stenosis risk. This gene was previously shown to lead to elevated fasting glucose levels. We found no significant association between loci implicated in hypertension or hypercholesterolemia and presence of stenosis.

Conclusions Our study suggests an association between atherosclerosis and a gene implicated in increased fasting glucose levels, but not with blood pressure- or lipid-related genes, in patients with recent cerebral ischemia.

Introduction

During the past years many large gene discovery studies have been undertaken, uncovering new genes and loci that are associated with various disorders and traits. For metabolic traits such as blood pressure, lipid and glucose levels, very large cohorts of over 100,000 participants have shown many new loci[1-6]. In these studies, genotyping has generally been broad but not dense, rendering the genetic variants found in these large studies likely not to be the true functional variant, but closely linked to it[7].

To find causative genetic variants, one possibility is to perform a dense genotyping and sequencing of the implicated locus. This is time-consuming and may not reveal a truly functional variant. Apart from uncovering functional genetic variants, it may therefore also be useful to study newfound genes and loci with respect to clinical parameters, to solidify their association with the studied traits. This increases knowledge on functional pathways in which the genetic variations could exert their functions, and leads to better comprehension of results of large-scale genetic studies.

Dyslipidemia, diabetes mellitus and hypertension are widely known to increase the risk of cerebrovascular disorders. Many single nucleotide polymorphisms (SNPs) have been found to be associated with these metabolic parameters, but studies of these variants with regard to clinically relevant parameters such as carotid artery stenosis have not been performed yet, despite that fact that a stenosis can have a causal relation to ischemic stroke or transient ischemic attack (TIA)[8]. Also, it is still unclear which metabolic traits are important in the occurrence of atherosclerosis, of which stenosis is a marker. Therefore, we investigated the associations between recently uncovered loci associated with metabolic traits and a clinically relevant parameter, stenosis in the craniocervical arteries, in a large hospital-based cohort of patients with recent ischemic stroke or TIA.

Methods

Study population

We included consecutive patients with ischemic stroke or TIA, treated at the Erasmus MC University Medical Center, between December 2005 and April 2010. All patients participated in the Erasmus Stroke Study (ESS), which has been described previously[9,10]. Briefly, the ESS is an ongoing registry of all patients with neurovascular disorders treated at our hospital, and includes elaborate clinical and radiological data. From all patients blood is drawn for DNA isolation.

Ischemic stroke was defined as a sudden focal neurological deficit with a presumed vascular origin lasting ≥24 hours, with brain imaging showing no abnormalities or typical signs of infarction. TIA was defined similarly, but with a duration of symptoms of <24 hours.

All patients gave written informed consent; the study was approved by the Medical Ethics Committee of the Erasmus MC University Medical Center.

Imaging

A CT-angiography (CTA) from the aortic arch to the intracranial vessels was routinely performed in all patients, according to a standard protocol. The CTA was scored for presence of calcifications and the

degree of stenosis, per vessel segment. The stenosis degree was determined for the brachiocephalic trunk, subclavian artery, common carotid artery, carotid bifurcation, internal carotid artery, carotid siphon and vertebral artery, all according to the North American Symptomatic Carotid Endarterectomy Trial (NASCET)[11] criteria. Stenosis degrees are often categorized as per the trial protocol: 0-29%, 30-49%, 50-69% and over 70%[32]. We chose a threshold of 30% stenosis to resemble presence of atherosclerosis. For the purpose of this study, we determined whether there was a stenosis ≥30% in any of the studied vessels (stenosis present), or no stenosis ≥30% in any of the scored vessels (stenosis absent). Presence of a stenosis was considered a marker for presence of atherosclerosis.

Genotyping

Genotyping was performed on the Illumina Metabochip. This chip was designed to fine-map regions that were discovered in high throughput studies with metabolic traits, including blood pressure and hypertension, lipid levels and hypercholesterolemia, glucose levels and diabetes, and BMI. It contains dense mapping of known loci, as well as single SNPs, with a total of 196,725 SNPs. Most loci contain SNPs in very high linkage disequilibrium.

Statistical analysis

Quality control was performed, taking into account Hardy Weinberg equilibrium, call rate, minor allele frequency, sex mismatch and identity by state. After quality control 163,735 SNPs remained. We performed a principal component analysis to evaluate the genetic structure of the sample. According to the first two principle components, our population was made up of at least three ethnic groups with the largest group made up of white participants.

We fitted an additive model to determine associations between SNPs and presence of stenosis by means of multiple logistic regression analysis, adjusted for the first three principle components to account for genetic stratification. This reduced inflation of test statistics (lambda) to 0.97.

All analyses were performed in the GenABEL R package[12].

The threshold corresponding to a 0.05 with Bonferroni correction for 163,735 independent tests, $3x10^{-7}$, was considered too conservative as the chip was designed for finemapping and contains mostly loci of several hundred SNPs that are highly correlated to each other. Therefore, the number of independent tests is substantially reduced, and this makes Bonferroni correction overconservative[33]. In the results section and tables we report p-values before permutation analysis. To estimate empirical significance, we performed permutation tests (1,000 replications) on the complete set of SNPs.

Results

From December 2005 until April 2010, 1299 patients with ischemic stroke or TIA were included in the Erasmus Stroke Study. From 256 patients DNA samples were unavailable, and CTA was unavailable in 166 patients. Reasons for not performing a CTA were very poor renal function, severe neurological

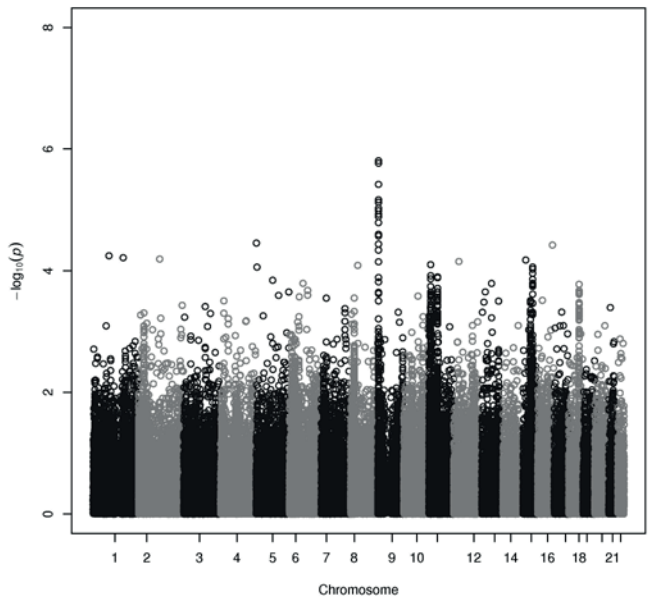
symptoms with short life expectancy and severe comorbidity with concomitant high surgical risk. From the remaining 877 patients, 149 were not genotyped for various practical reasons, leaving 728 patients for genotyping. After quality control of genotyping data, 700 patients were left and included in this study. The mean age of the study population was 62 years, 53% was female, and as expected in this patient group there was a high prevalence of cardiovascular risk factors (table 1).

Table 1. Baseline characteristics of the study population (N=700)

Mean age (years±SD)	**61.7±14.0**
Female, N(%)	370 (53)
Cerebral ischemia	
Transient ischemic attack, N(%)	303 (43)
Ischemic stroke, N(%)	397 (57)
Cardiovascular risk factors	
Hypertension, N(%)	378 (54)
Hypercholesterolemia, N(%)	245 (35)
Diabetes mellitus, N(%)	101 (14)
Current smoking, N(%)*	209 (31)
Atherosclerosis on CT-Angiography	
Stenosis ≥30%, N(%)†	337 (49)

*smoking status known in 665 patients
† scored for all vessels in 693 patients

Figure 1. Results of test for association between each SNP and stenosis in the craniocervical arteries ≥30%.



P values (–log$_{10}$(P)) are shown for each SNP according to its genomic position.

Table 2. SNPs most significantly associated to presence of stenosis ($p<10^{-4}$).

SNP	SNP location	Minor allele	MAF	Chromosome position	Odds ratio (95% CI)	P value	Closest gene*		Additional SNPs at $P<10^{-3}$	Associated trait
							Name	Distance		
Unnamed	Intronic	A	0.14	9:4251222	2.22 (1.60-3.08)	$1.56x10^{-6}$	GLIS3	Within gene	23	Increased fasting glucose[4]
rs7717939	Upstream	G	0.46	5:2277557	0.62 (0.50-0.78)	$3.50x10^{-5}$	IRX4	343	0	-
rs11865869	Intronic	G	0.22	16:7987404	0.58 (0.45-0.75)	$3.77x10^{-5}$	BCMO1	Within gene	0	-
rs12066353	Intronic	C	0.13	1:87293907	2.02 (1.43-2.84)	$5.66x10^{-5}$	HS2ST1	Within gene	0	-
rs10800152	Intronic	A	0.26	1:163968300	0.60 (0.47-0.77)	$6.10x10^{-5}$	TMCO1	Within gene	0	Glaucoma[29]
rs4251961	Upstream	G	0.38	2:113590938	1.57 (1.26-1.96)	$6.41x10^{-5}$	IL1RN	1	0	Inflammation
rs8024657	Upstream	A	0.36	15:35868908	1.60 (1.27-2.01)	$6.66x10^{-5}$	TMCO5	145	0	-
rs11049257	Upstream	G	0.20	12:28030272	0.56 (0.42-0.75)	$7.03x10^{-5}$	PTHLH	13	0	Atherosclerosis[30]
Unnamed	Intronic	C	0.14	11:10092608	0.53 (0.38-0.73)	$7.93x10^{-5}$	SBF2	Within gene	53	Increased HDL[1]
rs12544899	Upstream	G	0.23	8:38688488	0.59 (0.46-0.77)	$8.16x10^{-5}$	TACC1	75	0	Carcinogenesis[31]
Unnamed	Exonic	G	0.08	15:73099023	0.44 (0.29-0.66)	$8.69x10^{-5}$	SCAMP5	Within gene	23	-
rs13155847	Downstream	G	0.19	5:5374098	0.56 (0.42-0.75)	$8.71x10^{-5}$	ADAMTS16	0.7	0	-
rs10769288	Intronic	A	0.38	11:47685756	0.63 (0.50-0.80)	$1.23x10^{-4}$	AGBL2†	Within gene	54	†Increased BMI[2]
Unnamed	Downstream	G	0.29	18:56090278	1.58 (1.24-2.01)	$1.67x10^{-4}$	MC4R	87	30	Decreased HDL[5]

Stenosis is defined as stenosis >30% in any craniocervical artery.
MAF: Minor allele frequency
*distance in kilobase pairs
†locus continues into MTCH2 gene

In 337 patients (49%), a stenosis ≥30% was present. SNPs in several loci showed an association with the presence of a stenosis (figure 1). The QQ plot shows that although findings are not genomewide significant taking all SNPs together, there is evidence for a deviation from the expected null value when adjusting for stratification by principal components analysis. Particularly for chromosome 9, there is evidence for associations with multiple SNPs with p-values below 10^{-6}. Table 2 shows associations between SNPs with P values $<10^{-4}$ and presence of a stenosis ≥30%, expressed as odds ratios. This table includes five loci with a large number of SNPs associated with stenosis (GLIS3, $p=1.6x10^{-6}$; AGBL2, $p=1.2x10^{-4}$; SBF2, $p=7.9x10^{-5}$; SCAMP5, $p=8.7x10^{-5}$; and MC4R, $p=1.7x10^{-4}$). We found no association between SNPs in loci associated with blood pressure and hypertension, HDL or total cholesterol levels, diabetes mellitus, and risk of stenosis. After permutation testing the most significant locus was an intronic SNP (unnamed) in GLIS3, which was initially associated with fasting glucose, with a 2.2 (95%CI 1.6-3.1)-fold increase of stenosis risk (p=0.055).

Discussion

In this study of association between genes associated with metabolic traits and carotid and vertebral stenosis, we found several loci to be associated with occurrence of a stenosis. Only the glucose locus in GLIS3 remained significant after adjustment by means of permutation. Surprisingly, we found no associations for loci in several well-known cardiovascular risk factors such as lipid levels or blood pressure.

Before we interpret the results, some methodological issues have to be addressed. Firstly, our population size is small for a genetic association study. However, the number of SNPs is limited and they are mostly highly linked to each other, limiting the number of independent tests. We are not aware of a cohort with the same extensive CTA scoring that could be used as a replication cohort. Second, we had to exclude a considerable number of patients because CTA was unavailable due to comorbidity or short life expectancy. A strength of our study is the detailed scoring of the CTAs, enabling us to study stenosis in each vessel segment that can lead to cerebral ischemia. Also, this is the first study to use a chip containing all known genetic variations for metabolic traits with a common clinical parameter. This allowed us to study associations between SNPs for all common metabolic cardiovascular risk factors and a marker of atherosclerosis, craniocervical arterial stenosis.

GLIS3 encodes *GLIS family zinc finger 3*, which was found to have a critical role in the development of pancreatic b cells and regulation of mature b cells[16]. Deficiency of the GLIS3 product leads to hyperglycemia and hypoinsulinemia, and reduced b cell response to glucose[16,17]. Previous genetic studies have found SNPs in the GLIS3 locus to be associated with increased fasting glucose levels[4] and diabetes mellitus type 1 and 2 [18,19]. In our analysis we did not find an association between loci associated with diabetes mellitus and stenosis. Diabetes mellitus has been shown to increase the risk of stenosis and high-grade stenosis in the carotid artery[20,21].

The carotid intima-media thickness (IMT) is a surrogate marker for atherosclerosis, mostly for subclinical atherosclerosis. It can be used to assess relations between risk factors and severity of early atherosclerosis. Craniocervical stenosis degree is a more advanced form of atherosclerosis than IMT, but represents the same process.
Our finding that the GLIS3 gene is associated with craniocervical stenosis corresponds to earlier studies which indicate increased IMT in patients with elevated fasting glucose levels[13,14,34,35]. Also, a longitudinal study demonstrated that development of increased IMT after ten years of follow-up was associated with higher levels of fasting glucose at baseline[36]. Recently, genetic determinants of fasting glucose levels were shown to be associated with IMT as well, indicating a possible causal role for elevated fasting glucose in atherosclerosis[15].
Apart from candidate-gene studies there have been some less-hypothesis driven studies on genetic determinants of carotid IMT, without significant results[24,25] .

Earlier studies on genetic risk of atherosclerosis showed the 9p21 locus to be associated with coronary artery atherosclerosis and cerebrovascular disease[26,27], but not with carotid IMT[28]. In our study, we did not find an association with SNPs in the 9p21 locus that are commonly used to construct haplotypes. The locus with highest significance in our study, GLIS3, is located on chromosome 9p24.2, which is situated over 17 Mb pairs from the 9p21 locus and is therefore unlikely to co-segregate.

In this targeted genomic association study we found that only a locus associated with fasting glucose levels was associated with presence of a stenosis in the craniocervical arteries. We found no evidence for an association between occurrence of stenosis and previously suspected traits such as loci for blood pressure or lipid levels.

