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GENETIC ASSOCIATION STUDIES IN STROKE

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As time has past, I've had the privilege to get to know some remarkable people and good friends. Sometimes time, distance and our daily goings-on make our get-togethers less frequent. I dedicate this book to you all, and to the fascinating friends I hope to meet in the future.

At times, reading a thesis may become monotonous or tiresome. On such occasions it may prove heartening to rest the thoughts on something else. If this would happen to you as you reed this thesis, I would like to invite you to the world of Claes Hylinger, a personal favourite of mine:

Det är en torsdag morgon
och solen skiner klart
och molnen gå, på himlen blå
med god och stadig fart.
Claes Hylinger,
Nya dagar och nätter

ABSTRACT

Stroke is the third most common cause of death and the most common cause of disability in adults in developed countries. It is a complex disease in which genetic and environmental factors make about equal contributions. A significant proportion of the environmental component remains to be elucidated and little is known about which genes that are involved. There are two main stroke types; ischemic and hemorrhagic. Both these types have several different etiological subtypes.

The specific aim of the present work was to perform clinical association studies to test the hypothesis that hemostatic and inflammatory gene polymorphisms, and/or plasma levels of the respective proteins, are associated with stroke, and to investigate whether associations differ between stroke subtypes.

The studies on ischemic stroke were based on the Sahlgrenska Academy Study on Ischemic Stroke (SAHLSIS), in which great emphasize has been put on phenotyping by physical examination and neuroimaging. The study comprises 600 consecutive ischemic stroke patients presenting with ischemic stroke before the age of 70 years and 600 matched population-based controls. Stroke patients were classified according to the main etiological subtypes of ischemic stroke, i.e. large-vessel disease (LVD), small-vessel disease (SVD), cardioembolic stroke (CE stroke) and cryptogenic stroke. The study on aneurysmal subarachnoid hemorrhage (aSAH) was based on patients admitted to the Neurointensive Care Unit, Sahlgrenska. A total of 183 patients with a confirmed aneurysmal origin of the SAH were included. Two matched population-based controls were recruited for each case. Genotyping was performed using 5´nuclease assays (TaqMan) and plasma levels of proteins were determined by immunological methods.

Family history of stroke showed independent association to all ischemic stroke subtypes, except CE stroke. In our first genetic association study, the fibrinolytic pathway was studied. A reduced risk of ischemic stroke was observed for a genotype combination indicating a high gene expression level of both tissue-type plasminogen activator (tPA) and plasminogen activator inhibitor type 1 (PAI-1). This association was not detected in aSAH. However, an increased risk of aSAH was found for subjects carrying the coagulation factor XIII 34Leu allele. This variant has been shown to influence fibrinolysis by affecting the fibrin network. Family history of myocardial infarction (MI) only showed association to one ischemic stroke subtype, i.e. LVD. The explanation for this may be that atherosclerosis is a common denominator for MI and LVD. In support for this hypothesis, increased plasma levels of the inflammatory marker C-reactive protein was only found in the LVD group. This is in contrast to the fibrinolytic pathway. Plasma levels of tPA, PAI-1 and the fibrinolytic inhibitor TAFI were increased in all ischemic stroke subtypes.

In conclusion, the results support a genetic contribution in stroke. This genetic contribution seems to differ between subtypes, which highlights the importance of subtype classification in stroke research. Furthermore, the findings suggest that inflammatory factors may be of more importance for developing LVD, while the fibrinolytic pathway seems to be involved in all ischemic stroke subtypes.

Key words: stroke, ischemic stroke subtypes, subarachnoid hemorrhage, genetics, polymorphism, fibrinolysis, tPA, PAI-1, TAFI, CRP, factor XIII

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LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I Jood K, Ladenvall C, Rosengren A, Blomstrand C, Jern C. Family history in ischemic stroke before 70 years of age: The Sahlgrenska Academy Study on Ischemic Stroke (SAHLSIS).
Stroke 2005;36:1383-1387.

II Jood K, Ladenvall P, Tjarnlund-Wolf A, Ladenvall C, Andersson M, Nilsson S, Blomstrand C, Jern C. Fibrinolytic gene polymorphism and ischemic stroke.
Stroke 2005;36:2077-2081.

III Ladenvall C, Gils A, Jood K, Blomstrand C, Declerck PJ, Jern C. Thrombin activatable fibrinolysis inhibitor activation peptide shows association with all major subtypes of ischemic stroke and with TAFI gene variation.
Arterioscler Thromb Vasc Biol 2007;27:955-962.

IV Ladenvall C, Jood K, Blomstrand C, Nilsson S, Jern C, Ladenvall P. Serum C-reactive protein concentration and genotype in relation to ischemic stroke subtype.
Stroke 2006;37:2018-2023.

V Ladenvall C, Csajbok L, Nylén K, Jood K, Nellgård B, Jern C. Association between factor XIII single nucleotide polymorphisms and aneurysmal subarachnoid hemorrhage.
In manuscript.

CONTENTS

ABBREVIATIONS

A	Adenine
ANOVA	Analysis of variance
AP	released activation peptide
aSAH	Aneurysmal subarachnoid hemorrhage
BMI	Body mass index
bp	Base pair
C	Cytosine
CI	Confidence interval
CE stroke	Cardioembolic stroke
CRP	C-reactive protein
CT	Computer tomography
DNA	Deoxyribonucleic acid
ECG	Electrocardiogram
EM	Expectation maximization
ELISA	Enzyme-linked immunosorbent assay
FXIII	Coagulation factor XIII
G	Guanine
GOSE	The extended Glasgow outcome scale
GWA	Genome-wide association
Hcy	Homocysteine
hsCRP	high sensitive C-reactive protein
ICH	Intracerebral hemorrhage
LACI	Lacunar infarct
LD	Linkage disequilibrium
LVD	Large vessel disease
MAF	Minor allele frequency
MI	Myocardial infarction
MRI	Magnetic resonance imaging
mRS	The modified Rankin scale
NCBI	National Center for Biotechnology Information
NICU	Neurointensive Care Unit
OCSP	Oxfordshire Community Stroke Project
OR	Odds ratio
PACI	Partial arterial circulation infarct
PAI-1	Plasminogen activator inhibitor type 1
PCR	Polymerase chain reaction
POCI	Posterior circulation infarct
RNA	Ribonucleic acid
SAH	Subarachnoid hemorrhage
SAHLSIS	Sahlgrenska Academy Study on Ischemic Stroke
SD	Standard deviation
SNP	Single nucleotide polymorphism
SVD	Small vessel disease

T	Thymine
TACI	Total arterial circulation infarct
TAFI	Thrombin activatable fibrinolysis inhibitor
TIA	Transient ischemic attack
TOAST	Trial of Org 10172 in Acute Stroke Treatment
tPA	tissue-type plasminogen activator
UTR	Untranslated region
WHO	World Health Organisation
WHR	Waist to hip ratio

INTRODUCTION

Stroke is a very common neurological disease. According to the World Health Organisation (WHO), it was the second most frequent cause of mortality worldwide in 1990, and the third most common cause of mortality in more developed countries [Sarti 2000]. It accounts for approximately 10% of all deaths in the world and is also a leading cause of adult disability [Murray 1997, WHO 2004]. The incidence and mortality differs between populations and geographical regions [Sarti 2000, Truelsen 2003]. Though early stroke case-fatality has been falling since the early 1950s [Feigin 2003], there is a trend towards stabilising or increasing stroke incidence, probably because of an ageing population [Feigin 2003] and because of increased prevalence of some of the classic risk factors [Medin 2004]. However, it has also been speculated that the increased incidence may reflect technological developments that allow the detection of less severe strokes [Medin 2004]. In Sweden, some 25,000 individuals suffer a first stroke each year, and approximately 9,000 suffer a subsequent stroke [Norrving 2007]. Despite significant improvements in the management of stroke patients during the last decades [Truelsen 2003, Feigin 2003, Socialstyrelsen 2005], about 20% die during the first month after the event, and another third of those who survive remain severely disabled after 6-12 months [Stegmayr 1994]. Identification and management of new risk factors to improve prevention remains an important strategy to reduce the human and economic burden of stroke [Warlow 2003].