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Chapter 3.2

Variation in the C-reactive protein gene is associated with serum levels of CRP in patients with acute ischemic stroke

Abstract

Background and purpose: Elevated levels of C-reactive protein (CRP) are found in up to three quarters of patients with acute ischemic stroke and are associated with poor outcome. We investigated whether haplotypes representing common variations in the CRP gene are associated with levels of CRP in patients with acute ischemic stroke.

Methods: We included 185 patients with ischemic stroke in whom CRP was measured within 24h of symptom onset. Common haplotypes within the CRP gene were determined by 3 genotype-tagging single nucleotide polymorphisms (SNPs).

Results: Four haplotypes with frequencies >5% covered 99.2% of the genetic variation. Haplotype 4 (CCG, frequency 8.3%) was associated with a 20.6 mg/l (95% CI, 9.8-30.4) stronger increase in CRP level as compared with haplotype 1 (CTC, frequency 33.7%).

Conclusion: Variation in the CRP gene is associated with levels of CRP in acute ischemic stroke.

Background

C-reactive protein (CRP) is a strong acute-phase reactant. Elevated levels of serum CRP are found in up to three quarters of patients with acute ischemic stroke and are associated with poor outcome[1-6]. This leads to interest in the use of CRP as a biomarker of prognosis of ischemic stroke. Moreover, CRP may contribute to secondary brain damage after focal cerebral ischemia, possibly via a complement-mediated exacerbation of tissue injury[7].

The CRP concentrations are under genetic influence. Family and twin studies found that additive genetic factors account for 27-40% of the variance in CRP, indicating a role of DNA sequence variation in determining serum protein levels[8]. Furthermore, there is accumulating evidence for an association between single nucleotide polymorphisms (SNPs) in the CRP gene and levels of CRP[9-10].

This led us to hypothesize that levels of CRP in the acute phase of ischemic stroke are under genetic influence as well, and that haplotypes associated with higher CRP levels are associated with an increased risk of poor outcome after acute ischemic stroke.

Only limited and inconsistent data are available on the association between SNPs in the CRP gene and CRP concentrations in patients with acute ischemic stroke[3,11].

In the present study, we investigated whether haplotypes representing common variations in the CRP gene are associated with levels of CRP in patients with acute ischemic stroke.

Methods

Study population

Patients were derived from the Erasmus Stroke Study, a project aimed at collecting clinical information, blood samples and DNA of all patients with neurovascular diseases admitted to the Erasmus MC University Medical Center.

For the present study, we evaluated all patients with ischemic stroke admitted between December 2005 and December 2008 in whom venous blood was collected within 24h of symptom onset.

Written informed consent was obtained from all patients, signed by the participants or a first-degree relative, as approved by the Institutional Ethics Committee.

Baseline characteristics

Baseline clinical information was extracted from the trial records. This included a quantification of stroke severity according to the National Instituted of Health Stroke Scale (NIHSS)[12], ischemic stroke subtype according to the TOAST classification[13], and cardiovascular risk factors.

Measurement of CRP

CRP levels were determined by means of the Roche Modular Assay. The range of measurement is 1-285 mg/l with a variation coefficient of 4.6%.

CRP polymorphism Genotyping

In the Seattle SNPs program for Genomic applications, 31 SNPs were identified in the CRP gene on chromosome 1q21 (http://www.pga.gs.washington.edu/data/crp, 'visual haplotype' option).

In 23 unrelated individuals of European descent, these SNPs formed 4 well-defined haplotypes with frequencies of more than 5%. By selecting 3 tagging SNPs, the total common variation in the CRP gene is described. We selected the following tagging SNPs: 1184 C>T (rs1130864), 2042 C>T (rs1205) and 2911 C>G (rs3093068).

DNA was extracted according to standard procedures and stored at -20°C. Genotypes were determined in genomic DNA of 2 ng with the Taqman allelic discrimination assay (Applied Biosystems, Foster City, California, USA) and end-point readings of fluorescence were performed on Taqman Prism 7900HT (Applied Biosystems).

Statistical Analysis

SNPs were tested for Hardy-Weinberg equilibrium with a X^2 test. The association between CRP haplotypes and levels of CRP were assessed with the non-parametric haplo.score function of Haplo Stats (http://cran-r.project.org/scr/contrib/Descriptions/haplostats). We adjusted for age, sex and stroke severity on admission by means of multiple linear regression.

Results

Between December 2005 and December 2008, 561 patients with ischemic stroke were included in the Erasmus Stroke Study. Of these patients, 376 were excluded for the present study because of CRP measurement was not accomplished within 24h of stroke onset (n=140), DNA was not available (226) of genotyping failed for 2 or 3 out of 3 SNPs (n=10).

In the total study population, the mean age was 64 (SD 15), 53% were male and the median NIHSS score was 4 (range 0-22) (table 1). Mean CRP level was 10 mg/l (SD 27). Median time from onset of symptoms to CRP measurement was approximately 7h.

Table 1. Clinical characteristics of the patients (n=185)

Demographics	
Age, years (median±IQR)	64±15
Sex, male	98 (53)
Cardiovascular risk factors	
Hypertension	98 (53)
Atrial fibrillation	22(12)
Diabetes Mellitus	29(16)
Current cigarette smoking	35(19)
Hypercholesterolemia	85(46)

Table 1. (Continued)

Medical history	
Previous stroke	50(27)
Previous myocardial infarction	31(17)
Peripheral vascular disease	13(7)
Stroke subtype	
Large vessel disease	28(15)
Cardiac source of embolism	30(16)
Small vessel occlusion	27(15)
Other determined etiology	21(11)
Undetermined/negative evaluation	79(43)
Stroke severity	
NIHSS score (median (range))	4 (0-22)
Physical examination	
Systolic blood pressure on admission (median±IQR)	167±34
Diastolic blood pressure on admission (median±IQR)	86±20
Laboratory assessments	
Time from stroke onset to measurement of CRP, hours (median (range))	6.6 (3-13.9)
Treatment	
Treatment with rt-PA	36(19)
Shown are n(%), unless otherwise specified	

All SNPs were in Hardy-Weinberg equilibrium. The 1184T-allele was present in 25.9%, the 2042T-allele was present in 34.5% and the 2911G-allele in 8.6% of 370 chromosomes.

Four common haplotypes were identified (table 2). The remaining haplotypes (CTG, TCG, TTC) were present in less than 5%.

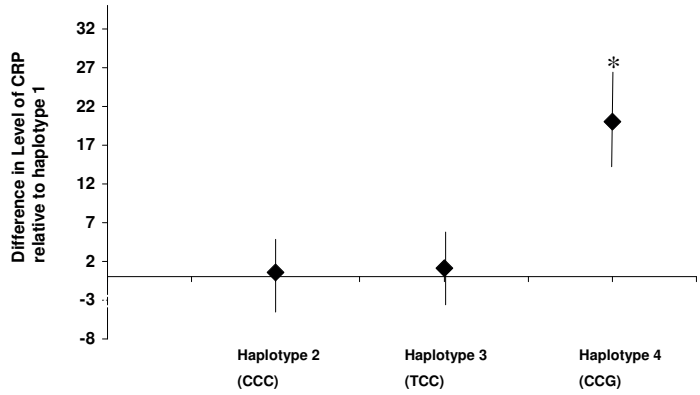
Table 2. Frequency of haplotypes and association between CRP haplotypes and levels of CRP

Haplotype	Haplotype frequency, %	Haplo score	P value*
1 (CTC)	33.7	-0.75	0.45
2 (CCC)	31.7	-0.01	0.98
3 (TCC)	25.5	0.41	0.68
4 (CCG)	8.3	4.49	<0.01

*p values were obtained using haplo score, after 2,377 simulations. The frequencies refer to the reference (Seattle) population.

Haplotype 4 (CCG) was associated with a significant stronger increase in CRP levels (20.6 mg/l; 95% CI, 9.8-30.4) relative to the reference haplotype 1 (CTC) (table 2 and fig. 1). Adjustment of age, sex and NIHSS score did not attenuate this association (fig. 1).

Figure 1. Relative effects of CRP gene haplotypes on levels of CRP (mg/l).



**p<0.01. Regression coefficients were adjusted for age, sex and NIHSS score on admission. Coefficients reflect the mean difference in levels of CRP relative to haplotype 1 (CTC).*

Discussion

In this study, we found that SNPs in the CRP gene were associated with levels of CRP within 24h of ischemic stroke onset. Haplotype 4 (CCG) was associated with significant higher levels of CRP than haplotype 1 (CTC).

Our results provide further proof that, apart from environmental factors, genetic variations may influence levels of CRP. Several studies found an association between CRP haplotypes and levels of CRP in diseases in which inflammation is involved[9,14,15]. The direction of the haplotype-CRP level associations are also supported by previous studies that have used the same polymorphisms to reconstruct the haplotypes[9,14,15]. Two previous studies that investigated the association between SNPs in the CRP gene and levels of CRP in patients with acute ischemic stroke cannot be compared directly with ours, because only single SNPs were studies[3,11].

The present study has some limitations. First, the study compromised a small number of patients. As a result, the precision of our estimated is limited. Furthermore, because of this small number of patients we could not adjust the association between CRP haplotypes and levels of CRP for vascular risk factors, like diabetes mellitus and smoking, which might have influenced the levels of CRP. Second, our results did not include information about ethnicity. Allele frequencies have been reported to differ between ethnic groups. However, the haplotype structures are similar and therefore the direction and size of changes in CRP levels associated with most SNPs might be similar regardless of ethnic group[15].

A third issue that warrants consideration in this study is that DNA was lacking for 226 patients who were admitted within 24h of symptom onset. On the other hand, comparison of the excluded patients with the study population showed no significant differences with regard to baseline characteristics or case fatality. Hence, it is not likely that it has substantially influenced the results.

Finally, our study focused on CRP levels within 24h of stroke onset. As CRP may peak several days after ischemic stroke, CRP measured at later time points that in our study may better reflect the magnitude of the inflammatory reaction. Furthermore, we did not have levels of CRP prior to ischemic stroke or several months after the stroke. It would be interesting to compare the association between CRP haplotypes and these levels with that of stroke-free participants. On the other hand, a previous study did not find an association between CRP genotypes and baseline levels of CRP nor between CRP genotypes and levels of CRP in stroke-free participants[10].

Increases in CRP levels following ischemic stroke may reflect a systemic inflammatory response, the extent of tissue injury, or concurrent infections. Interestingly, in animal models of focal cerebral ischemia, CRP increased secondary brain damage through activation of the complement system[7,16]. These findings suggest that understanding genotype-phenotype associations of CRP polymorphisms may provide a genetic basis for the future development of tailored therapeutic strategies in ischemic stroke.

In conclusion, variation in the CRP gene is associated with levels of CRP in acute ischemic stroke. Further studies are needed to investigate the relation between variation in the CRP gene, levels of CRP and clinical outcome after acute ischemic stroke.

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Chapter 3.3

Haplotypes of the FGG gene affect levels of fibrinogen gamma' but are not related to risk of ischemic or hemorrhagic stroke

Abstract

Introduction: Plasma levels of fibrinogen gamma' are associated with stroke. Fibrinogen gamma' is an isoform of fibrinogen that is produced after alternative mRNA processing of the FGG transcript. Genetic variations in the FGG gene are associated with decreased levels of fibrinogen gamma' and also with an increased risk of thromboembolic disorders. For ischemic and hemorrhagic stroke these genetic associations have been studied sparsely, with conflicting results. We investigated associations of FGG haplotypes with fibrinogen gamma' levels and risk of stroke.

Methods: We included 157 patients with ischemic stroke, 38 with intracerebral hemorrhage and 155 controls. Three common haplotypes were discriminated with 2 tagging SNPs, rs2066865 and rs1049636. In all participants, fibrinogen gamma' levels and total fibrinogen levels were measured.

Results: In the total group, individuals with Haplotype 2 (H2) had lower fibrinogen gamma' levels and a lower gamma'/total fibrinogen ratio, compared with H1 (301.5 vs 368.3 mg/l, p=1.5e-8 and 0.085 vs 0.102, p=6.7e-10, respectively). Similar differences were seen in (ischemic and hemorrhagic) stroke patients and controls. H3 gave significantly higher levels of the gamma' ratio (ratio 0.118; p=0.002 vs H1) in controls and patients with ischemic stroke, but not in patients with intracerebral hemorrhage. We found no associations between haplotypes and risk of ischemic or hemorrhagic stroke.

Conclusions: Genetic variations in the FGG gene are associated with levels of fibrinogen gamma'. We found no association between FGG haplotypes and risk of ischemic or hemorrhagic stroke.

Introduction

Fibrinogen is a glycoprotein that is essential for the formation of clots, since it is converted to insoluble fibrin in the last step of the coagulation cascade. The fibrinogen molecule consists of three pairs of polypeptide chains, Aα, Bβ and γ[1], encoded by three genes clustered on chromosome 4, denoted FGA, FGB and FGG, respectively[2]. The FGG gene gives rise to the normal γ chain, γA, but also to an alternative γ chain, known as γ′. Fibrinogen γ′ is a result of alternative mRNA processing[3,4]. Most γ′-containing fibrinogen molecules are heterodimers with one γA and one γ′ chain[5].

Fibrinogen γ′ has pro- and antithrombotic properties (for a review, see Uitte de Willige et al[6]) and has been linked to cardiovascular diseases such as myocardial infarction and ischemic stroke[7-11]. Also, we have previously demonstrated that fibrinogen γ′ is associated with outcome in ischemic stroke[11]. It has been shown that a common haplotype of FGG, mostly referred to as haplotype H2, is associated with a lower level of γ′, whereas no clear effect was seen for the other haplotypes[9,12,13]. These data were obtained in healthy individuals and patients with cardiovascular diseases in the chronic phase after an acute event. Limited information is available on the influence of genetic variation on levels of γ′ in the acute phase of ischemic stroke, and no studies have yet been performed in this field in patients with hemorrhagic stroke. Several studies have demonstrated that FGG haplotypes or single nucleotide polymorphisms (SNPs) confer an increased risk of arterial and venous thrombotic disorders[7,13-17]. An association between FGG haplotypes and ischemic stroke was reported in a number of studies[9,15,18], while another failed to demonstrate such an association[19].

As yet, little is known on how FGG variations affect the level of fibrinogen γ′ in stroke patients. Our study was aimed to investigate the influence of FGG haplotypes on levels of fibrinogen γ′ and outcome in stroke patients and control persons, and the relation between these haplotypes and risk of stroke, ischemic as well as hemorrhagic.

Methods

Study population

Patients and controls were derived from the Erasmus Stroke Study (ESS), a large prospective registry of all patients with neurovascular disorders treated at the Erasmus MC University Medical Center[11]. The ESS also includes population-based controls, mostly friends and spouses of patients, but no relatives. All participants gave written informed consent. The study was approved by the Medical Ethics Committee of the Erasmus MC University Medical Center.

For this study we included 200 patients with ischemic stroke, 55 patients with intracerebral hemorrhage and 156 stroke-free controls. To avoid bias due to the influence of ethnic differences between patients, we restricted the analysis to Caucasians (85% of participants). This left a total of 350 participants: 157 patients with ischemic stroke, 38 with intracerebral hemorrhage and 155 control persons.

Ischemic stroke was defined as the occurrence of a sudden neurological deficit of presumed vascular origin lasting >24 hours, with a brain CT scan showing no abnormalities or typical signs of cerebral

ischemia. Hemorrhagic stroke was defined as the occurrence of a sudden neurological deficit with a brain CT showing signs of intracerebral hemorrhage, concordant with clinical symptoms. Functional outcome in all patients was assessed at discharge with the modified Rankin Scale (mRS)[20] score, which reflects the level of handicap and ability to perform activities of daily living independently. It ranges from 0 (no symptoms) to 6 (death). We dichotomized the mRS as favorable (≤2) or unfavorable (>2).

Fibrinogen level assessments

Blood was drawn at a median of six days (interquartile range 3-19 days) after the occurrence of stroke in patients. Citrated plasma was centrifuged at 1700 g and stored at -80°C within two hours of collection until analysis. Levels of total fibrinogen were determined using an automated clotting assay according to Clauss. Levels of fibrinogen γ' were determined by ELISA as described earlier[11]. Briefly, mouse anti-human γ' fibrinogen antibodies were used for catching, and HRP-conjugated rabbit anti-human fibrinogen was used for tagging.

Gene selection and genotyping

The Seattle SNPs program for Genomic Applications[21] has identified three haplotype tagging SNPs in the FGG gene that tag the genetic variation in the FGG gene in Caucasians. SNP rs2066865 tags haplotype (H)2, rs1049636 tags H3 and rs2066860 tags H4. Of these, H4 is rare (<5% of the population), and has been shown to have a similar effect on fibrinogen γ' levels as the haplotype with common alleles for all SNPs (H1)[9]. Therefore we only selected rs2066865 and rs1049636 to form three haplotypes, of which H1 was assigned to those with common alleles for both SNPs and was used as reference haplotype.

DNA was isolated using Magnapure (Roche), and genotyping was performed using DNA samples stored at -80°C until analysis. Genotypes were determined in 2 ng genomic DNA using Taqman allelic discrimination assays (Applied Biosystems, Foster City, CA, USA). Primer and probe sequences are available upon request.

Haplotype assembly and statistical analyses

Haplotypes were inferred using the haplo.stats package in the statistical program R (http://cran.r-project.org/web/packages/haplo.stats/index.html). This method computes the probability of haplotype pairs for each individual. It then models the phenotype as a function of the inferred haplotype pair, weighed by their estimated probability, to account for haplotype ambiguity[22].

Haplotype frequencies were estimated using the *haplo.em* function, which uses an expectation maximization algorithm. Associations between haplotypes and levels of fibrinogen γ', occurrence of stroke and outcome were tested using the *haplo.glm* function. This function uses a generalized linear model, and computes the regression of a trait on haplotypes and covariates. Hardy Weinberg equilibrium for SNPs was tested using a X^2 test. Analyses were adjusted for age and sex. A probability value <0.05 was considered significant.

Results

Both SNPs were successfully genotyped in >98% of the individuals. Baseline characteristics of participants are shown in table 1. The allele distribution of both SNPs was in Hardy Weinberg equilibrium.

Table 1. Baseline characteristics of study population and haplotype frequencies.

			Control persons (N = 155)	Ischemic stroke (N=157)	Intracerebral hemorrhage (N=38)
Age, years (mean ± SD)			60±12	64±12	62±14
Female, %			51	46	42
	rs2066865	rs1049636			
H1	C	T	44.5%	42.6%	49.2%
H2	T	T	24.1%	30.7%	22.5%
H3	C	C	31.5%	26.8%	28.4%

Genetic variations in FGG and fibrinogen γ' levels

Levels of γ' and the ratio of γ' over total fibrinogen according to haplotypes of the FGG gene in patients and control persons are presented in table 2. There was no difference in total fibrinogen levels between haplotypes. In all participants, regardless of case/control status, presence of H2 gave a significant decrease in levels of γ', and γ'/total fibrinogen ratios when compared with H1. Haplotype 3, on the other hand, gave increased levels of fibrinogen γ' and relative γ' levels in the total group, and in the subgroups of control persons and patients with ischemic stroke. Patients with intracerebral hemorrhage showed a significant decrease of the γ'/total fibrinogen ratio with presence of H2, although this effect seems smaller that in the other subgroups, but they showed no increase in ratio with H3.

The absolute difference in fibrinogen γ' level was similar for haplotype 2 and haplotype 1, both in patients and control persons. The absolute increase in γ' levels in those with H3 compared with H1 was present only in patients, and not in control persons.

Table 2: Mean levels of fibrinogen γ' and γ'/total fibrinogen ratio according to haplotypes.

		Fibrinogen γ'	P value*	γ'/total fibrinogen ratio	P value*
All participants	H1	368.3 mg/l	Ref	0.102	Ref
	H2	301.5 mg/l	1.53e-8	0.085	6.72e-10
	H3	390.1 mg/l	0.06	0.111	0.001
Control persons	H1	348.3 mg/l	Ref	0.098	Ref
	H2	277.0 mg/l	7.8e-5	0.084	2.4e-4
	H3	337.6 mg/l	0.56	0.106	0.04
Ischemic stroke	H1	378.0 mg/l	Ref	0.104	Ref
	H2	311.8 mg/l	4.9e-4	0.085	3.9e-5
	H3	409.1 mg/l	0.03	0.118	0.002
Intracerebral hemorrhage	H1	400.7 mg/l	Ref	0.107	Ref
	H2	347.2 mg/l	0.20	0.083	0.021
	H3	454.0 mg/l	0.21	0.107	0.94

*P values for difference between haplotypes and haplotype 1, adjusted for age and sex, multiple linear regression

Genetic variations in FGG and risk of stroke and outcome

There we no associations between the FGG gene haplotypes and risk of intracerebral hemorrhage. However, we did observe slight differences in risk of ischemic stroke in different haplotype groups, with H2 having a slightly higher risk and H3 a slightly lower risk of ischemic stroke in comparison with H1, although results were not significant (OR 1.32, 95% CI 0.88-1.99 and OR 0.91, 95%CI 0.61-1.36, respectively).

We found no associations between FGG haplotypes and outcome after ischemic stroke or intracerebral hemorrhage. However, there were slight differences between both types of stroke. In patients with ischemic stroke, H2 tended to be related to a higher risk of poor functional outcome compared with H1 (mRS >2; OR 1.36, 95%CI 0.42-4.39), and H3 showed no change in outcome (OR 0.98, 95%CI 0.32-3.03). In patients with intracerebral hemorrhage, both H2 and H3 led to a slightly lower chance of unfavorable functional outcome compared with H1 (H2: OR 0.21, 95%CI 0.04-1.04; H3: OR 0.86, 95%CI 0.22-4.50).

Discussion

In this study we have found that levels of fibrinogen γ' and the γ'/total fibrinogen ratio are influenced by genetic variations in the FGG gene. Stroke patients as well as healthy controls with H2 had significantly lower levels of γ', whereas individuals with H3 had significantly higher levels of γ' compared with those with the most common haplotype.

We found no association between FGG haplotypes and risk of ischemic or hemorrhagic stroke. Haplotype H2 showed a nonsignificant association with unfavorable outcome in ischemic stroke patients, and favorable outcome in patients with intracerebral hemorrhage.

Before interpreting the results of our study, some methodological issues have to be discussed. A strength of our study is the use of haplotype probabilities in our analyses, which allowed us to also study individuals in whom one or both alleles were missing, limiting loss of data. This was possible because the statistical package calculated probabilities of each haplotype for each individual and used these weighed probabilities in the analyses. Also, this is the first study assessing the relationship between FGG variations and fibrinogen γ' levels in patients with intracerebral hemorrhage, and it extends the limited knowledge on this topic in ischemic stroke.

A drawback of our study is that our sample size is relatively small for exploring associations between genetic variation and clinical outcome. However, we did find highly significant associations between haplotypes and levels of fibrinogen γ'. Secondly, we only included Caucasians in our study since genetic variations may differ between ethnicities. Therefore, it remains to be shown whether our results apply to other ethnic groups. Thirdly, in the group of patients with ischemic stroke many patients had a favorable outcome, limiting power to detect associations between genetic variation and outcome.

The relation between variations in the FGG gene and the risk of ischemic stroke has been evaluated in a limited number of studies. In a large population-based study, no association between FGG haplotypes and risk of stroke was found, which is in line with our findings. However, this study did show an association with stroke risk of a combined haplotype of the FGG and FGA genes[19]. Jood et al found an association between our H2 (called A2 in their study) and ischemic stroke[18]. Also, this haplotype was associated with occurrence of silent brain infarcts in a study by van Oijen et al[23]. The relation between FGG haplotypes and risk of hemorrhagic stroke has not been investigated previously.
Many studies have found lower fibrinogen γ' levels in individuals with H2[12,13,15]. However, these studies were all performed in the chronic phase of the disease, and only one previous study was performed in the acute phase of ischemic stroke[9]. We confirm the finding of Cheung *et al* that H2 is related to lower γ' levels and ratios in the acute phase after stroke. This is relevant, because we have previously shown that fibrinogen γ' levels in the acute phase of ischemic stroke are associated with functional short-term outcome[11]. The genetic influence on levels may thus influence outcome. Our observation that γ' levels were higher in persons with H3 is in agreement with a previously published finding of the SNP tagging H3 being related to increased levels of γ'[15].

Our H2-tagging SNP rs2066865 has been considered the functional SNP in lowering γ' levels, as it has been shown to influence the rate of γ' transcription based on its location in the gene[12,13]. By lowering the fibrinogen γ' level, genetic variation in the FGG gene may influence risk of stroke, as lower γ' ratios are associated with higher risk of ischemic stroke. However, this has not been prospectively proven, our knowledge on this is based on retrospective studies.
The mechanism underlying the association of H3 with higher fibrinogen γ' levels is as yet unknown. Further studies are required to find the functional genetic variant, and its influence on elevating γ' levels.

In conclusion, we have shown that common variations in the FGG gene affect levels of fibrinogen γ' in healthy persons and in patients with ischemic or hemorrhagic stroke. Future studies should unravel whether the strong genetic impact on fibrinogen γ' levels influences the outcome of stroke.

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Chapter 4

Hemostatic risk factors for stroke

Chapter 4.1

γ'/total fibrinogen ratio is associated with short-term outcome in ischemic stroke

Abstract

Background: Fibrinogen γ' (γ') is a natural isoform of fibrinogen, and alters the rate of formation and the properties of clots. It could therefore affect outcome after ischemic stroke. The prognostic significance of γ' fibrinogen levels is, however, still unclear.

Objectives: To assess levels of γ' in ischemic stroke, and its association with short-term outcome.

Patients/Methods: We included 200 ischemic stroke patients and 156 control persons. Total fibrinogen and γ' levels were measured; outcome at discharge was assessed by means of the modified Rankin Scale score (defined as unfavorable when >2). We compared levels between patients and controls using multiple linear regression analysis, and logistic regression analysis was used to assess the relationship between levels and outcome. All analyses were adjusted for age and sex.

Results: Mean γ' levels were significantly higher in patients with ischemic stroke than in controls (0.37 vs 0.32 g/l, p<0.001), and patients also had a higher γ'/total fibrinogen ratio (0.102 vs 0.096, p=0.19). The γ'/total fibrinogen ratio is associated with unfavorable outcome in patients with ischemic stroke (OR per unit increase of γ'/total fibrinogen ratio 1.27, 95%CI 1.09-1.47).

Conclusions: Our study shows that patients with ischemic stroke have increased levels of fibrinogen γ' and suggests a trend towards an increased γ'/total fibrinogen ratio in ischemic stroke. Increased fibrinogen γ' relative to total fibrinogen levels are associated with unfavorable outcome in the early phase after stroke.

Introduction

Human plasma fibrinogen is an important protein in the hemostatic system, in which soluble fibrinogen is converted to insoluble fibrin, leading to clot formation. Fibrinogen is a heterogeneous protein, existing as several natural isoforms. The fibrinogen variant containing γ' is one such isoform, comprising 10±3% of the circulating fibrinogen molecules[1]. In the systemic circulation, γ' is mostly present as a heterodimeric protein with the common γA form (γA/ γ'), while <1% is present in the γ'/ γ' form[2]. Fibrinogen γ' is a result of alternative mRNA processing, in which 20 amino acids replace the 4 amino acids at the C-terminal of γA.[3,4] The extension of fibrinogen γ' contains additional binding sites for thrombin (hence the alternative name antithrombin I for γ'-containing fibrinogen molecules)[5] and for the factor XIII B-subunit[6], enhancing binding of factor XIII. The platelet binding site for integrin $\alpha_{IIB}\beta_3$ is disrupted, which deducts platelet binding capacity[7].

Recently, we and others reported that an elevated γ' level or γ'/total fibrinogen ratio is associated with cardiovascular disease[1,8-12], especially in the acute phase of ischemic stroke. Results of our previous studies suggest that fibrinogen γ' levels as well as the γ'/total fibrinogen ratio alter during an acute phase reaction[1,8]. This may lead to a different clot formation rate and to different characteristics of the fibrin network, and may therefore contribute to the pathology of thrombotic diseases and stroke.

The role of fibrinogen γ' and the γ'/total fibrinogen ratio in the precipitation and course of ischemic stroke is as yet unclear. To our knowledge, no studies have been published on fibrinogen γ' in relation to outcome of cardiovascular disease, ischemic stroke in particular.

We aimed to investigate the differences in fibrinogen γ' levels and γ'/total fibrinogen ratio, between patients with ischemic stroke and control persons. Furthermore, we studied whether there is an association between these levels and short-term outcome in ischemic stroke.

Materials and Methods

Study population

All patients and controls participated in the Erasmus Stroke Study (ESS), which is an ongoing prospective registry of all patients with transient ischemic attack (TIA) or stroke treated at Erasmus MC University Medical Center Rotterdam since December 2005. From all patients, detailed clinical and radiological data, blood samples and DNA are collected. The ESS also includes population-based control persons, mostly friends and spouses of patients, but no family members. All participants in the ESS provided informed consent. The study was approved by the Medical Ethics Committee of the Erasmus MC University Medical Center.

For this case-control study we included 200 patients with ischemic stroke, and 156 age- and sex matched stroke-free control persons. In all patients, citrated blood was collected and centrifuged at 4000 rpm for 15 minutes. Citrated plasma was stored at -80°C within 2 hours from collection. Blood was drawn 8 days (interquartile range 3-22 days) from the date of onset, either in the stroke unit or the outpatient clinic.

Total fibrinogen and fibrinogen γ' antigen measurements

We determined total fibrinogen levels according to Von Clauss[13] on a fully-automated blood coagulation analyzer (Sysmex CA-1500 system, Siemens Healthcare Diagnostics, Breda, the Netherlands). Normal Reference Plasma was used as reference plasma in this assay (Precision BioLogic, Dartmouth, Canada), the intra-assay coefficient of variation was 3.5%.