Stroke

A stroke occurs when the blood supply to part of the brain is interrupted, and the subsequent shortage of oxygen and nutrients cause damage to the brain tissue. The acute disruption is usually caused by a clot blocking a blood vessel (ischemic stroke) or by a ruptured blood vessel (hemorrhagic stroke). The effects of both types of stroke depend on which part of the brain that is injured and on how severely it is affected. Thus, patients with the same cause of stroke can have differing clinical symptoms. In the same way, patients with the same clinical handicap can have different underlying pathologies. Thus, stroke may be classified as a syndrome, and not as a single disease. To accurately classify the stroke, modern neuroimaging, either with computer tomography (CT) or magnetic resonance imaging (MRI) is required.

The traditional definition of stroke by the WHO is “rapidly developing clinical signs of focal (at times global) disturbance of cerebral function, lasting more than 24 hours or leading to death with no apparent cause other than that of vascular origin” [WHO MONICA 1988]. The most common symptom of a stroke is sudden weakness or numbness of the face, arm or leg, most often on one side of the body. Other symptoms include: difficulty speaking or understanding speech, visual disturbance, difficulty walking, dizziness, loss of balance or coordination and severe headache with sudden onset or unconsciousness. Symptoms lasting

less than 24 hours are called transient ischemic attacks (TIA). Most frequently, TIAs last only seconds or minutes and the lesions can generally not be detected by modern neuroimaging. Still, TIAs have the same causes as stroke and thus may serve to indicate that a person is at increased risk of stroke.

Risk factors for stroke

Because stroke is pathologically heterogeneous it can be expected that the risk factor profiles leading to the different types and subtypes of stroke vary [Arboix 2000, Schulz 2003]. However, many large prospective studies on risk factors were performed before it was feasible to differentiate between the main types, let alone the various subtypes of ischemic stroke [Leys 2004, Goldstein 2006].

Age, gender, race, ethnicity, and heredity have been identified as markers of risk for stroke [Brass 1995, Hassan 2000, Goldstein 2006]. Although these factors cannot be modified, their presence helps identifying those at greatest risk, in whom treatment of modifiable risk factors can be initiated. High blood pressure, hypertension, is the most prevalent and modifiable risk factor for stroke [Leys 2004, Kuller 2000]. A number of other modifiable risk factors have been identified and include cigarette smoking, diabetes mellitus, certain cardiac conditions, obesity, hypercholesterolemia and physical inactivity [Goldstein 2006]. Individuals who have had a TIA also have a much higher risk of suffering a subsequent stroke [Kuller 2000]. In recent years there has been considerable interest in identifying novel risk factors for stroke [Warlow 2003]. Examples of these are infection, hemostatic factors, inflammatory markers, plasma homocysteine and various genetic polymorphisms. However, because of small sample sizes, differing inclusion criteria between studies, and other methodological issues, data on the impact of these novel risk factors on stroke are still limited [Hankey 2006].

Ischemic stroke

The majority of strokes (approximately 85% of strokes) are ischemic and occur when a blood vessel becomes occluded and the blood supply to part of the brain is totally or partially blocked. In the majority of ischemic strokes, intravascular thrombus formation plays an important role for vessel occlusion. The thrombus commonly forms around atherosclerotic plaques where it gradually narrows the lumen of the affected artery (stenosis). Even though stenosis may lead to complete occlusion, the ischemic effect of stenosis in pre-cerebral arteries is reduced because of collateral flow in the Circle of Willis. However, these plaques may become unstable and rupture, causing emboli to pass through to other parts of the brain, occluding other cerebral arteries. Likewise, clots that form in a part of the body other than the brain can travel through blood vessels and become trapped in a brain artery. These emboli often originate from the heart. Using a classification system such as the Trial of Org 10172 in Acute Stroke Treatment (TOAST) [Adams 1993], ischemic strokes can thus be subtyped into the main etiological subtypes large-vessel disease (LVD), small-vessel disease (SVD) and cardioembolic stroke (CE), based on the presumed

pathophysiology (Figure 1). Another way to group the strokes is by clinical presentation. The Oxfordshire Community Stroke Project (OCSP) classification [Bamford 1991] separates the strokes into the clinical subtypes total arterial circulation infarct (TACI), partial arterial circulation infarct (PACI), posterior circulation infarct (POCI) and lacunar infarct (LACI).

Large vessel disease (LVD)

The proportion of LVD in a stroke population largely depends on age, sex and ethnicity. For instance, it has been shown that LVD is 2-4 times more common in men than in women [Petty 1999]. In European populations it as been shown to be the cause in some 15-25% of ischemic strokes [Kolominsky-Rabas 2001, Jerrard-Dunne 2003]. LVD is used to denote significant atherosclerotic narrowing (>70% measured by the ECST method [European Carotid Surgery Trialists´ Collaborative group 1998]) and occlusion in large and medium sized precerebral and cerebral arteries. The plaques normally develop close to branching points and in places of confluence, such as the carotid bifurcation. Artery-to-artery embolization is regarded as the most common stroke mechanism, together with stenosis and hemodynamic mechanisms [Rovira 2005]. As stated above, the effect of stenosis and hemodynamic mechanisms can be relieved by collateral flow. Thus, the mere presence of atherosclerotic lesions does not imply causality and in order to accurately classify a LVD, clinical presentation and location of the lesion must be considered. Potential sources of cardiogenic embolism must also be excluded.

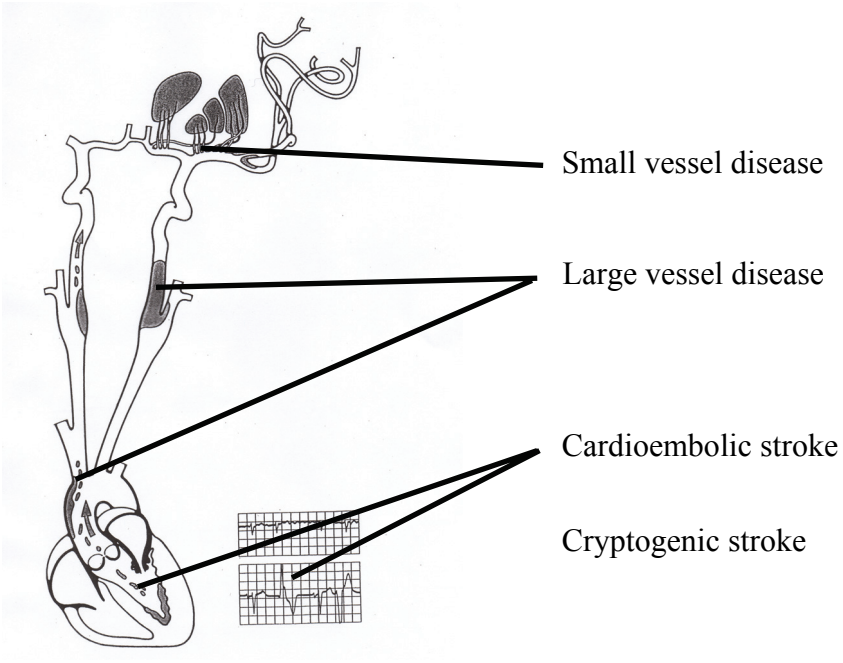

Figure 1. The main etiologic ischemic stroke subtypes.