Fibrinogen γ' antigen levels were measured with an enzyme-linked immunosorbent assay as described previously[9], with minor modifications. Briefly, plastic 96-well microtiter plates (Nunc maxisorp, Roskilde, Denmark) were coated with mouse anti-human γ' fibrinogen (2.G2.H9; Millipore, Billerica, MA, USA) and then incubated overnight at 4°C. Wells were blocked with bovine serum albumin for 1 hour at room temperature. Plasma samples were added to each well in duplicate. After 1 hour incubation at room temperature, horseradish peroxidase-conjugated rabbit anti-human fibrinogen (DAKO A/S, Glostrup, Denmark) was added to tag the immobilized patient fibrinogen γ', and incubated for 1 hour at room temperature. Color was developed with Tetramethylbenzidine substrate solution (BioMérieux, Marcy l'Etiole, France) and was terminated with H_2SO_4 after 15 minutes. The plate was read at 450 nm spectrophotometrically. Wells were washed 3 times between all incubation steps. Pooled normal plasma calibrated against purified human γ' fibrinogen was used as calibrator[1]. The intra-assay variation was less than 10%.

Definitions

Ischemic stroke was defined as focal neurological deficit of presumed vascular origin, lasting >24 hours or leading to death within 24 hours, with brain imaging showing no abnormalities or typical signs of brain infarction. Hypertension was defined as the use of antihypertensive drugs before the event, hypercholesterolemia as the use of cholesterol lowering drugs before the event. Diabetes mellitus was defined as the use of oral antidiabetic drugs and/or insulin before the event. All strokes were classified according to TOAST criteria[14] based on all available information.

Functional short-term outcome was assessed by means of the modified Rankin Scale (mRS)[15] score at time of discharge from the stroke unit for hospitalized patients, and from the outpatient clinic for ambulant patients. The mRS is a score for handicap and level of dependency. The scale ranges from 0 (no symptoms) to 6 (dead), with each increase denoting more severe disability. At a score of 2, patients have neurological symptoms affecting their daily life but are still able to live completely independent; at a score of 3 patients cannot live independently due to symptoms. The mRS was dichotomized between scores of 2 and 3, as favorable (=<2, independent living) or unfavorable (>2, dependent or institutionalized living).

Statistical analysis

Differences between baseline characteristics of patients and controls were assessed with Student's t-test. Differences in fibrinogen levels between patient groups and controls were assessed using Student's t-test; adjustment for confounders was performed using analysis of variance (ANOVA). Associations between

fibrinogen levels and outcome were assessed using multiple logistic regression analysis and expressed as odds ratio (OR) with 95% confidence intervals (CI). Analyses were also performed in 2 subgroups of equal size, based on time from event to blood drawing. This allowed us to study levels of γ' in the acute and subacute stage after ischemic stroke. All analyses were adjusted for age and sex. Statistical analyses were performed using SPSS 15.0 for Windows (SPSS Inc., Chicago, Illinois). A probability value <0.05 was considered significant.

Results

Baseline characteristics of patients and controls are shown in table 1. As expected, prevalence of cardiovascular risk factors (smoking, hypertension and hypercholesterolemia) was different between patients and controls.

Table 1. Characteristics of the study population.

		Patients	Controls	p-value
N		200	156	
Age (years), mean (SD)		62 (13)	59 (12)	0.04
Female, %		45	51	0.29
Current smoking, %		36	24	0.02
Hypertension, %		55	29	<0.001
Hypercholesterolemia, %		34	18	0.001
Diabetes Mellitus, %		17	13	0.37
Body Mass Index, mean (SD)		26.0 (4.2)	26.7 (4.5)	0.22
TOAST classification N(%)	Large artery atherosclerosis	39 (20)		
	Cardioembolism	21 (11)		
	Small vessel occlusion	57 (29)		
	Other etiology	12 (6)		
	Undetermined etiology	71 (36)		
Time from onset of symptoms to blood drawing (days), median (IQR)		7.5 (3-22)		
Modified Rankin Scale at discharge, median (IQR)		1 (1-2)		

Levels of total fibrinogen and fibrinogen γ' were significantly higher in patients compared with controls, after adjustment for age and sex (table 2a). Levels of the γ'/total fibrinogen ratio were similar in patients and controls.

The median time from onset of symptoms to blood drawing was 7.5 days. Two subgroups of patients were formed, by dividing patients in 2 equal groups based on time from onset of symptoms to blood drawing, the acute phase and the subacute phase. Differences in γ'/total fibrinogen ratio between patients and controls were most pronounced in the group with blood drawing in the acute phase

of stroke (table 2b). In this group γ'/total fibrinogen ratio was significantly increased, in line with our previous results[8]. In the group of patients in the subacute phase of ischemic stroke, the γ' ratio was not different from controls.

Table 2a. Levels of total fibrinogen, fibrinogen γ' and γ'/total fibrinogen ratio in patients and controls.

	Controls	Patients	Model 1, p-value*	Model 2, p-value†	Model 3, p-value‡
Total fibrinogen (g/l)	3.37 (0.72)	3.69 (0.96)	<0.001	0.001	<0.001
Fibrinogen γ' (g/l)	0.32 (0.13)	0.37 (0.15)	<0.001	<0.001	0.002
γ'/total fibrinogen ratio	0.096 (0.028)	0.102 (0.036)	0.19	0.30	0.495

Presented are means (SD).
*Model 1: adjustment for age and sex
†Model 2: adjustment for age, sex and time to blood drawing
‡Model 3: adjustment for age, sex, time to blood drawing, current smoking, hypertension and hypercholesterolemia

Table 2b. Levels of total fibrinogen, fibrinogen γ' and γ'/total fibrinogen ratio in subgroups of patients and controls. Subgroups: patients were divided in 2 equal groups of 100 patients based on time from event to blood drawing.

	0-7 days	>= 8 days	0-7 days vs controls, p-value*	>= 8 days vs controls, p-value*
Total fibrinogen (g/l)	3.76 (1.11)	3.62 (0.78)	<0.001	0.033
Fibrinogen γ' (g/l)	0.40 (0.17)	0.34 (0.12)	<0.001	0.194
γ'/total fibrinogen ratio	0.110 (0.040)	0.095 (0.029)	0.015	0.64

Presented are means (SD).
*p value after adjustment for age and sex

Outcome was unfavorable (modified Rankin Scale score >2) in 13.5% of patients with ischemic stroke. These patients could no longer live independently due to neurological symptoms, had severe disabilities, or died.
Levels of fibrinogen, fibrinogen γ' and γ'/total fibrinogen ratio with respect to favorable or unfavorable outcome are shown in table 3. After adjustment for confounders, γ' and total fibrinogen levels and the γ'/total fibrinogen ratio were significantly higher in patients with unfavorable outcome, compared with patients with a favorable outcome.

Increased fibrinogen γ' levels and γ'/total fibrinogen ratio were significantly associated with unfavorable outcome (OR per unit increase γ' 1.48, 95%CI 1.15-1.91 and OR per unit increase γ' ratio 1.27, 95%CI 1.09-1.47; both adjusted for age, sex and time to blood drawing; table 4). The γ'/total fibrinogen ratio adjusts fibrinogen γ' levels for total fibrinogen levels, the prognostic effect of fibrinogen γ' on outcome was thus independent of total fibrinogen level. Effects were similar in subgroups based on time to blood drawing, showing that the γ'/total fibrinogen ratio was associated with unfavorable outcome in the first week

as well as later in the course of ischemic stroke (OR 1.25, 95%CI 1.05-1.49 and OR 1.20, 95%CI 0.84-1.70, respectively).

Table 3. Levels of total fibrinogen, fibrinogen γ' and γ'/total fibrinogen ratio in patients with favorable or unfavorable outcome

	Favorable outcome (modified Rankin Scale ≤ 2)	Unfavorable outcome (modified Rankin Scale >2)	Model 1*, p-value	Model 2†, p-value
N	173	27		
Total fibrinogen, g/l (SD)	3.64 (0.89)	3.97 (1.34)	0.112	0.013
fibrinogen γ', g/l (SD)	0.36 (0.14)	0.47 (0.18)	<0.001	<0.001
γ'/total fibrinogen ratio, SD	0.098 (0.029)	0.129 (0.059)	<0.001	0.006

*Model 1: adjusted for age and sex
†Model 2: adjusted for age, sex, current smoking, hypertension and days to blood drawing

Table 4. Associations between total fibrinogen levels, fibrinogen γ' levels and γ'/total fibrinogen ratio and outcome of ischemic stroke. OR (95% CI) for unfavorable outcome (modified Rankin Scale >2) per unit increase in levels

Unfavorable outcome, N (%)	27 (13,5)	
	Model 1*	**Model 2†**
Total fibrinogen (g/l)	1.35 (0.90-2.01)	1.31 (0.87-1.96)
Fibrinogen γ' (dg/l)	1.52 (1.18-1.96)	1.48 (1.15-1.91)
γ'/total fibrinogen ratio (%)	1.28 (1.10-1.48)	1.27 (1.09-1.47)

* Model 1: adjusted for age and sex
† Model 2: adjusted for age, sex and time to blood drawing

Discussion

Our study shows that levels of total fibrinogen, fibrinogen γ' and the γ'/total fibrinogen ratio are increased in the acute phase of ischemic stroke. Furthermore, this is the first study to show that increased relative levels of fibrinogen γ' are significantly associated with unfavorable outcome. The γ'/total fibrinogen ratio was associated with unfavorable outcome both in the acute and subacute phase after ischemic stroke.

Some methodological issues of our study have to be discussed. Strengths of our study are the extent and quality of clinical data and availability of short-term follow-up, which enabled us to study fibrinogen γ' in relation to outcome of stroke. Limitations of our study are the relatively small number of patients, and the low rate of unfavorable outcome among our patients. However, this study provided sufficient precision to allow meaningful conclusions about the relationship between fibrinogen γ' and outcome. We observed that the γ' levels were increased most in patients who were presented at the hospital soon after the onset of stroke. In general, patients with severe stroke were admitted to the hospital

sooner after the onset of complaints than patients with milder stroke. Therefore, we cannot distinguish between the effects of the acute phase reaction that was initiated by the stroke, and the severity of stroke. Still, adjustment in the analysis for the time since onset of complaints has only a minor effect on the associations of γ' levels with outcome of stroke. After adjustment for cardiovascular risk factors, levels of fibrinogen γ' were still significantly increased in patients, but the γ' ratio was similar in patients and controls. Also, when looking at associations with outcome, the absolute γ' level had a stronger association with outcome than the γ'/total fibrinogen ratio. This may indicate that the absolute level of fibrinogen γ' is perhaps more important than the γ'/total fibrinogen ratio.

Our results confirm those of our previous studies that showed increased total fibrinogen and fibrinogen γ' levels in the acute phase of ischemic stroke[8]. Also, earlier studies have found a relation between higher post-stroke total fibrinogen levels and poor outcome measured on the modified Rankin Scale[16]. We found that higher total fibrinogen levels are associated with unfavorable outcome as well, but in addition we found that the association between increased fibrinogen γ' levels and unfavorable outcome after ischemic stroke is independent of total fibrinogen levels.

Fibrinogen γ' has antithrombotic properties; one of these is a high affinity binding site for thrombin, which may result in lower levels of circulating thrombin available for clot formation[17]. Also, γ' fibrinogen has a disrupted binding site for platelet integrin $\alpha_{IIb}\beta_3$, which diminishes its capacity to facilitate platelet aggregation[7]. On the other hand, fibrin formed from γ'-containing fibrinogen has a finer, more tightly branched network and smaller pores than fibrin formed from γA fibrinogen[18,19]. *In vitro* studies have reported that fibrinogen γ'-rich clots show a lower clot lysis rate than clots formed from γA fibrinogen[20,21]. Increased total fibrinogen levels have been shown to increase risk of cardiovascular disease[22-24]. Several studies in the convalescent phase of thrombotic diseases show a decreased fibrinogen γ' level, which is thought to resemble the level before occurrence of an event. When considering fibrinogen γ' as a protein with mostly antithrombotic properties, it is possible that increased baseline fibrinogen levels, combined with a low fibrinogen γ'/total fibrinogen ratio, could predispose to atherothrombotic disease. In the acute phase of thrombotic diseases, the fibrinogen γ' level then increases, as was seen in previous studies[1] as well as our study. Based on findings from previous studies and our study, it is still too early to use fibrinogen γ' assays for diagnostic or prognostic evaluation in acute stroke, for which further studies are needed.

In conclusion, we have found that levels of fibrinogen γ' are increased in the acute phase of ischemic stroke. Furthermore, increased levels of fibrinogen γ' are associated with unfavorable outcome. Future research should be aimed at unraveling the role of fibrinogen γ' in the pathogenesis and prognosis of cardiovascular diseases such as ischemic stroke, in which the genetic background of fibrinogen γ' levels may be of particular interest.

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Chapter 4.2

Fibrinogen γ′ levels in patients with intracerebral hemorrhage

Abstract

Background: The fibrinogen γ' variant (γ') has both antithrombotic and prothrombotic properties when compared to normal fibrinogen. It may therefore be of relevance in intracerebral hemorrhage and intraventricular extension of the bleeding.

Objective: To study the role of γ' in intracerebral hemorrhage, and in intraventricular extension of the hemorrhage.

Patients/Methods: We performed a case-control study in 156 controls and 55 patients with intracerebral hemorrhage, with and without intraventricular extension. Levels of fibrinogen γ' and the γ'/total fibrinogen ratio were measured in all participants.

Results: Levels of γ' were increased in patients with intracerebral hemorrhage when compared with controls (0.40 vs 0.32 g/l, p<0.001). The γ'/total fibrinogen ratio was similar in patients and controls (0.092 vs 0.096 p=0.42). There was evidence for an unfavorable outcome in patients with fibrinogen levels in the highest tertile compared with the lowest tertile (OR 4.0, 95%CI 1.1-15.2), and a nonsignificant trend toward unfavorable outcome with higher levels of γ' (p-value for trend = 0.06).

Conclusions: Our study shows that absolute levels of fibrinogen γ' are increased in patients with intracerebral hemorrhage, but relative levels are similar in patients and controls, suggesting that the absolute rise in γ' is an acute phase response.

Introduction

Fibrinogen is one of the central proteins in hemostasis, and several isoforms are present in the blood. One of these isoforms is fibrinogen γ' (γ'), which contains an altered γ chain that is present in 8 to 15% of fibrinogen molecules[1]. Fibrinogen γ' is produced after alternative mRNA processing of the γ chain gene product[2,3]. It has antithrombotic properties such as a binding site for thrombin[4] and a disrupted binding site for platelet integrin $\alpha_{IIB}\beta_3$ [5], as well as a high-affinity binding site for factor XIII:B, which is considered prothrombotic.

Intracerebral hemorrhage (ICH), resulting from rupture of blood vessels in the brain, is a severe neurologic disorder leading to a high rate of mortality and disability[6]. Hemostasis plays an important role in the pathophysiology of hemorrhagic disorders, such as ICH, and plays a role in hematoma growth[6]. Larger hematomas are associated with extension of the bleeding into the brain ventricles (intraventricular extension, IVE)[7]. IVE is associated with an unfavorable prognosis, resulting in a higher mortality[8].

In several cardiovascular disorders, such as myocardial infarction and ischemic stroke, a pathogenic role of γ' has been suggested but not yet proven[9,10]. The role of γ' in hemorrhagic disorders such as ICH has, however, not yet been studied. We therefore aimed to determine the association between γ' levels and the γ'/total fibrinogen ratio and occurrence of ICH, and with intraventricular extension.

Methods

We performed a case-control study in 55 consecutive patients admitted to our hospital with non-traumatic intracerebral hemorrhage between July 2006 and July 2009, and 156 age- and sex matched stroke-free controls. Participants had no history of coagulation disorders. All individuals participated in the Erasmus Stroke Study (ESS), a hospital-based registry of patients with cerebrovascular disorders treated at our hospital. The ESS also includes population-based controls, mostly friends and spouses of patients, but no family. All participants provided written informed consent. The institutional Medical Ethics Committee approved the study.

Upon admission, all patients underwent a plain brain CT scan to confirm the diagnosis and to determine the presence or absence of intraventricular extension of the hemorrhage. Functional outcome was evaluated at discharge by means of the modified Rankin Scale (mRS)[11], which is a score for handicap and disability ranging from 0 (no symptoms) to 6 (death). At a score of 2 patients have disabilities interfering with activities but are still able to live completely independent; at a score of 3 patients are dependent for activities of daily living. Unfavorable functional outcome was defined as mRS>2, signifying loss of independency.

Citrated blood was drawn 3-6 days after admission and centrifuged at 1700 g for 15 minutes. Citrated plasma was frozen at -80°C within 2 hours from collection and stored until analysis.

Laboratory analyses

Total fibrinogen levels were measured according to von Clauss on a fully-automated coagulation analyzer (Sysmex CA-1500 system, Siemens Healthcare Diagnostics, Breda, the Netherlands). Fibrinogen

γ′ antigen levels were measured with an enzyme-linked immunosorbent assay as described previously[12] using anti-γ′ fibrinogen antibodies for catching and HRP-labeled rabbit anti-fibrinogen antibodies for tagging.

Statistical analysis

Apart from fibrinogen γ′ levels we also determined the γ′/total fibrinogen ratio, to adjust for total fibrinogen levels, which are known to increase as results of an acute phase reactions[13]. Differences in γ′ levels and γ′/total fibrinogen ratio between patients and controls and within patient subgroups were assessed by means of multiple linear regression, adjusted for age and sex. The relation between γ′ levels and the γ′/total fibrinogen ratio and functional outcome was assessed using logistic regression and expressed as odds ratio (OR) per tertile increase in fibrinogen level, the lowest tertile being the reference category. All statistical analyses were performed using SPSS 17.0 for Windows (SPSS Inc., Chicago, Illinois). A probability value <0.05 was considered significant.

Results

Median age was 63 years in patients and 60 years in controls (difference not significant), 55% of patients and 49% of controls were men (not significant). Of all patients, 8 (15%) had intraventricular extension of the hemorrhage.

Total fibrinogen and γ′ were significantly higher in patients than in controls (table 1). Table 1 also shows levels in patients with and without intraventricular extension of the hemorrhage. We found no difference in the γ′/total fibrinogen ratio between patients and controls. However, the subgroup of patients with IVE had a significantly higher γ′/total fibrinogen ratio than controls and patients without IVE.

Table 1. Levels of total fibrinogen, fibrinogen γ′ and γ′/total fibrinogen ratio in patients and controls

	Patients			Controls				
	All N = 55	No IVE N = 47	IVE present N = 8	N = 156	P value*	P value†	P value‡	P value§
Total fibrinogen (g/l)	4.41 (1.21)	4.41 (1.23)	4.40 (1.13)	3.37 (0.72)	<0.001	0.95	<0.001	<0.001
Fibrinogen γ′ (g/l)	0.40 (0.14)	0.38 (0.14)	0.51 (0.13)	0.32 (0.13)	<0.001	0.02	0.01	<0.001
γ′/total fibrinogen ratio	0.092 (0.031)	0.087 (0.032)	0.119 (0.032)	0.096 (0.028)	0.42	0.01	0.08	0.03

Presented are means (SD). All analyses are adjusted for age and sex. *P value between all patients and controls; †P value between patients with and without IVE; ‡P value between patients without IVE and controls; §P value between patients with IVE and controls

The median modified Rankin Scale score at discharge was 2 (interquartile range 2-4). Patients with fibrinogen levels in the highest tertile had a significantly higher probability of unfavorable outcome, compared with patients in the lowest tertile. There were also signs of a linear relationship (p-value for

trend over tertiles 0.12, table 2). Levels of fibrinogen γ' seemed to be higher in patients with unfavorable outcome, but not statistically significant. These risks were not attenuated when patients with IVE were left out of the analysis.

Table 2. Risk of unfavorable functional outcome (mRS >2) and levels of fibrinogen and fibrinogen γ' and the γ'/total fibrinogen ratio, in tertiles of the distribution.

	Tertiles			
	1	**2**	**3**	**p-trend**
Fibrinogen				
Distribution	<3.8 g/l	3.8 – 4.4 g/l	>4.4 g/l	
All patients	1.00 (ref)	1.56 (0.41-5.95)	4.00 (1.05-15.21)	0.12
Only patients without IVE	1.00 (ref)	1.35 (0.32-5.71)	2.62 (0.66-10.48)	0.39
Fibrinogen γ'				
Distribution	<0.33 g/l	0.33 – 0.44 g/l	>0.44 g/l	
All patients	1.00 (ref)	5.60 (1.36-23.06)	2.80 (0.71-11.10)	0.06
Only patients without IVE	1.00 (ref)	6.24 (1.44-27.06)	2.60 (0.56-12.02)	0.05
γ'/total fibrinogen ratio				
Distribution	<0.078	0.078 – 0.101	>0.101	
All patients	1.00 (ref)	0.80 (0.21-3.00)	1.72 (0.47-6.32)	0.50
Only patients without IVE	1.00 (ref)	0.83 (0.21-3.35)	2.25 (0.54-9.45)	0.38

Shown are OR (95%CI)

Discussion

In this case-control study, we found that levels of fibrinogen γ' were strongly elevated in patients with intracerebral hemorrhage as compared to control persons. In the total patient group, the fibrinogen γ'/total fibrinogen ratio was similar to controls, but subgroup analyses revealed an increased γ'/total fibrinogen ratio in patients with intraventricular extension of the hemorrhage, while in patients without IVE, this γ'/total fibrinogen ratio was somewhat lower, although not statistically significant, than in controls.

Strengths of our study are the novelty of studying fibrinogen γ' in intracerebral hemorrhage, and the availability of a functional outcome measure. A methodological concern of our study is the small number of patients. Intracerebral hemorrhage, a rare disorder when compared to ischemic stroke, has a high mortality and many patients die before arriving in the hospital or within hours from admission[6,14]. This makes any study of patients with intracerebral hemorrhage, including our own, vulnerable to bias as many patients are not included. A second point is the case control design of the study, which makes it difficult to study causal relationships between fibrinogen γ' and ICH.

Although the anti- and prothrombotic properties of γ' have been studied quite extensively, most clinical research in this field has been done in thromboembolic disorders such as venous thrombosis,

myocardial infarction and ischemic stroke[10,12,15-17]. No studies have yet been performed focusing on the antithrombotic properties of γ' in relation to hemorrhage.
The γ' chain has a binding site for thrombin, with both antithrombotic or prothrombotic consequences, which have been subject of discussion[4,18,19]. Despite this, thrombin binding to the γ' chain overall seems to be antithrombotic, reducing the free thrombin content and inhibiting thrombin-mediated platelet activation[20,21]. Also, the binding of thrombin leads to a selective inhibition of the intrinsic coagulation pathway[19]. An additional antithrombotic property of γ' is the decreased binding to platelet integrin $\alpha_{IIB}\beta_3$ by the γ' chain, leading to a diminished platelet aggregation activity[22]. Given the antithrombotic properties of γ', one might hypothesize that γ' might also affect the risk and outcome of (intracerebral) hemorrhage. Thus far, no studies have been done on this particular subject.

In our study we found that γ' levels were indeed increased in patients with intracerebral hemorrhage. However, due to the case control design we can't rule out that this finding is the result of an acute phase response in ICH patients[9].
Previous studies have shown a stronger acute phase response in patients with blood in the intraventricular or subarachnoid space[23-25]. The relevance of intraventricular blood may play a role in our finding that relative fibrinogen γ' levels seem to increase significantly only in patients with extension of bleeding into the ventricles. However, in our study the presence or absence of IVE did not affect total fibrinogen levels. The reason for this discrepancy remains unknown, but it has been hypothesized that the direct contact between blood and cerebrospinal fluid might play a role[23].
Our finding that patients with high levels of total fibrinogen had a significantly higher chance of unfavorable outcome is in line with findings from previous studies[26,27]. The prognostic value of fibrinogen γ' in patients with ICH is still unclear. The risk estimates we found for unfavorable outcome in higher tertiles of fibrinogen γ', was present in all patients, also after removing patients with IVE from the analysis. Larger studies are required to explore the prognostic value of fibrinogen γ' in patients with ICH.
Any hemorrhage leads to consumption of coagulation factors; for intracerebral hemorrhage, literature is very limited with very little knowledge on consumption in this category of patients. The volume of the hemorrhage is usually small, varying depending on the method used, between 30-50 ml[28,29]. Considering the small size of the hemorrhage, systemic consumption of coagulation factors will be minimal and not affect the results that we measured in our study.

In conclusion, the level of fibrinogen γ' is increased in patients with intracerebral hemorrhage compared with controls; however, the ratio of γ'/total fibrinogen ratio is similar, suggesting that the rise in fibrinogen γ' may reflect an acute phase response.

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Chapter 4.3

Von Willebrand Factor levels are strongly associated with atherosclerosis in patients with ischemic stroke

Abstract

Background: Increased levels of von Willebrand Factor (VWF) are associated with increased risk of ischemic stroke, albeit its mechanism is yet unknown. One might hypothesize that there is a role for atherosclerosis in this association, as it is known that levels of VWF increase due to endothelial dysfunction. Therefore, we investigated the association between levels of VWF antigen (VWF:Ag) and presence of atherosclerosis in patients with ischemic stroke or transient ischemic attack (TIA).

Methods: We included 985 consecutive patients with ischemic stroke or TIA, in whom we performed CT angiography of the craniocervical vessels and measured VWF:Ag levels. Presence of calcifications and stenosis degree were scored for all arterial segments from the supra-aortic arteries to the intracranial vessels. All events were classified according to TOAST criteria. Analyses were adjusted for potential confounders including blood group and cardiovascular risk factors.

Results: Levels of VWF:Ag were significantly increased in the presence of any calcification or stenosis. Levels of VWF:Ag rose linearly with increasing number of calcifications (P=0.008), with increasing degree of stenosis in the carotid artery (P<0.001), and with increasing degree of the most severe stenosis in any of the arteries (P<0.001). Patients with a large artery atherosclerosis etiology of ischemic stroke or TIA had significantly increased levels of VWF:Ag compared with other stroke subtypes (P<0.001).

Conclusions: Our study shows a positive association between calcifications and stenosis degree in the craniocervical arteries of patients with ischemic stroke or TIA, and levels of VWF:Ag. Furthermore, levels of VWF:Ag are highest in patients with large artery atherosclerosis. This suggests that atherosclerosis leads to increased levels of VWF:Ag, thereby contributing to the association between VWF:Ag levels and risk of ischemic stroke.

Introduction

Von Willebrand Factor (VWF) plays a crucial role in platelet adhesion and aggregation, the initial steps in thrombus formation. VWF is a multimeric plasma protein that is produced by endothelial cells and megakaryocytes[1,2]. In the event of endothelial damage, plasma levels of VWF increase, and VWF levels may thus serve as a marker of endothelial damage or dysfunction[3]. Endothelial dysfunction is the first phase in the development of atherosclerotic plaques[4], making plasma VWF levels a marker of early atherosclerosis.

Previous studies have shown increased levels of VWF to be associated with an increased risk of coronary heart disease and stroke[5-10].The increase of VWF may in part be genetically determined, but also acquired cardiovascular factors are known to increase VWF levels[29]. In ischemic stroke, a particular association with large artery atherosclerosis and cardioembolic stroke was found[11]. Prospective studies have identified VWF levels as a predictor of stroke[12,13]. However, the mechanism by which increased VWF levels lead to stroke is still unclear. It is still debated whether VWF levels are a consequence of arterial thrombosis or atherosclerosis, or merely a marker of atherosclerosis, which is encountered in nearly all patients with ischemic stroke. As there is a relation to endothelial activation and an association has been found with a large vessel subtype of ischemic stroke, there may be an association between VWF and atherosclerosis.

Atherosclerosis plays are major role in cardiovascular diseases, including ischemic stroke. The relation between levels of VWF and the degree of atherosclerosis in patients with cerebral ischemia has not been well studied. Therefore, we aimed to study the association between levels of VWF and the degree of atherosclerosis in the craniocervical arteries in patients with ischemic stroke or transient ischemic attack (TIA), and with etiologic subtypes of cerebral ischemia.

Methods

Study population

For the current study, we included all consecutive patients with ischemic stroke or TIA from the Erasmus Stroke Study, an ongoing registry of patients with cerebrovascular diseases treated at our hospital, from December 2005[30]. Ischemic stroke was defined as a focal neurological deficit of presumed vascular origin lasting ≥24 hours or leading to death within 24 hours, with brain imaging studies showing no abnormalities or typical signs of infarction. TIA was defined as a focal neurological deficit of presumed vascular origin lasting less than 24 hours, with imaging studies showing no abnormalities.

Based on all available information patients were classified according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST)[14] criteria. This is an etiological classification model for patients with ischemic stroke or TIA. Large artery atherosclerosis was defined as cerebral ischemia due to a stenosis ≥70% in an ipsilateral artery. A cardioembolic cause was defined as presence of atrial fibrillation, atrial flutter, mechanical valve, or other definite cardioembolic causes. Small vessel occlusion was defined as presence of a lacunar infarct on imaging studies, with concordant clinical symptoms. Other causes of ischemia were hematopoietic malignancies, arterial dissection, vasculitis and other rare causes of

cerebral ischemia. Patients who did not fit either of the categories or who fit multiple categories were classified as cerebral ischemia of undetermined etiology.
Hypertension and hypercholesterolemia were defined as the use of antihypertensive or cholesterol lowering drugs, respectively, before the inclusion event. Diabetes mellitus was defined as the use of oral and/or parenteral antidiabetic drugs before the event. Smoking status was assessed at the time of stroke or TIA and dichotomized as current smoking or not. Patients were considered to have a history of ischemic heart disease when they had a documented myocardial infarction, angina pectoris or cardiac revascularization therapy. Peripheral arterial disease was defined as a history of intermittent claudication, peripheral vascular surgery or amputation due to lower limb ischemia.
All participants provided written informed consent. The study was approved by the Medical Ethics Committee of the Erasmus MC University Medical Center.