Small vessel disease (SVD)
SVD is the cause of about 25% of all first-ever ischemic strokes [Warlow 2003]. The most frequent pathologies related to SVD are atherosclerosis and lipohyalinosis, limited to the small deep perforating end-arteries supplying the deep white matter, basal ganglia, thalamus and brain stem [de Jong 2002]. The pathology has traditionally been strongly associated with hypertension. Infarcts are usually small (<1.5 cm in diameter) and often asymptomatic. Typically the clinical symptoms are related to size and location and manifest themselves as so called lacunar syndromes [Bamford 1987, Bamford 1991]. Of note is that not all patients with lacunar syndromes have SVD. Vasculitis, haematological diseases, monogenic disorders and other unusual forms of stroke may also cause small deep infarcts [Gan 1997, Arboix 2004]. Lacunar syndromes may also arise from artery-to-artery embolism or cardioembolism. Thus, potential embolic sources (cardiac, stenosis in large extracranial arteries) must be absent for correct classification.

Cardioembolic stroke (CE stroke)
CE strokes account for about one forth of all ischemic strokes. They occur when embolic material originating from thrombi in the heart occludes cerebral arteries, and are in general severe and prone to early and long-term recurrence [Ferro 2003]. The median volume of infarcts caused by cardiogenic embolism is more than twice the median volume of infarcts caused by artery-to-artery embolism [Timsit 1993]. Atrial fibrillation is the most common source of cardiac emboli, but several other atrial, valvular and ventricular conditions may result in embolism. Because the embolic blockage is sudden in onset, symptoms are usually maximal at start. Also, symptoms may regress rapidly as the embolus is degraded, or evolve into simultaneous or sequential strokes in different arterial territories as the partially degraded emboli moves to one or several different locations. Symptoms may also dissolve altogether. Haemorrhagic transformation of an ischemic infarct also points to a cardiac origin of the stroke [Ferro 2003]. In contrast to other ischemic stroke subtypes, CE stroke may be prevented by anticoagulation [Saxena 2004].

Cryptogenic stroke
In several instances the underlying mechanism of stroke can not be determined with certainty, even after an extensive evaluation. These cryptogenic strokes may account for a quarter of ischemic strokes and are more common in the young.

Other causes of ischemic stroke
Less common causes of ischemic stroke include arterial dissections [Schievink 2001], vasculitis [Ferro 1998], the antiphospholipid syndrome [Levine 2002], hematological diseases [Tatlisumak 1996] and rare monogenic disorders [Natowicz 1987, Hassan 2000]. Sometimes the underlying mechanism of an ischemic stroke remains unknown, either because of cryptogenic strokes, or because two or more potential causes of stroke were identified. The cause may also remain unknown in patients in whom the evaluation was cursory.

Hemorrhagic stroke

In hemorrhagic stroke the underlying cause is usually a rupture of a cerebral artery. Apart from hampering the brain's blood supply, the presence of blood in the brain also causes swelling. The surrounding tissues of the brain resist the expansion of the bleeding, and both swelling and hematomas compress and distort cerebral tissue. Based on the origin and site of the bleeding, hemorrhagic strokes are divided into intracerebral hemorrhage (ICH) and subarachnoid hemorrhage (SAH). ICH and SAH have partly different underlying pathology, risk factors, clinical presentation and management. Because ICH was not included in the present studies, this thesis focuses on SAH.

SAH is caused by bleeding into the subarachnoid space surrounding the brain. It is fatal in up to 50% of patients and causes permanent disability in one third of survivors [van Gijn 2001]. The most common (85% of cases) non-traumatic source is a ruptured aneurysm. Most commonly the patient experiences an explosive headache, often followed by unresponsiveness and neurological deficits. Ten percent of SAHs occur in patients with non-aneurysmal perimesencephalic hemorrhage, a benign condition in which the blood is limited to the area of the midbrain. Less common causes of SAH include vasculitic damage to arteries, other disorders affecting the vessels, and bleeding into various tumors [van Gijn 2001].

In comparison with ischemic stroke, SAH occur more frequently in women. In a systematic review of eight longitudinal and 10 case-control studies, the only modifiable risk factors that emerged for SAH were cigarette smoking, hypertension and heavy drinking [Teunissen 1996]. Geographic region also has an influence on the risk, with countries such as Finland and Japan presenting higher incidence rates than other parts of the world [van Gijn 2001]. It has also been shown that first degree relatives of patients with SAH have an increased risk of being struck with the same disease [Gaist 2000, Teasdale 2005].

Genetics

The word "genetics" was first suggested in 1905 by William Bateson (1861-1926) (from the Greek genno: to give birth) to describe the study of inheritance and the science of variation. Three years later, Wilhelm Johannsen (1857-1927) used the word "gene" to describe the units of hereditary information and made a distinction between genotype and phenotype [Churchill 1974]. The word *phenotype* refers to an observed quality of an organism, while the *genotype* describes the inherited instructions an organism carries, which may or may not be expressed [Churchill 1974]. Thus the phenomenon of phenotype was investigated decades before James D. Watson (1928-) and Francis Crick (1916-2004) resolved the structure of deoxyribonucleic acid (*DNA*) [Watson 1953]. DNA is an information macromolecule that stores genetic code in all living species. For humans, the DNA is packed into 23 pairs of double-stranded, linear *chromosomes*. (Figure 2). These are long sequences of *nucleotides* of four

different kinds: adenine (A), guanine (G), cytosine (C) and thymine (T), a four letter alphabet of life. Nucleotides on opposite strands of the DNA pair specifically. Because of the physical properties of the nucleotides, A specifically binds to T and C specifically binds to G (the Watson-Crick rules). The nucleotides are sometimes referred to as *bases*, and all together the 23 chromosome pairs, the human *genome*, contain approximately 3.08 billion *base pairs* (bp) [Abdellah 2004].

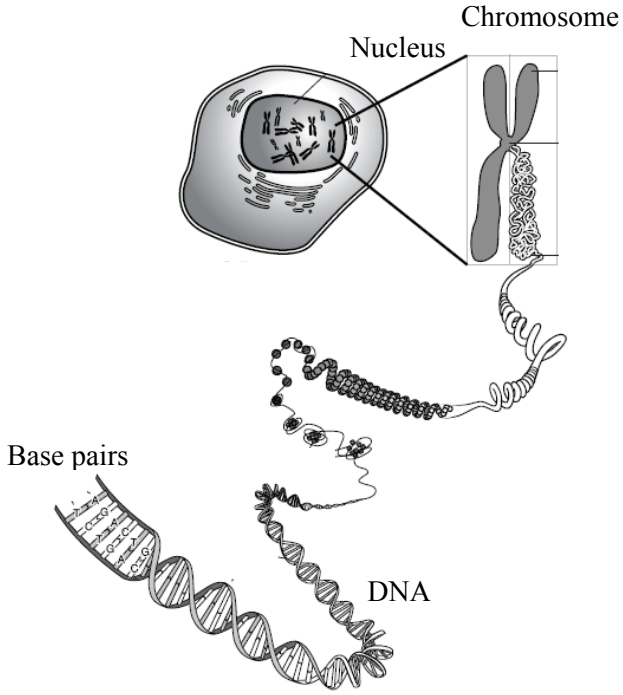

Figure 2. Level of DNA organisation.
Source: Access Excellence at the National Health Museum, USA;
http://www.accessexcellence.org/RC/VL/GG/chromosome.html.

The genome can be separated into coding regions and non-coding regions. The coding regions, which represent 1.2% of the genome, are unevenly distributed across the chromosomes [Abdellah 2004]. These coding regions are what we today commonly refer to as *genes*, and are separated into *exons*, which carry the instructions for making proteins, and non-coding *introns* [Pearson 2006]. At present, the number of protein-encoding genes in the human genome is estimated to 20,000 – 25,000 [Abdellah 2004]. Most human genes have multiple exons (average 10 per gene), and introns are frequently much longer than flanking exons [Abdellah 2004].

Francis Crick is also known for use of the term *central dogma* to summarize the idea that genetic information flow in cells is essentially one-way, from DNA to ribonucleic acid (RNA) to protein. Thus, proteins are essentially what maintain all processes in living organisms, the DNA contains the information necessary to construct these building blocks and RNA is used to transcribe this information.

Single nucleotide polymorphisms and haplotypes

During evolution changes in the nucleotide composition of the DNA occur, most often due to spontaneous errors in DNA replication and repair [Strachan & Read 2004a]. When these changes have a phenotypic effect they are referred to as *mutations* and, most likely, have arisen within, or near, a gene. However, many changes in nucleotide composition pass unnoticed, or may cause small or even beneficial effects. These mutations give rise to variants of genes, or *alleles*. A well defined position on a chromosome is called a *locus*. When not used to designate the overall genetic composition of an individual, *genotype* is often used to denote the pair of alleles at a specific locus. The smallest change in nucleotide composition is the mutation of a single nucleotide. When these mutations are inherited and accumulate in a population they are referred to as single nucleotide polymorphisms (*SNP*), meaning that different alleles exist at a specific nucleotide position in the DNA. Traditionally, a requirement for a mutated allele to be referred to as a SNP is that it should be present in more than 1% of all alleles in a population. At present, some 11,9 million SNPs have been reported to the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/). Of these, approximately 6,3 million have been validated, and more than 5 million have a minor allele frequency (MAF) above 10% [Carlson 2004]. Thus, on average there is one SNP in each 300-1000 bases in the genome.

If a particular SNP is more common among people with a particular phenotype or disease, that SNP can be used as a marker to locate and identify the genetic variant involved in the disease. However, testing all the common SNPs would be extremely laborious and expensive. To circumvent this, the HapMap project was launched [HapMap Consortium 2003]. This project takes advantage of the fact that SNPs that are near each other tend to be inherited together, a phenomenon called *linkage disequilibrium* (LD).

LD exists because of the shared ancestry of contemporary chromosomes. When a new mutation arises, it is initially bound to a unique chromosome on which it occurred, marked by a distinct combination of genetic variants. With time, recombination and mutation act to erode this association, but do so slowly. A block of alleles on the same chromosomal segment and from the same parental origin is called a *haplotype*. The strong associations between SNPs within a haplotype have a practical value: genotyping only a few, carefully chosen SNPs in the region will provide enough information to predict much of the information about the remainder of the common SNPs in that region [HapMap Consortium 2003]. As a result, only a few of these *tagSNPs* are required to identify each of the common haplotypes in a region. For instance, in the ENCODE region of the HapMap, one in five common SNPs in the population with European ancestry (CEU) has 20 or more perfect proxies, and it has been estimated that only 250,000 to 500,000 tagSNPs contain most of the information about the patterns of genetic variation in the human genome [HapMap Consortium 2005]. Various methods have been developed to define haplotype structure [Niu 2004], and to

select maximally informative subsets of tagSNPs, to uniquely identify these haplotypes [Stram 2004].

Monogenic and multifactorial inheritance

When the genotype of a single locus is both necessary and sufficient to give rise to a specific trait, such a trait is called monogenic or *Mendelian* (to honour the monk Gregor Mendel (1822- 1884)). Several thousand such traits are known in man and information on them is readily available online in the OMIM database (http://www.ncbi.nlm.nih.goc/omim/). Most human traits are governed by genes at more than one locus. Such non-mendelian, *multifactorial*, traits may depend on two, three or many loci, with great or small contribution from environmental factors. For dichotomous traits the underlying loci are envisaged as *susceptibility genes*, while for continuous traits they are seen as *quantitative trait loci* (QTLs) [Strachan & Read 2004b]. Because the contribution of each gene in a complex trait is relatively minor, identification of each of the genes that ultimately determine a complex trait is a major challenge.

Genetics in stroke

Stroke is both a heterogenous and a multifactorial disease, in which heritable and environmental factors equally contribute [Hassan 2000, Dichgans 2007]. The heritable component has been investigated in family [Floßmann 2004], twin [de Faire 1975, Brass 1992, Bak 2002] and animal studies [Jeffs 1997]. Twin studies provide the most reliable evidence of a genetic component in complex diseases, as they are least confounded by environmental factors. In twins, concordance rates were reported to be about 1.6 times greater in monozygotic twins than in dizygotic twins. However, most of these studies have been relatively small and have not differentiated between stroke types [Floßmann 2004].

Cohort and case-control studies on family history of stroke support a hereditary component in both ischemic and hemorrhagic stroke. However, study designs and possible publication and recall bias have made it difficult to reliably estimate the strength of the association [Floßmann 2004]. Most studies combined ischemic and hemorrhagic stroke and failed to differentiate between the various ischemic stroke subtypes, assuming that heritability for stroke would be similar in all types and subtypes [Floßmann 2004]. Recent data have suggested that a family history of stroke is a risk factor for SAH [Kissela 2002], LVD and SVD, but not for CE stroke or stroke of undetermined etiology [Polychronopoulos 2002, Jerrard-Dunne 2003]. In all subtypes, the family history effect was stronger in patients with a young age of onset [Schulz 2004]. Concern has been raised that part of the increased risk may be explained by heritability of common intermediate phenotypes, such as hypertension [Lindgren 2005, Floßmann 2005]. Of note is also that some recent studies suggest that there are sex-specific differences in stroke heritability, with women being about 50% more likely to have a maternal than a paternal history of stroke [Touzé 2007]. No similar effect was detected in men. This mother-to-daughter mechanism is hard to explain by

classical genetic mechanisms, but could perhaps be explained by non-genetic factors or by transmitted epigenetic factors [Touzé 2007]. Although the field of epigenetics is intriguing, and part of the hereditary component in stroke may be epigenetic, the work presented in this thesis deals exclusively with traditional genetics.