Blood samples, VWF measurement and blood group assessment

Blood sampling was performed early after onset of symptoms (median six days, interquartile range 3-15 days), in the stroke unit or the outpatient clinic. Citrated blood was centrifuged at 1700g for 15 minutes at room temperature, and stored at -80°C within 2 hours from collection. DNA was isolated from blood using MagNA Pure (Roche Diagnostics) and stored at -80°C until analysis.
VWF antigen (VWF:Ag) levels were determined with an in-house ELISA. In brief, plates were coated with anti-human VWF antibodies (DakoCytomation, Glostrop, Denmark); after incubation plasma samples were added. Then HRP-conjugated anti-human VWF antibodies were added (DakoCytomation, Glostrop, Denmark). Plates were read with BioTek's microplate reader (BioTek). Reference standard plasma was used as a calibrator. The intra-assay coefficient of variation was 3.2%.
Blood groups were assessed with a standard measurement of blood group antibodies in 605 patients. In the remaining 380 patients, we genotyped rs687289, which can be used to assess blood group O or non-O status, using custom TaqMan Genotyping Assays (Applied Biosystems, Foster City, CA, USA). Endpoint fluorescence was measured on the ABI 7900HT instruments (Applied Biosystems, Foster City, CA, USA) and clustered according to genotype using SDS 2.1 software (Applied Biosystems, Foster City, CA, USA)[31]. Genotyping was successful in 98% of all patients.

CT angiography

A CT angiography (CTA) from the aorta to the intracranial vessels was performed routinely according to a standard protocol. Vessels were analyzed separately: aortic arch, brachiocephalic trunk, subclavian artery, common carotid artery, carotid bifurcation, internal carotid artery, carotid siphon and vertebral artery. For each vessel segment the presence or absence of calcifications was assessed. The number of vessel segments with calcifications was added up to form a calcification score, with a minimum of 0 and a maximum of 13.
The stenosis degree for each artery was measured according to the North American Symptomatic Carotid Endarterectomy Trial (NASCET) criteria[15]. The highest degree of stenosis in the left and right

carotid bifurcation or internal carotid artery were added up and divided by two in order to calculate a mean stenosis degree for the bifurcation / internal carotid artery. Also, the highest stenosis degree in any of the scored arteries per participant was categorized as no stenosis, <30% stenosis or ≥30% stenosis.

Statistical analysis

Levels of VWF:Ag were not normally distributed and therefore logtransformed for analyses. In the results, geometric means and standard deviations (SD) are shown.

Correlation between levels of VWF:Ag and time from event to blood drawing was assessed with Spearman correlation. Associations between presence of calcifications, mean stenosis degree, maximum stenosis degree and VWF:Ag were assessed using linear regression analysis. We evaluated the relation between calcification score, maximum stenosis degree and VWF levels by means of multivariate linear regression, with calcification score and maximum stenosis degree as a continuous variable (per point increase in score or 10% increase in stenosis), and in tertiles.

Differences in VWF:Ag level between TOAST subtypes were assessed using ANOVA. Associations between VWF:Ag levels and TOAST subtypes were assessed using a logistic regression analysis, where the risk for one subtype was compared with risk of all other subtypes combined.

After unadjusted analyses, we adjusted for potential confounders: age, sex, ABO blood group, smoking, hypertension, hypercholesterolemia, and diabetes mellitus.

All analyses were performed using SPSS version 17.0 (IBM, Somers, NY, USA). A P value <0.05 was considered to indicate statistical significance.

Results

From December 2005 until December 2010, we included 1524 consecutive patients with ischemic stroke or TIA in the ESS. For 1362 patients CTA was available, reasons for not performing CTA were very poor renal function, significant comorbidity with resultant very short life expectancy and very severe stroke with likely fatal outcome. Plasma samples were available from 1005 patients. Of those, DNA samples were missing in 20 patients, resulting in a study population of 985 patients. Baseline characteristics are shown in table 1. Mean age was almost 62 years, with 47% females. As expected in our cohort of patients with ischemic stroke or TIA, hypertension, hypercholesterolemia and smoking were highly prevalent.

The mean VWF:Ag level in our patients was 1.43 IU/ml (SD 0.42 IU/ml). Patients with bloodgroup O had significantly lower levels of VWF:Ag compared with those with a non-O bloodgroup (1.24 IU/ml vs 1.62 IU/ml, p<0.001). We found no association between VWF:Ag levels and time from event to blood drawing (P=0.2).

Calcifications and VWF:Ag plasma levels

For 967 patients (98.2%) a calcification score was available, with a mean ± SD score of 3.9 ± 3.8. No calcifications were found in 303 patients (31%). Patients with presence of any calcification had

significantly higher levels of VWF:Ag compared with those without calcifications (1.42 vs 1.21 IU/ml, P<0.001, after adjustment for confounders P=0.008).

We found a significant association between VWF:Ag levels and the calcification score, vWG:Ag levels rose linearly in increasing tertiles of the calcification score; these associations were not attenuated after adjustment for potential confounders (figure 1 and table 2).

Table 1. Baseline characteristics of the study population (N=985)

Age, years	61.8 (13.6)
Female	462 (47)
Current cigarette smoking	299 (30)
Body mass index, kg/m^2	27.3 (13.1)
Hypertension	531 (54)
Hypercholesterolemia	354 (36)
Diabetes mellitus	140 (14)
History of	
Atrial fibrillation	61 (6)
Ischemic heart disease	186 (19)
Peripheral arterial disease	46 (5)
Bloodgroup	
O	445 (45)
Non-O	534 (54)
Missing	6 (1)
Duration of symptoms	
TIA (<24 hours)	452 (46)
Ischemic stroke (≥24 hours)	533 (54)
TOAST classification	
Large artery atherosclerosis	167 (17)
Cardioembolism	117 (12)
Small vessel occlusion	194 (20)
Other determined etiology	55 (6)
Undetermined etiology	452 (46)

Data are presented as N(%), unless for age and body mass index, where mean(SD) are shown.

Table 2. Association between calcification score and levels of VWF, in tertiles and per point increase in calcification score

	Mean level VWF, IU/ml (SD)	Coefficient	
		Unadjusted	Adjusted†
Tertiles:			
T1: ≤1 calcification	1.37 (0.61)		
T2: 2-5 calcifications	1.55 (0.66)		
T3: ≥6 calcifications	1.77 (0.67)		
Continuous*		0.03 (P<0.001)	0.02 (P<0.001)

*mean difference in ln(VWF:Ag) level per point increase in calcification score
†adjusted for age, sex, bloodgroup, current smoking, hypertension, hypercholesterolemia and diabetes mellitus

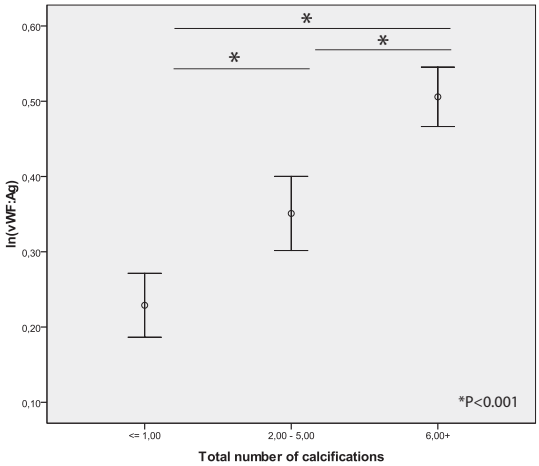

Figure 1. Levels of mean ln(VWF:Ag) with corresponding 95%CI per tertile of total number of calcifications.

Mean carotid artery stenosis degree and VWF:Ag plasma levels

For four participants data were unavailable to calculate mean stenosis degrees in the carotid artery. In the remaining patients, the mean stenosis degree was 12.0% (95%CI 10.7-13.3%). A total of 593 patients (60.4%) had no stenosis in the left and right carotid bifurcation or internal carotid artery.

Increases in mean stenosis degree in the carotid bifurcation and internal carotid artery was associated with an increase in VWF:Ag (table 3).

Maximum stenosis degree in any artery and VWF:Ag plasma levels

In 32 patients (3.2%) the scan was incomplete, rendering calculation of maximum stenosis degree in all arteries combined impossible. For the remaining 953 patients, the mean maximum stenosis degree in any of the scored arteries was 33.2% (95%CI 30.9-35.5%). In 365 patients (38.3%) there was no stenosis in any of the arteries, whereas 88 patients (9.2%) had an occlusion of one of the arteries. Levels of VWF:Ag rose linearly with increasing tertiles of the maximum stenosis degree. An increase in the maximum stenosis degree led to higher VWF:Ag levels (table 3).

Patients without a stenosis in any of the arteries had significantly lower VWF:Ag levels compared with patients with a stenosis in any of the arteries (geometric mean 1.28 vs 1.53 IU/ml, P<0.001).

Etiologic subtypes of stroke or TIA and levels of VWF:Ag

Levels of VWF:Ag differed significantly between subtypes of stroke (P<0.001, after adjustment for confounders P=0.002). Levels of VWF:Ag were significantly increased in patients with a large artery atherosclerosis or cardioembolic subtype of ischemic stroke or TIA, compared with the other subtypes (1.64 vs 1.39 IU/ml, P<0.001; and 1.56 vs 1.42 IU/ml, P=0.02, respectively). Patients with a small vessel occlusion etiology had significantly lower levels of VWF:Ag than patients in other etiologic groups (OR for small vessel occlusion per 0.1 IU/ml increase in VWF:Ag 0.94, 95%CI 0.90-0.98).

Table 3. Association between mean carotid stenosis degree and maximum stenosis degree and levels of VWF:Ag, in tertiles and per 10% increase in stenosis

	Mean level VWF (IU/ml)	Coefficient	
		Unadjusted	Adjusted†
Mean carotid stenosis degree tertiles:			
T1: 0%	1.46		
T2: 1-8%	1.62		
T3: ≥9%	1.73		
Continuous*		0.04 (P<0.001)	0.02 (P<0.001)
Maximum stenosis degree tertiles:			
T1: 0%	1.39		
T2: 1-46%	1.60		
T3: ≥47%	1.70		
Continuous*		0.02 (P<0.001)	0.01 (P<0.001)

* mean difference in ln(VWF:Ag) level per 10% increase in stenosis degree
†adjusted for age, sex, bloodgroup, current smoking, hypertension, hypercholesterolemia and diabetes mellitus

Discussion

The underlying mechanism of the association between levels of von Willebrand Factor and risk of ischemic stroke is unknown, but may be associated with atherosclerosis. In this study we investigated associations between levels of VWF and atherosclerosis in craniocervical arteries in patients with ischemic stroke or TIA. We found strong associations between presence of and the number of calcifications, stenosis degree in the carotid artery and other craniocervical arteries, and levels of VWF. Also, we found levels of VWF to be highest in patients with a large artery atherosclerosis subtype of ischemic stroke or TIA.

Before we discuss the results of our study, some methodological issues have to be addressed. This is, to the best of our knowledge, the first study to investigate VWF levels and atherosclerosis in patients with ischemic stroke or TIA. Strengths of our study are the large number of patients of all ages, with availability of well-documented clinical information, extensive scoring of CT angiography of the craniocervical vessels, and plasma of nearly all consecutive patients. A drawback of our study is that we have no stroke-free control persons, to compare our results to. We consider it unethical to perform CT angiography in a large number of healthy persons, which would expose them to radiation and contrast agents. Furthermore, for several patients the CTA was incomplete, resulting in exclusion of those patients from one or more subanalyses in our study. However, this number was very small, at most 3.2% of patients. Last, due to the cross-sectional design of this study we were unable to study the temporal relationship between levels of VWF:Ag and severity of atherosclerosis.

Our study showed that levels of VWF:Ag increase with increasing levels of calcifications or stenosis. This leaves us with two possible hypotheses of cause and consequence: are levels of VWF increased due to

presence of atherosclerosis, and with that, endothelial dysfunction? Or do increased levels of VWF lead to atherosclerosis, and thereby increased risk of ischemic stroke?
It has been well established that endothelial activation or dysfunction leads to an increase of plasma levels of VWF:Ag, due to release from activated endothelial cells. Also, in our study we found that a majority of patients with ischemic stroke or TIA had no signs of atherosclerosis on CTA, but did have slightly increased levels of VWF:Ag compared with levels of VWF:Ag in population-based studies[32]. This makes a causal role for atherosclerosis in increasing VWF:Ag levels less likely.
Moreover, when looking at etiologic subtypes of ischemic stroke and TIA, we found large artery atherosclerosis to be associated with the highest levels of VWF:Ag. This constitutes a group of patients with severe atherosclerosis of the cervical arteries.
In patients with a small vessel occlusion etiology of ischemic stroke or TIA, levels of VWF:Ag were significantly lower than in other subtypes. In this group large artery atherosclerosis does not play a role in the etiology of ischemia, however, the small arterioles of the brain are presumed to be affected due to presence of vascular risk factors[16]. This may also lead to endothelial activation, however, there might be a relation only to certain subtypes of lacunar infarcts[17].
Taking all into account, it seems most likely that the presence of atherosclerosis leads to increased levels of VWF:Ag. This would explain the previously found association between increased levels of VWF:Ag and risk of stroke, as atherosclerosis is an important risk factor for ischemic stroke and TIA.
We used two different markers of atherosclerosis, calcifications and stenosis degree. Both are reflections of the same process, albeit in different points of time. For both calcifications and stenosis, we found similar associations with levels of VWF:Ag. This strengthens our finding of a positive association between VWF and presence of atherosclerosis.

It has been postulated that VWF may have a pathogenetic role in atherosclerosis by increasing platelet adhesion and subsequent plaque formation. Therefore, a number of studies have investigated the development of atherosclerosis in pigs and mice with a deficiency of VWF, showing less atherosclerosis in animals with an absence of VWF[21-23]. However, two postmortem studies of individuals with severe von Willebrand's Disease (vWD), showed advanced atherosclerotic lesions[24,25]. In addition, individuals with type 3 Von Willebrand disease, which is characterized by the lack of VWF in plasma, had normal atherosclerosis compared with an age-matched control group[27].
Other studies have investigated the role of VWF in carotid atherosclerosis by measuring the intima-media thickness, a surrogate marker for carotid atherosclerosis, showing no differences between patients with vWD and healthy controls[26-28].Together with the postportem studies, this suggests that VWF does not play a role in development of atherosclerosis.

Concluding, our study in patients with ischemic stroke or TIA has shown that levels of von Willebrand Factor are strongly increased in the presence of calcifications or stenosis in the craniocervical arteries. Furthermore, levels of VWF are increased most strongly in patients with a large artery atherosclerosis subtype of ischemic stroke or TIA. The association between VWF and atherosclerosis could explain the known relationship between increased levels of VWF and risk of stroke.

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Chapter 5

Genereal Discussion

Stroke is a major disabling disorder, with a huge impact on patients' lives. Unfortunately, many patients cannot be given the answer to a burning question: what was the cause of their stroke? Stroke is a multifactorial disease in which both genetic and non-genetic factors interact[1]. Many of these risk factors are still unknown, largely because they have only limited influence and are therefore difficult to trace.
In this thesis, I have explored potential new risk factors for stroke, focusing on common genetic and hemostatic variants. In this chapter, I will briefly summarize the main findings, discuss methodological issues and scientific and clinical implications of my results, and provide recommendations for future research.