Monogenic stroke

By use of linkage mapping in large affected families, several monogenic disorders that can cause stroke have been identified [Natowicz 1987, Hassan 2000]. Many of these are systemic disorders where stroke is only one part of the clinical syndrome, but in some stroke is the only clinical manifestation. These mendelian conditions are overall infrequent, but should be considered when common courses have been ruled out, especially in the young [Dichgans 2007]. Most single-gene disorders are associated with a specific stroke subtype, which together with genetic tests and systemic features can help in settle diagnose. A detailed description of all these disorders is beyond the scope of this thesis, but a few examples are given below.

CADASIL

Cerebral Autosomal Dominant Arteriopathy with Subcortical Infartcs and Leucoencephalopathy (CADASIL) is an autosomal dominant condition caused by mutations in the Notch3 gene [Joutel 1996]. In Scotland and Finland the probable mutation prevalence has been estimated to be around 4 per 100.000 adults [Kalimo 2002, Razvi 2005]. Symptoms normally appear between 30 and 50 years of age and the clinical phenotype comprises recurrent small vessel strokes and TIAs, progressive cognitive impairment, mood disturbances and migraine with aura [Hassan 2000, Opherk 2004]. Notch3 encodes a cell-surface receptor, which has a role in arterial development and is expressed on vascular smooth muscle cells. The majority of mutations are located in exons 3-6, predominantly in exon 4 [Markus 2002, Peters 2005], and lead to either a gain or loss of a cysteine residue [Hassan 2000]. CADASIL mutations cause an abnormal accumulation of Notch3 at the cytoplasmic membrane of vascular smooth-muscle cells and sensitive methods to diagnose CADASIL using immunostaining of skin biopsy samples with monoclonal antibody specific for Notch3 have been developed [Joutel 2001].

Fabry's disease

Fabry´s disease is an X-linked systemic disorder resulting from deficient or absent activity of the lysosomal enzyme alpha-galactosidase A. This enzymatic defect leads to the systemic accumulation of globotriaoslyceramide (Gb3) and related glycosphingolipids in the vasculature, myocardium, skin, eye and renal epithelium [Clarke 2007]. In a large series of young patients (18-55 years) with cryptogenic stroke, 4.9% of men and 2.4% of women were shown to carry a functionally relevant mutation in the alpha-galactosidase gene (GLA) [Rolfs 2005]. Treatment with recombinant alpha-galactosidase is effective in reducing

globotriaosylceramide deposition, and improving some of the symptoms [Wilcox 2004].

Sickle cell disease
Sickle-cell disease is the most common cause of stroke in children [Switzer 2006]. It is caused by homozygosity of a beta-globulin A-to-T mutation in the sixth codon of the beta-globulin gene (HbS), or by a heterozygous state combined with another abnormal hemoglobin allele such as haemoglobin C [Dichgans 2007]. The mutation cause red blood cells to polymerize upon deoxygenation, and as a consequence hemoglobin proteins stick to each other, giving the cell a rigid surface and sickle shape. The process damages the red blood cell membrane, and can cause the cells to become stuck in blood vessels, promoting thrombosis. The risk of stroke in sickle-cell disease seems to be strongly affected by modifier genes [Sebastiani 2005, Steinberg 2006]. By applying a Bayesian network to a large number of SNPs, Sebastiani et al. identified 31 SNPs in 12 genes that were shown to interact with haemoglobin in modulating stroke risk. Remarkably, a predictive model was constructed that was able to predict stroke occurrence in a second population with 98% accuracy [Sebastiani 2005].

MELAS
Mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) are syndromes often caused by defects in the mitochondrial genome, which is inherited purely from the mother [Thambisetty 2004]. MELAS is associated with various symptoms, but cases have been reported in whom stroke has been the sole manifestation [Martinez-Fernandez 2001].

Multifactorial stroke

In contrast to the mendelian forms of stroke, for the more common polygenic trait it can be expected that the contribution from each single susceptibility locus is relatively small [Casas 2004]. Furthermore, the effect of each underlying gene may depend upon interaction with other loci and with environmental and life-style factors, as reported for some of the mendelian forms of stroke. To further complicate the issue, many conventional risk factors such as diabetes mellitus, hypertension and cardiovascular diseases, are themselves complex genetic diseases that may interact with environmental exposures. Still, on the individual level genetic variants may interact in an additive or multiplicative manner with other genetic variants and with environmental exposures, thus making certain individuals more vulnerable to certain exposures. This would help explain the observation that some individuals with traditional risk factors for all types of stroke, such as cigarette smoking and hypertension may develop SVD and no signs of LVD, or the converse pattern, or both. It may also explain why recurrent strokes most frequently belong to the same subtype as the first event [Jackson 2005]. Accurate stroke subtyping thus appears crucial in order to elucidate the genetic components. However, most previous studies have suffered from small sample size, lack of adequate phenotyping, and poor case-control matching

[Dichgans 2007]. As a result, attempts to identify the underlying genes have been largely disappointing.

Strategies for identifying genetic factors in multifactorial stroke

The most popular approach for identifying genes in human polygenic ischemic stroke has been the candidate gene approach using case-control methodologies. More recently this has been extended to family-based association studies. Linkage-based approaches have been used less frequently. In the future genome-wide association studies are likely to become more widely used.

Linkage studies

In linkage studies, the aim is to map a possible disease locus by studying how genetic markers have segregated in large multigenerational pedigrees with many affected family members in relation to the affection status of the pedigree members. Genetic markers (normally microsatellites) covering the whole genome are used to genotype patients and affected family members. An advantage of linkage analysis is that it is performed in a hypothesis-free manner and does not require any prior knowledge of the underlying disease mechanism. The method also is insensitive to spurious results due to problems with population stratification. For several reasons the linkage approach is difficult to apply in the search of genes contributing to stroke. Because of the late-onset of the disease the collection of information from other family members becomes difficult, and the affection status of siblings and offspring uncertain. The polygenic nature of stroke, and shared environmental exposures, also contributes to the difficulties with the linkage approach, because linkage is unable to detect genes with minimal or modest effect on stroke risk [Hassan 2002, Gulcher 2005].

Candidate gene allelic association studies

The major approach used to find stroke genes has been the candidate gene approach using case-control methodologies. This is a hypothesis-driven approach in which genes that may be involved in the pathogenesis of stroke are tested for association. One or more markers covering the gene are genotyped in a set of cases and controls, to look for allelic variants that are over- or underrepresented in cases compared with controls. To be reliably detected, small relative risks require large samples sizes, in the magnitude of 1,000 patients or more [Dichgans 2005]. However, few studies have achieved such numbers. It can be speculated that differences in sample characteristics and limitations in study designs may explain why most reports of significant associations have not been replicated. This has raised concerns on the validity of association studies and complex genetics in general and lead to publications suggesting standard criteria for genetic association studies in stroke [Dichgans 2005, Chanock 2007]. A description of these criteria is presented in Table 1. When appropriately designed, association studies remain a powerful tool to identify genetic factors for stroke.

Table 1.
Methodological criteria for candidate gene association studies in ischemic stroke that enhance the significance of an association finding. Adapted from [Dichgans 2005, Chanock 2007].