Main findings

New genetic risk factors

Many studies have investigated the role of genetic risk factors in the etiology of stroke. The large majority of these studies was hypothesis-driven, focusing on a candidate gene[2]. Most of these have not been robustly replicated in prospective studies. With the advance of high-throughput sequencing, it became possible to study the entire genome for small, common gene variations that are associated with stroke[3]. These studies are known as genomewide association studies (GWAS) .
With such a GWAS, we identified two single nucleotide polymorphisms (SNPs) in the NINJ2 locus that are associated with risk of ischemic stroke. This locus had not previously been linked to stroke or vascular risk factors. Although the results were highly significant, the underlying pathophysiological mechanism by which this gene might influence the risk of cerebral ischemia remained unclear. Therefore, we performed in-depth analyses of correlations between the uncovered SNPs and clinical and radiological phenotypes in patients who experienced an ischemic stroke or TIA. We observed that patients who had at least one risk allele of one of the two identified SNPs had multiple, short episodes of ischemia, and were likely to have an atherosclerotic cause of the ischemia. This was further supported by the finding that both SNPs were associated with presence of atherosclerotic stenosis and calcification in the craniocervical vessels.
We also performed a genomewide association study in patients with intracerebral hemorrhage, in which we uncovered two loci, CAMTA1 and ATP6V1H, that were associated with hemorrhagic stroke. Both loci have not been studied before in the context of cerebrovascular disease, and their influence on stroke risk remains to be explored.
To further unravel how genetic variants could be causally related to ischemic stroke, we studied associations between metabolic traits and atherosclerosis. From many genes that have previously been shown to be associated with blood pressure, glucose metabolism and lipid spectrum, we found only the GLIS3 gene to be associated with craniocervical artery stenosis. Genetic variants in GLIS3 have been implicated in increased fasting glucose levels, which is a risk factor for the development of atherosclerotic plaques.

Inflammatory and hemostatic factors

During a stroke, damage to the brain parenchyma induces inflammation[4]. This results in an influx of inflammatory proteins, which in itself increases damage to the brain[5]. To further unravel the influence of inflammation on brain parenchyma damage, we studied genetic influence on levels of C-reactive protein (CRP). We found that common haplotypes in the CRP gene increase CRP levels in the acute phase of stroke, implying that rise of CRP levels is not only influenced by infarct size. This suggests that the extent of the inflammatory response after brain ischemia is triggered by CRP levels, and not worsened by the extent of the damage.

Fibrinogen is a major player in hemostasis, as it is the last step in the coagulation cascade. Fibrinogen gamma' is a common isoform of fibrinogen, present in 8-15% of all fibrinogen molecules[6]. Gamma' fibrinogen has both anti- and prothrombotic properties and is therefore of interest in thromboembolic disorders and hemorrhagic disease alike[7-10]. We showed that levels of fibrinogen gamma' are increased in the acute phase of stroke compared to healthy controls. Also, the gamma' levels had prognostic value in patients with ischemic stroke, as higher levels were associated with unfavorable short-term outcome. In patients with hemorrhagic stroke, higher levels of gamma' were associated with intraventricular extension of the bleeding.

Levels of gamma' are regulated by genetic variation in the FGG gene, coding for the fibrinogen gamma chain[11]. Haplotypes of the FGG gene were also associated with acute phase levels of fibrinogen gamma', which may be of relevance as we have earlier shown these acute phase levels to be related to short-term outcome.

Von Willebrand factor (vWF) is another important hemostatic protein, which becomes elevated in plasma in the event of endothelial dysfunction. Also, increased levels of vWF lead to increased risk of stroke. We found that levels of vWF are associated with presence of calcifications and the degree of stenosis in craniocervical arteries in patients with ischemic stroke or TIA. This suggests a relation between presence of atherosclerosis and increased VWF levels, making VWF a biomarker for atherosclerosis. This might explain why increased levels of vWF lead to increased risk of stroke.

Methodological considerations relevant for the studies in this thesis

For all studies in this thesis, I used data from the Erasmus Stroke Study, a large hospital-based registry of patients with neurovascular disorders. Patients were included after they had a TIA or stroke, and blood, DNA and very detailed clinical information were collected. This implies that all our findings originate from a hospital-based series, and cannot be easily generalized to the general population.

Genetic studies

Genomewide association studies are hypothesis-generating, which means that they are designed to uncover new genes that were previously unsuspected. However, they are also hypothesis-driven: common SNPs confer risk of common diseases. After all, there must be a common factor that is shared

between patients who suffer a stroke, but otherwise have a different risk profile.
Large-scale genetic studies like ours, require many participants to detect rare genetic variants associated with disease and to avoid false-positive or false-negative associations[12,13]. Currently, large consortia including >10.000 individuals are being assembled to perform such studies[14-16]. For stroke this is rather difficult as the number of studies that include stroke patients – and the number of patients per study – are limited, and therefore a huge number of cohorts are required to participate.
The multiple testing problem in these studies, due to performing over a million tests for an equal number of SNPs, is usually tackled by applying Bonferroni correction. This method takes the number of independent tests that were performed into account[17]. However, many SNPs on most used chips are not independent from each other. Therefore, simply dividing the desired alpha value by the number of tests performed may not be optimal. It could be better to divide by the number of haplotypes or loci tested, taking into account linkage between markers.
For our study on genes for metabolic traits and atherosclerosis, we used a permutation analysis to work around the multiple testing problem. As our number of patients was very small and the correct p-value to use is unknown, we performed 1,000 permutations of the data. This simulates a larger sample size and could provide valuable information on true versus false associations in our study.

A major problem of GWAS is that SNPs, which are found in such studies, hardly ever represent a functional gene that has directly caused the disease[18]. Most GWAS have identified common genetic variants that were located far away from genes, or if they were close to or within a gene, it had no known biological relevance to the trait studied[19]. Therefore, uncovering new genetic variants is only the beginning of finding new biological pathways leading to complex diseases. This quest for functionality of the SNPs is what should ultimately follow after GWAS that have been replicated. For this purpose, animal models could be used to study the phenotype which occurs by knocking out the gene involved; fine mapping of the found locus could be performed[20], or the phenotype of patients with the found SNPs which may confer an indication of the underlying functional pathway can be investigated. All methods are time-consuming, and the majority are expensive. Nearly all GWAS performed until today did not yield any functional variants yet[19].
Still, there are success stories too. For age-related macular edema and inflammatory bowel disease, new etiologic insights have been uncovered and these may lead to new, targeted treatments[21,22]. For stroke, a very heterogenic disease, I am less optimistic that a functional variant will be found that sheds new light on biologic pathways or lead to new treatments. However, as our current treatment options are disappointingly limited, it is definitely worth a good search.

Many large-scale studies are population-based and have a long follow-up for "stroke" in general, sometimes further characterized as ischemic or hemorrhagic. A drawback is that we have no further information on stroke subtypes, which is of importance as stroke is very heterogeneous. In our hospital-based studies, we could use etiologic subtypes of ischemic stroke or TIA, to more accurately assess

associations between traits or genetics and particular aspects of stroke risk, for example atherosclerosis or cardioembolism.

Hemostatic studies

A large advantage of our study database was the availability of very extensive clinical data from a large group of patients. The major drawback for our hemostatic studies was that blood samples had been drawn mostly in the acute phase of stroke or TIA. Hemostatic proteins increase promptly when metabolic stress occurs, such as after stroke. This specifically applies to fibrinogen, which is upregulated in response to elevated IL-6 levels[23]. Due to this acute phase phenomenon, we were unable to make any statements about fibrinogen gamma' as a risk factor for stroke, as we had no information on the pre-stroke values.

In our studies, we noted longitudinal changes in levels of hemostatic proteins, and examined whether it was useful to adjust for the time between the event and the blood sampling. However, this did not change results of our studies, most likely because the large majority of cases were included within 2 weeks after the event. Nonetheless, this merely accounts for differences within the study population, and does not ameliorate the problem that we have no information on baseline levels, before stroke or TIA.

Future solutions could be to use a prospective study such as the population-based Rotterdam Study with a blood sampling at baseline and follow-up, or blood sampling in the acute phase and again in the convalescent phase (after three to six months). This would give new opportunities to study the behavior of hemostatic proteins such as fibrinogen gamma'. Unfortunately we had no blood samples from both the acute phase and the convalescent phase of our patients. However, we did include patients in a late phase after stroke or TIA (months after the event). We were unable to use them as "late cases", as most of these patients did not seek medical attention until the moment we saw them, which implies that they had mild symptoms. As we are inclined to believe that mild symptoms come with a limited acute phase response, we cannot tell whether the level of gamma' would have been influenced by a passed acute phase response, or by the magnitude of the acute phase response.

CT-Angiography

The use of CT-Angiography (CTA) has become common practice to assess atherosclerosis in patients with stroke or TIA. It is a sensitive and specific method, allows direct measurement of the stenosis degree, and gives information on plaque characteristics such as presence calcifications or ulcerations[24-27].

In this thesis, CTA measures were examined in several studies. All CTAs were scored for presence of calcifications and stenosis degree per vessel segment scanned, typically from the aortic arch to the intracranial vessels. Assessing presence of calcifications is relatively easy, as it stands out as a hyperintense focus in the vessel wall. Assessing degree of stenosis is on the opposite of a spectrum of difficulties. Both commonly used methods, ECST and NASCET[28,29], assess the degree of stenosis in the direction perpendicular to the vessel direction. They do not take into account the amount of outward remodeling of the artery, and uneven surfaces of the plaques which make stenosis degrees differ depending on the angle

in which it is measured. Therefore, it would be better to measure stenosis degrees based on the surface of the lumen that is occluded as a percentage of the total lumen. At present, this technology is under development.

Scientific and clinical implications

Finding new genetic factors that are associated with stroke offers new opportunities. Genes that were disregarded at first, now provide a new focus of etiologic research. Are those genes causal, are they linked to other causal genes, and how do the causal genes or genetic variants exert their effect? New functional pathways will have an impact on clinical and scientific speculations. It may well be that the common hypothesis of atherosclerosis leading to thrombosis and thereby stroke is not the full story. Many genes that have shown to be associated with ischemic stroke in the GWAS, albeit not statistically significant, were involved in neuronal repair, suggesting a role for ischemic preconditioning and response to ischemic injury, as has previously been found in myocardial ischemia[30].

Another possible application of the new genetic knowledge derived from large-scale genetic studies is personalized medicine. Robust risk genes can be determined in healthy persons to predict risk of future disease, leading to a recommendation of primary prevention through medication or lifestyle modifications. This may also be used in secondary prevention, to assess risk of recurrent disease leading to treatment adjustments. There is, however, still a long way to go before this will be reality. Once identified, risk genes have to be robustly replicated and proven as a risk predictor. Consequently, interventions have to be developed to modify the risk of disease.

Elevated fibrinogen levels have been recognized as a risk factor for stroke for several decades[31,32]. However, it has never been added to a primary prevention risk profile, probably because it remains without consequences: we need fibrinogen to form clots, and there are no drugs to decrease fibrinogen levels. The existence of fibrinogen gamma' has also been known for quite a while[33]. Interest in it in thromboembolic disorders has only grown recently. Multiple studies have shown fibrinogen gamma' to be a risk factor for venous thrombosis, myocardial infarction, and probably also for ischemic stroke[34-37]. It has even been proffered to add fibrinogen gamma' to cardiovascular risk assessments, as it was independent of other vascular risk factors in predicting risk of stroke[38].

Clots with a high concentration of gamma' have shown to be less responsive to fibrinolysis in vitro[39-41]. When translated to practice, it is possible that patients with high gamma' levels have clots that do not respond very well to recombinant tissue plasminogen activator (rtPA), as used in acute ischemic stroke. Research should point out whether it is useful to adjust the dose of rtPA for those patients.

Recommendations for future research

Fibrinogen

There have been no prospective studies on fibrinogen gamma' in ischemic stroke. It is therefore still unknown whether gamma' can be used as a prognostic marker to predict risk of future ischemic stroke, as has already been shown for myocardial infarction in the prospective Framingham Heart Study[38]. It would be of great interest to study the relation between gamma' and stroke in a large prospective study such as the Rotterdam Study, which has a vast number of participants and ongoing follow-up for stroke. Until there are prospective studies, it is also impossible to make a statement about fibrinogen gamma' in causality of stroke.

Apart from studying fibrinogen gamma' as a prognostic marker, it would be intriguing to study clot characteristics in patients. In vitro studies have demonstrated that clots formed from fibrinogen with high concentrations of gamma' chains have smaller pores, thinner fibers and more extensively crosslinked fibrin[9,39,42]. Moreover, these clots were more resistant to lysis. However, actual clots from patients with thromboembolic disease have never been studied. The advance of current interventions, including mechanical clot removal in ischemic stroke, offers the possibility to determine whether plasma levels of fibrinogen gamma' are associated with the same clot characteristics in vivo, thereby adding evidence to the possible causal role of fibrinogen gamma'.

Genetics

Genetics is one of the most rapidly evolving areas of research at this moment. Merely five years after the introduction of GWAS, they are already regarded as useless when the number of participants does not meet a particular threshold (thousands). New methods are introduced continuously, with whole exome and genome sequencing as a particularly promising technique[43]. However, the chances of employing this in ischemic stroke in general are minimal, as its phenotype is too heterogeneous. Very specific subtypes could deliver results; unfortunately, specification of stroke subtypes is very difficult. In intracerebral hemorrhage, whole exome sequencing could be more promising as the phenotype is more robust when imaging studies are used to confirm the diagnosis. A very welcome aspect of these studies is that only a few patients are needed, instead of vast consortia.

A less complex but very practical development is the 1000 Genomes project, that will hopefully elaborate our knowledge on rare SNPs in particular[44]. This allows more extensive imputation of genetic data, increasing the chances of finding associations between SNPs and complex diseases, especially for infrequent SNPs.

Gene-finding studies are a first step, but next steps should always follow; how do found SNPs lead to functional genes and to disease? Both for ischemic and hemorrhagic stroke, we have uncovered new loci and genes to be associated with the disease, but their mechanism of action is as yet unknown. For ischemic stroke, results have been replicated in an independent cohort, but not in several other cohorts. For hemorrhagic stroke, replication is yet to follow. When findings are validated, functionality should be studied to elaborate on current knowledge but in particular to develop new treatments.

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Chapter 6

Summary/Samenvatting

Summary

Stroke is a common cardiovascular disorder, and the first cause of disability and second cause of death in industrialized countries. Risk factors for stroke include hypertension, hypercholesterolemia and diabetes mellitus, as well as a positive family history. However, on the basis of known risk factors, a large proportion of stroke cases cannot be explained, stimulating research into yet unknown risk factors. Over the last years, genetic research has taken a leap forward, enabling detailed studies into genetic factors in complex diseases such as stroke. Genetics are important, but it is perhaps more important to study gene products, for instance coagulation and metabolic factors, which exert the actual risk effect.

This thesis focuses on new risk factors in stroke, in particular genetic factors contributing to risk of stroke, and effects of changes in hemostasis proteins on the occurrence and prognosis of stroke.

Chapter 1 describes the background for the research presented in this thesis.

In **Chapter 2.1**, a large-scale genetic study uncovering new genetic risk loci for cerebral infarction is presented. We describe two single nucleotide polymorphisms (SNPs) in a new locus, NINJ2, that is associated with risk of ischemic stroke, in particular an atherothrombotic subtype. In **Chapter 2.2**, I continue with this study by looking at clinical and radiological phenotypes of patients with the risk alleles of SNPs from the previous chapter. The study suggests that the alleles are associated with presence of calcifications and stenosis on vessel imaging in patients with ischemic stroke, confirming the earlier finding of an association with atherothrombotic stroke. **Chapter 2.3** describes a new multi-cohort study and meta analysis of new genetic loci in patients with intracerebral hemorrhage, uncovering the ATP6V1H locus as a risk locus. Results of this study await replication in other studies.

From genetics I continue with effects of genes on gene products. In **Chapter 3.1**, I describe a candidate gene study on the effect of various metabolic traits on presence of atherosclerosis in the craniocervical vessels. A gene associated with fasting glucose and diabetes mellitus, GLIS3, was associated with severity of stenosis and calcifications. **Chapter 3.2** describes a study in patients with acute ischemic stroke, which showed that common genetic variations in the C-reactive protein (CRP) gene alter the level of CRP in the acute phase. That common variation in genes influence levels of proteins in the acute phase is also shown in **Chapter 3.3.** There, a study is presented on fibrinogen gamma' levels, which in acute stroke depend on the individual's haplotype of the fibrinogen gamma' gene.

From genetic influence I move on to the proteins themselves, focusing on hemostatic proteins. In **Chapter 4.1,** I describe the results of a study on levels of fibrinogen gamma' in ischemic stroke, and its influence on functional outcome. We found higher levels of fibrinogen gamma' to be associated with a higher probability of unfavorable functional outcome. Fibrinogen gamma' was also studied in patients with intracerebral hemorrhage in **Chapter 4.2**, where we showed that levels of fibrinogen gamma' are increased in patients with intracerebral hemorrhage although relative levels of gamma' are not, probably reflecting an acute phase phenomenon. **Chapter 4.3** describes a study on von Willebrand factor (VWF) in patients with ischemic stroke, and its association with atherosclerosis in those patients. We found that VWF levels are associated with severity of carotid stenosis, and the number of calcifications in the craniocervical vessels.

In **Chapter 5,** I try to place our findings into a broader perspective, discussing methodologic difficulties in our studies as well as the relevance of our findings and possibilities for future research.

Samenvatting

Wereldwijd zijn hart- en vaatziekten de belangrijkste doodsoorzaak, waarbij een beroerte de tweede belangrijkste doodsoorzaak is. Daarnaast is het de belangrijkste oorzaak van invaliditeit in de westerse wereld. Er zijn twee typen beroerte, een herseninfarct of een hersenbloeding. Ondanks kennis over factoren die het risico op een beroerte verhogen, zoals hypertensie, hypercholesterolemie en diabetes mellitus, is bij een groot deel van de patiënten niet duidelijk waarom ze een beroerte hebben doorgemaakt. Dit is de drijfveer om nieuwe risicofactoren te ontdekken.

De laatste jaren heeft grootschalig genetisch onderzoek bij complexe aandoeningen een vlucht genomen, zo ook bij cardiovasculaire aandoeningen. Alhoewel de genetica de basis vormt voor het functioneren van systemen in het lichaam, zijn de genproducten belangrijker, zoals bijvoorbeeld stollingseiwitten. Zij voeren immers de functies uit die zijn vastgelegd in het DNA.

In dit proefschrift wordt onderzoek beschreven naar nieuwe risicofactoren voor beroertes. Hierbij heb ik met name gekeken naar genetische factoren voor zowel een herseninfarct als een hersenbloeding en naar de effecten van veranderingen in hemostase-eiwitten op het optreden van, en de prognose bij een beroerte. Het onderzoek heb ik uitgevoerd in de Erasmus Stroke Study, een groot, prospectief cohort van patiënten met neurovasculaire ziekten uit het Erasmus MC. Van alle patiënten is gedetailleerde klinische informatie, bloed en DNA verzameld. Inmiddels zijn er meer dan 2000 patiënten geïncludeerd.

Na een introductie in **hoofdstuk 1** beschrijf ik in het volgende hoofdstuk studies die gericht zijn op het vinden van nieuwe genetische varianten die het risico op een beroerte verhogen. In **hoofdstuk 2.1** beschrijf ik het onderzoek in een grote groep patiënten met een herseninfarct, die wordt vergeleken met een grote groep mensen zonder een herseninfarct. In deze grootschalige, internationale studie is gekeken welke verschillen er zijn in het gehele DNA tussen deze twee groepen, wat een aanwijzing kan zijn dat dit een risicofactor is voor het ontstaan van een herseninfarct. Wij hebben een locatie op chromosoom 12 gevonden welke geassocieerd bleek te zijn met een verhoogd risico op een herseninfarct. In **hoofdstuk 2.2** gaan we verder met dit onderzoek, en toon ik aan dat personen met de risico-allelen van de eerder beschreven single nucleotide polymorphisms (SNPs) vaker een TIA hebben dan een herseninfarct, vaak meerdere aanvallen en dat er vaak een atherotrombotische oorzaak is voor de ischemie. In **hoofdstuk 2.3** beschrijf ik een studie waarin een groep patiënten met een hersenbloeding is vergeleken met gezonde mensen, weer over het gehele DNA. Wij vonden dat het ATP6V1H gen is geassocieerd met het risico op het krijgen van een hersenbloeding, nadat we drie verschillende populaties hebben samengevoegd en een meta-analyse van deze data hebben uitgevoerd.

De genen zijn een basis, maar de functie wordt uitgevoerd door producten van de genen, zoals eiwitten. In hoofdstuk 3 ga ik in op de invloed van genetica op bekende risicofactoren voor een beroerte, zoals atherosclerose, en parameters voor ontsteking en stolling. In **hoofdstuk 3.1** beschrijf ik een kandidaat-gen studie waarbij is gekeken naar genen die betrokken zijn bij metabole risicofactoren, en de invloed hiervan op de mate van atherosclerose bij patiënten met een TIA of herseninfarct. Het blijkt dat van deze risicofactoren alleen een gen dat geassocieerd is met een verhoogd nuchter glucose, het risico op vernauwingen in de halsslagaders vergroot, evenals de kans op aanwezigheid van calcificaties in deze

vaten. De invloed van haplotypes in het c-reactive protein (CRP) gen op levels van CRP bij patiënten met en acuut herseninfarct beschrijf ik in **hoofdstuk 3.2**. Hier hebben wij gevonden dat veelvoorkomende genetische varianten in het CRP gen de hoogte van het CRP na een beroerte beïnvloeden. In **hoofdstuk 3.3** beschrijf ik een studie naar de invloed van genetische variatie in het FGG gen op de hoogte van fibrinogeen gamma' in het bloed bij patiënten met een beroerte. Wij toonden aan dat zowel bij gezonde mensen als bij patiënten met een herseninfarct, een veelvoorkomende variatie in dat gen een hoger niveau geeft van fibrinogeen gamma', wat daardoor van invloed is op de kans op een beroerte maar ook op herstel van een beroerte, wat ik verderop in het proefschrift wordt beschrijf.

Het volgende hoofdstuk is gericht op hemostasefactoren en hun invloed op het optreden van een beroerte en de prognose hiervan. In **hoofdstuk 4.1** kijk ik naar de hoogte van fibrinogeen gamma' bij patiënten in de acute en subacute fase na een herseninfarct. Ik laat hierin zien dat het niveau hiervan significant stijgt, en tevens kan dienen als prognostische factor waarbij een hoger level van fibrinogeen gamma' aanleiding geeft tot een minder gunstige uitkomst. Ook bij patiënten met een hersenbloeding tonen wij aan dat het niveau van fibrinogeen gamma' verhoogd is (**hoofstuk 4.2**). Bij deze groep is echter het relatieve level van fibrinogeen gamma' ten opzichte van al het fibrinogeen niet verhoogd, leidend tot de hypothese dat de stijging in fibrinogeen gamma' in ieder geval zeker een acute fase component heeft. In **hoofdstuk 4.3** heb ik de relatie tussen de hoogte van von Willebrand Factor (VWF) en atherosclerose in de halsslagaders onderzocht. In dit hoofdstuk laat ik zien dat een hogere stenosegraad of aantal calcificaties in de slagaders samenhangt met een verhoogd VWF. Dit zou een van de factoren kunnen zijn die kunnen verklaren waarom een verhoogd VWF is geassocieerd met een verhoogd risico op een beroerte.

In **hoofdstuk 5** plaats ik onze bevindingen in een breder perspectief. Daarnaast bespreek ik methodologische problemen in onze studies, de relevantie van onze bevindingen, en doe ik suggesties voor toekomstig onderzoek.

Epilogue

List of publications
Acknowledgements/Dankwoord
About the author
Portfolio

Manuscripts based on studies described in this thesis

Chapter 2.1

Ikram MA, Seshadri S, Bis JC, Fornage M, DeStefano AL, Aulchenko YS, Debette S, Lumley T, Folsom AR, **van den Herik EG**, Bos MJ, Beiser A, Cushman M, Launer LJ, Shahar E, Struchalin M, Du Y, Glazer NL, Rosamond WD, Rivadeneira F, Kelly-Hayes M, Lopez OL, Coresh J, Hofman A, DeCarli C, Heckbert SR, Koudstaal PJ, Yang Q, Smith NL, Kase CS, Rice K, Haritunians T, Roks G, de Kort PL, Taylor KD, de Lau LM, Oostra BA, Uitterlinden AG, Rotter JI, Boerwinkle E, Psaty BM, Mosley TH, van Duijn CM, Breteler MM, Longstreth WT Jr, Wolf PA. Genomewide association studies of stroke.
N Engl J Med. 2009;360(17):1718-28.

Chapter 2.2

van den Herik EG, de Lau LML, Mohamad A, Ikram MA, Koudstaal PJ. Association of two SNPs from genomewide association studies with clinical phenotypes of cerebral ischemia.
Int J Stroke, in press.

Chapter 2.3

van den Herik EG, Struchalin MV, de Lau LML, de Kort PLM, Schmidt R, Breteler MMB, Hofman A, van Duijn CM, Koudstaal PJ, Ikram MA. Genomewide association studies in intracerebral hemorrhage.
In preparation.

Chapter 3.1

van den Herik EG, Struchalin MV, de Lau LML, den Hertog HM, Fonville S, Koudstaal PJ, van Duijn CM. Associations between recently discovered genetic variations in metabolic traits and arterial stenosis in patients with recent cerebral ischemia.
In preparation.

Chapter 3.2

den Hertog HM, **van den Herik EG**, Dippel DWJ, Koudstaal PJ, de Maat MPM. Variation in the C-reactive protein gene is associated with serum levels of CRP in patients with acute ischemic stroke. *Cerebrovasc Dis 2010;29(4):372-5.*

Chapter 3.3

van den Herik EG, Smalberg J, de Lau LML, Leebeek FWG, Koudstaal PJ, de Maat MPM. Haplotypes of the FGG gene affect levels of fibrinogen gamma' but are not related to risk of ischemic or hemorrhagic stroke. *Submitted.*

Chapter 4.1

van den Herik EG, Cheung EYL, de Lau LML, den Hertog HM, Leebeek FWG, Dippel DWJ, Koudstaal PJ, de Maat MPM. Gamma'/total fibrinogen ratio is associated with short-term outcome in ischemic stroke. *Thromb Haemost. 2011;105(3):430-4.*

Chapter 4.2

van den Herik EG, Cheung EYL, de Lau LML, den Hertog HM, Leebeek FWG, Dippel DWJ, Koudstaal PJ, de Maat MPM. Fibrinogen γ' levels in patients with intracerebral hemorrhage.
Thromb Res. 2011 Sep 12, epub ahead of print, doi 10.1016/j.thromres.2011.08.017

Chapter 4.3

van den Herik EG, Sonneveld MAH, van Loon JE, de Lau LML, de Maat MPM, Koudstaal PJ, Leebeek FWG. Von Willebrand Factor levels are associated with atherosclerosis in craniocervical arteries in patients with ischemic stroke or TIA.
In preparation.

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About the author

Evita van den Herik was born on October 8th, 1984 in Leidschendam, the Netherlands. She attended secondary school in the bilingual VWO department of O.S.G. Wolfert van Borselen in Rotterdam, from which she graduated in 2002. In October 2002 she received her International Baccalaureate degree in English. The same year she started her training in medicine at the Erasmus University Rotterdam. During her study, she performed research at the department of Neurology under prof.dr. P.J. Koudstaal on hemostasis and inflammation in ischemic stroke. In 2008 she graduated from medical school and subsequently started the work described in this thesis under supervision of prof.dr. P.J. Koudstaal and dr. L.M.L. de Lau. From October 2010 she started her residency in Neurology at the Erasmus MC (prof.dr. P.A.E. Sillevis Smitt), combined with finishing the research described in this thesis.

PhD portfolio

	ECTS
Courses COEUR	
Congenital heart disease	1.5
Neurovascular and peripheral vascular diseases	1.5
Molecular biology in atherosclerosis and cardiovascular research	1.5
Pathophysiology of ischemic heart disease	1.5
Vascular medicine	1
Clinical cardiovascular epidemiology	1.5
Total	8.5
General courses	
SNP course V (Molmed)	1.4
Introduction to clinical research (NIHES)	0.9
Biostatistics for clinicians (NIHES)	1.0
Genetic analysis in clinical research (NIHES)	1.9
Minicursus CPO	0.3
Modern statistical methods (NIHES)	4.3
Basic course on R (Molmed)	1
Genomewide association analysis (NIHES)	1.9
Total	12.7
In depth courses Nederlandse Hartstichting (Papendal PhD courses)	
Thrombosis and Haemostasis	2
Vascular Biology	2
Total	4
Research seminars COEUR	
Gene and cell based therapies of cardiovascular disease	0.4
Imaging of carotid bifurcation atherosclerosis	0.4
Hypertension	0.4
NT-proBNP measurements in clinical practice	0.4
Total	1.6
Symposia and congresses	
European Stroke Conference 2009 (Stockholm), oral presentation	2
European Stroke Conference 2010 (Barcelona), oral presentation and two poster presentations	3
COEUR PhD day 2010 (Rotterdam), poster presentation	0.5
International Fibrinogen Workshop 2010 (Rotterdam), oral presentation and poster presentation	2.5
European Stroke Conference 2011 (Hamburg), oral presentation and poster presentation	2.5
Total	10.5
Teaching activities	
Supervision of A.Mohamad, keuzeonderzoek	1.7
Supervision of R.E.D. Lamers, keuzeonderzoek	1.5
Supervision of M.A.H. Sonneveld, keuzeonderzoek	1.5
Total	4.7
Total ECTS	42