Criteria	Comment
Hypothesis	The a priori hypothesis should be clearly stated.
Power calculation	Power calculations should be performed to demonstrate that the study is sufficiently powered to test all hypotheses of the study.
Previous studies	Overlap with previous studies should be indicated.
Case-control recruitment	Controls ethnically matched to cases. Allow possibility to account for population stratification. Careful accounting for potential bias in selection of subjects.
Phenotype assessment	Phenotype protocol should be specified and done according to standardized criteria, such as the TOAST system.
Conventional risk factors	Definitions and methods used to determine presence or absence of risk factors should be stated. If power is sufficient, gene-environmental interactions should be investigated.
Genotyping	Negative and positive control samples should be assessed along with study samples. Genotyping errors should be reported along with frequencies for all groups investigated. Indications whether if markers are in Hardy-Weinberg equilibrium should be stated. Investigators performing genotyping should be blinded to phenotype.
Statistics	Explicit information on statistical method and level of significance (odds ratios with 95% confidence intervals, or attributable risks) should be presented. Uncorrected p-values should be presented, but adjustment for multiple testing should still be performed.
Replication studies	Best way to convincingly demonstrate association is through replication in an independent sample. Replication study should have somewhat greater power than initial study. Results of replication studies should be reported even if the results are not significant.
Gene dose	Authors should check for a possible gene-dose effect. If absent, it should be discussed if results are explicable from a biological viewpoint.

Genome-wide association studies (GWA)

The last decade has seen tremendous advances in sequencing and genotyping technologies. This development has been a prerequisite for the completion of both the sequencing of the human genome and the mapping of human haplotypes. Now such technology offers the possibility of typing hundreds of thousands of SNPs simultaneously in genome-wide association (GWA) studies. This approach relaxes the need for a prior hypothesis in case-control studies and allows genes and genetic regions with unknown function to be tested. The great challenge with this transition to GWA studies is to separate true associations from the huge amount of false positives that will be produced [Dichgans 2007, Chanock 2007, Wellcome Trust 2007].

Recently, the Wellcome Trust Case Control Consortium showed that GWA studies are feasible. In a joint effort they examined 2,000 individuals for each of 7 major diseases and a shared set of 3,000 controls in the British population [Wellcome Trust 2007]. They were able to replicate some previous findings, and also discovered several new candidate loci for these diseases. The authors draw a number of conclusions that merit attention:

1) Importance of careful quality control. In such large data sets, small systematic differences may readily produce effects capable of obscuring the true associations being sought.
2) The novel variants that they uncovered were characterized by modest effect size (per-allele ORs between 1.2 and 1.5). The authors believe that those estimates are likely to be inflated.
3) Extensive replication will still be required to establish validity.
4) Strong evidence that GWA studies require even larger sample sizes than in their study. Less powered studies will likely miss several loci and risk producing false negative results.

With regard to stroke, the issue of subtyping and controlling for environmental exposure in both patients and controls is likely to be underestimated in present discussions. Future studies will therefore most likely have to be performed by multiple centres acting in concert to achieve sufficient power for studies on well characterized groups and subgroups of stroke patients and controls. When such a study has reported significant associations, confirmations in replication studies that are similarly designed and equally, or better, powered to detect associations will be required to confirm susceptibility loci.

Results from genetic studies in multifactorial stroke

Linkage studies

Linkage analysis has proven successful in identifying mendelian variants of stroke. However, it has hardly been applied to multifactorial stroke. The two studies that have been performed excluded SAH and used microsatellite markers. Both report linkage to chromosomal position 5q12 [Gretarsdottir 2002, Nilsson-Ardnor 2007]. The Icelandic study also reported linkage to position 13q12 when

stroke was combined with myocardial infarction (MI) [Helgadottir 2004]. In the 5q12 region the phosphodiesterase 4D gene (PDE4D) was further investigated, and an association between genetic variants of PDE4D and LVD and CE stroke was later reported [Gretarsdottir 2003]. Replication studies have used different genetic markers and phenotypes, and none of them constitute a true replication of the original finding [Rosand 2006]. Still, there are now several studies that support a role for PDE4D in ischemic stroke, particularly in CE stroke. However, results are inconsistent as there are studies that have failed to detect any association. This possibly reflects the underlying heterogeneity in stroke and suggests that the causal variant has yet to be identified [Woo 2006]. In the 13q12 region the Icelandic authors later identified the arachidonate 5-lipoxygenase-activating protein (ALOX5AP) gene, encoding 5-lipoxygenase activating protein (FLAP), and found that a 4-SNP haplotype in the gene (Hap A) conferred a nearly 2 times greater risk of MI and stroke [Helgadottir 2004]. The association was later replicated in the Scottish population [Helgadottir 2005], which shares a common ancestry with the Icelandic population. However, studies in other populations have not been able to replicate this association [Lohmussaar 2005, Meschia 2005, Zee 2006].

There is a large multicentered effort underway in the US called SWISS (siblings with ischemic stroke study), in which the aim is to collect 300 sibling-pairs concordant for ischemic stroke, and 200 of their unaffected siblings [Meschia 2002, Meschia 2006]. Because collection is still ongoing, no complete genome-wide linkage results have yet been published.

As regards SAH, there are a few linkage studies on intracranial aneurysm formation in extended pedigrees. Promising LOD scores have been reported, but no gene has yet been identified [Nahed 2007].

Candidate gene allelic association studies

The vast majority of candidate genes that have been investigated in stroke come from specific pathways where evidence has been accumulating of a role in stroke pathology. Very often candidates are chosen after an association has been shown in a thrombotic disease, such as MI. This may be a consequence of that more research is put into MI [Rothwell 2001, Bhatia 2005, Pendlebury 2007], but fails to reflect the more complex nature of stroke [Casas 2004]. Among the most widely investigated genes are those involved in fibrinolysis and coagulation, renin-angiotensin-aldosterone system, nitric oxid release, homocysteine metabolism, inflammation, lipid metabolism and extracellular remodelling. Additionally, several studies have attempted to replicate associations for the positional candidates PDE4D and ALOX5AP. The ALOX5AP result also contributed to an interest in investigating other genes involved in the leukotriene pathway.

The majority of the investigated polymorphisms are located in coding regions, or in other regions that are more likely to harbour functional SNPs, such as

promoters and 3´ untranslated regions (UTR). Some have a demonstrated biological effect, but in most cases it remains a laborious task to establish functionality. A thorough description of all investigated genes and polymorphisms is beyond the scope of this thesis. Below I shortly provide information on some of the most investigated genes. Information on genes involved in inflammation and the coagulation and fibrinolytic systems are given separately.

Renin-angiotensin-aldosterone system (RAAS)
The most extensively studied sequence variant in the RAAS is an insertion/deletion (I/D) polymorphism in the angiotensin converting enzyme (ACE). ACE produces angiotensin II and catabolises bradykinin, thereby affecting vascular tone. The deletion allelic variant is associated with higher plasma levels of ACE [Tiret 1992] and several meta-analyses have demonstrated that subjects homozygous for the deletion are at an increased risk for ischemic stroke [Sharma 1998, Casas 2004, Ariyaratnam 2007]. The effect estimates are small but significant (OR 1.21; 95% CI 1.08-1.35) [Casas 2004], but may perhaps be strengthened since the functional ACE variant remains elusive [Sayed-Tabatabaei 2006]. Other genes that have been investigated with conflicting results include angiotensinogen, angiotensin II type-1-receptor and aldosterone synthase [Dichgans 2007]. With regard to hemorrhagic stroke fewer studies have been performed, however associations between ACE DD and SAH have been reported [Krischek 2006].

Nitric oxide
Nitric oxide, NO, a product of the normal endothelium, has a variety of physiological effects to maintain endothelial function and an antithrombotic intravascular milieu [Loscalzo 1995]. In the brain, NO functions as a neuromodulator and appears to mediate aspects of learning and memory, but excess production of NO may lead to brain injury [Bredt 1999]. Several SNPs have been investigated in the endothelial NO synthase (eNOS) gene. Quite a few studies suggest that genetic variants in eNOS may confer an increased risk of ischemic stroke [Hassan 2004, Berger 2007], but the meta-analyses that have been performed have not been able to detect any association [Casas 2004]. A disputed finding in relation to SAH was that eNOS polymorphisms may distinguish between small and large ruptured aneurysms, despite a lack of association with SAH in the population at large [Khurana 2003]. The same authors later reported that eNOS polymorphisms may indicate what intracranial aneurysms are more prone to rupture [Khurana 2005]. These findings have not been replicated.

Homocysteine metabolism

The interest in homocysteine (Hcy) metabolism originated from the knowledge that several autosomal and dominant enzyme deficiencies can lead to homocysteinuria, with increased Hcy and arteriosclerosis [Hassan 2000]. Even moderate elevations of plasma Hcy levels are associated with an increased risk of both ischemic and hemorrhagic stroke [Wald 2002, Li 2003]. A common SNP in the gene encoding methylenetetrahydrofolate reductase (MTHFR), an important enzyme in Hcy metabolism, has been associated with lower enzymatic activity, higher Hcy levels and increased stroke risk [Li 2003, Casas 2004]. In a large meta-analysis, the mean difference in Hcy level between TT and CC genotypes of the C677T SNP were 1.93 μmol/L, and the observed OR for overall stroke was 1.26; 95% CI 1.14-1.40 for TT versus CC [Casas 2005]. There have been reports demonstrating that the impact of the SNP may be modulated by folate intake [Casas 2005]. Knowledge on the impact of genetic variation in other genes in this pathway is limited. However, a recent linkage study on Hcy level reported that the highest linkage peak was positioned at 11q23, and suggested that nicotinamide N-methyltransferase may be a positional candidate gene [Souto 2005].

Lipid metabolism

There are many genes involved in lipid metabolism that have been investigated in relation to stroke. The most well investigated gene is apolipoprotein E (apoE), which plays a major role in lipid transport and metabolism. There are 3 major isoforms of human apoE (apoE2, -E3, and -E4) that differ in amino acid sequence at 2 sites, the most common is apoE3. Compared with apoE3, individuals with apoE4 have been shown to have increased total cholesterol levels, whereas individuals with apoE2 have decreased levels [Eichner 2002]. A number of studies have observed an association with markers of atherosclerosis (eg, carotid intima-media thickness) [Humphries 2004], but studies of apoE and stroke have produced conflicting results. Support for a role of apoE4 in both SAH and ischemic stroke, particularly LVD, came from a recent meta-analysis [Sudlow 2006], in contrast to a previous meta-analysis which failed to provide evidence for increased stroke susceptibility [Casas 2004]. However, as stated by Sudlow, their results were based on a small numbers of cases and controls, and seem likely to be the combined result of publication and reporting bias.

Another rather well investigated gene involved in lipid metabolism is lipoprotein lipase (LPL). It is involved in the removal of cholesterol from the circulation, and polymorphisms in this gene have been associated with levels of high-density lipoprotein (HDL) cholesterol and triglycerides. The S allele of N291S is associated with elevated plasma triglycerides and reduced HDL cholesterol levels [Kastelein 1999], and the X allele of S447X with reduced plasma triglycerides and increased HDL cholesterol levels [Wittrup 1999]. This last SNP has been investigated in relation to stroke, but results have been inconclusive. Other genes involved in lipid metabolism that have been investigated include apolipoprotein A, apolipoprotein B, cyclooxygenase and paraoxonase.

Extracellular remodelling
SNPs in genes involved in maintaining the integrity of the extracellular matrix of the arterial wall have been proposed as the most likely candidate genes for SAH [Ruigrok 2005]. Accordingly, a large number of SNPs in functional and positional candidate genes have been tested, but mainly in relatively small samples. A few studies have reported significant associations, but no association has been consistently replicated and the majority have been negative. Some of the investigated genes are: collagen 1 and 3, lysyl oxidase, fibrillin 2, endoglin, metalloproteinase 1, 3, 9 and 12 and tissue inhibitors of metalloproteinases 1, 2 and 3 [Ruigrok 2005, Krischek 2006, Nahed 2007]. Some of these genes have also been investigated in relation to ischemic stroke, but associations have been absent or inconclusive.

The hemostatic systems in ischemic stroke

Normal hemostasis is maintained by a delicate balance between prothrombotic and antithrombotic processes, which are mediated by cellular components, soluble plasma proteins and endothelium derived factors [Rosenberg 1999]. In the past, studies have proposed excess coagulation factors, increased levels of fibrinolytic inhibitors, or both, as risk factors for stroke. Circulating levels are subject to considerable variation, and the acute-phase response that accompanies an acute stroke event may hinder the interpretation of levels in a case-control study. Prospective studies are not subject to the confounding influence of the acute phase reaction, but large numbers are required because stroke event rates are rather low, making this a resource-intensive task. In addition, circulating levels obtained from peripheral blood samples sometimes bear no relationship to local intracerebral levels of hemostatic proteins.

Coagulation

The coagulation cascade is initiated by the exposure of blood to tissue factor (TF) on the surface of damaged vessels. The factor VIIa–TF complex then activates both factors IX and X. Factor IXa also converts factor X to Xa in the presence of factor VIIIa, phospholipid (PL), and calcium (Ca^{2+}). Factor Xa then converts prothrombin (II) to thrombin (IIa) in the presence of factor Va, PL, and Ca^{2+}. Thrombin then converts fibrinogen to fibrin, which polymerizes to form the fibrin clot. The generation of thrombin is amplified by the feedback activation of factors V, VIII, and XI mediated by thrombin itself. In addition, thrombin activates factor XIII, which stabilizes the fibrin clot (Figure 3).

Coagulation gene polymorphisms and stroke
The procoagulant state caused by activated protein C resistance and the underlying factor V Leiden polymorphism (Arg506Gln) is an established risk factor for venous thrombosis, but the impact on stroke is still under debate. This coding SNP is located at the protein C cleavage site, and mutated factor V is less efficiently cleaved by protein C. As a result thrombin production is less inhibited, giving rise to a hypercoagulant state. This is perhaps the most commonly studied

SNP in relation to stroke, and support for a moderate role in ischemic stroke was shown in a recent meta-analysis (OR 1.33; 95% CI 1.12-1.58) [Casas 2004]. The same meta-analysis also concluded that the prothrombin (Factor II) G20210A SNP confers a similar increase in risk of ischemic stroke (OR 1.44; 95% CI 1.11-1.86). This rare SNP is located in the 3´UTR and alters mRNA stability. The uncommon allele is associated with elevated prothrombin levels and thrombin formation [Franco 1999].

A plethora of other genes involved in coagulation have been investigated in relation to ischemic stroke, but results have mainly been negative or inconclusive. In many cases the biological effect of the investigated SNP is relatively well understood. These genes include factor VII, factor XIII, fibrinogen, von Willebrand factor, thrombomodulin and platelet surface receptors, such as platelet glycoprotein IIb/IIIa [Voetsch 2003].

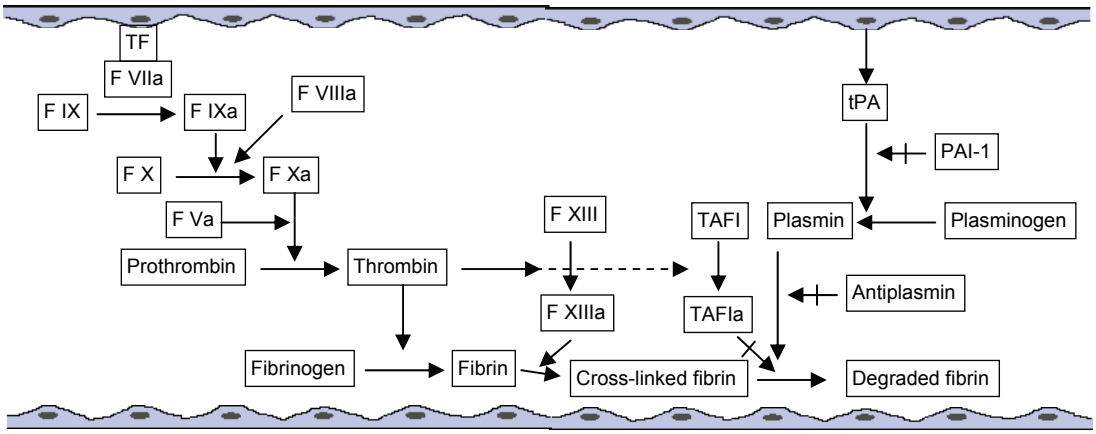

Figure 3. Overview of the coagulation and fibrinolytic systems

Fibrinolysis

Once coagulation has initiated, the fibrinolytic system is triggered to prevent extension of the clot beyond the site of injury. Tissue-type plasminogen activator (tPA) is secreted from endothelial cells and converts plasminogen to plasmin in the presence of fibrin. Plasmin then degrades the fibrin clot to soluble fibrinogen degradation products and also inactivates factors Va and VIIIa (Figure 3). The main inhibitor of plasmin is antiplasmin and the main inhibitor of tPA is plasminogen activator inhibitor type 1 (PAI-1), which is present in platelets and plasma. PAI-1 acts by rapidly forming a complex with tPA and in consequence the bulk of tPA in the blood is inactivated and bound to PAI-1. Thus, fibrinolysis is a local phenomenon in which the local release rate of tPA has to surmount the plasma concentrations of PAI-1 to achieve efficient lysis of the fibrin clot. Apart from PAI-1 and antiplasmin, excessive fibrinolysis is also prevented by the action of thrombin activatable fibrinolysis inhibitor (TAFI). Activated TAFI operates by continuously removing C-terminal lysine residues on plasmin-modified partially degraded fibrin, thereby attenuating a positive feedback loop in tPA-mediated activation of plasminogen.

The recognition that formation of thrombotic or thromboembolic intravascular occlusions is important in the pathogenesis of the majority of ischemic strokes, regardless of subtype, and the observation that early administration of thrombolytic agents results in recanalization of occluded arteries, have provided the basis for the development of recombinant tPA therapy in acute ischemic stroke. The efficacy of these drugs shows the potential of the fibrinolytic enzymes in relieving the effects of an occluding thrombus. However, in contrast, both case-control and prospective studies have shown that elevated tPA antigen levels are associated with an increased risk of ischemic stroke [Ridker 1994, Lindgren 1996, Johansson 2000]. Plasma levels of tPA antigen and activity are unrelated to the capacity to release tPA from the endothelium, and the bioavailability of active tPA for fibrinolysis [Jern 1994, Hrafnkelsdóttir 2004]. Instead, plasma tPA antigen is highly dependant on the plasma levels of PAI-1, as well as on the hepatic clearance of tPA [Hrafnkelsdóttir 2004]. Elevated tPA has therefore been suggested to be related to hypofibrinolysis. In line with this, plasma tPA antigen shows a negative correlation with plasma tPA activity [Wall 1995]. Because of technical difficulties, few studies have investigated tPA release rates *in vivo*. A recent small study on 9 ischemic stroke patients and 9 matched controls could not detect any significant differences in tPA release rates between patients and controls [Jood 2007]. However, impaired tPA release in both smokers and hypertensives, well-recognized risk indicators of both ischemic stroke and MI, have been reported [Hrafnkelsdóttir 1998, Newby 1999].

Our group has shown that genetic factors are associated with tPA release rates in man [Jern 1999]. Out of several identified SNPs at the tPA locus, the -7,351 C>T enhancer SNP showed closest association to tPA release rates [Ladenvall 2000]. Functional studies have shown that this SNP affects the binding of transcription factors, and that the T allele expresses less tPA compared with the C allele [Wolf 2005, Tjärnlund-Wolf 2006]. Thus, it can be hypothesised that this genetic variant may be used as a marker of tPA release rates and endogenous fibrinolytic capacity. This approach was used in a prospective study on MI, in which an increased risk for MI was observed for carriers of the T allele [Ladenvall 2002]. As regards ischemic stroke, an increased risk for lacunar stroke has been reported for subjects with the TT genotype [Jannes 2004], but others have not been able to detect an association [Attia 2007]. Furthermore, a recent study on tPA haplotypes in a Japanese population suggested that a tPA haplotype is a marker for ischemic stroke [Saito 2006]. However, they did not specifically study the -7,351 C>T SNP.

In concert with what has been reported for tPA antigen, studies have shown that plasma levels of PAI-1 and the tPA/PAI-1 complex are associated with an increased risk of ischemic stroke [Catto 1997, Johansson 2000]. At the PAI-1 locus, the -675 4G>5G promoter SNP has been demonstrated to be functional [Eriksson 1995]. A repressor binds less tightly to the 4G allele, which results in a higher transcriptional activity compared with the 5G allele. This SNP has been extensively studied and meta-analyses have concluded that the 4G allele may be

associated with a decreased risk of ischemic stroke, although the direction of the effect is inconsistent from study to study [Casas 2004, Attia 2007]. This contrasts with the effect that has been reported for MI [Eriksson 1995]. Also in contrast to the previously mentioned meta-analyses, a recent meta-analysis that included mainly the same studies as Attia failed to demonstrate a significant association between the PAI-1 4G>5G polymorphism and ischemic stroke [Tsantes 2007]. The authors conclude that further studies are warranted.

Less attention has been given to the recently discovered functional inhibitor TAFI. Some studies have demonstrated that plasma TAFI levels are augmented in the acute phase of ischemic stroke [Montaner 2003, Leebeek 2005] but, as reviewed by Leurs, associations with arterial diseases have been ambiguous [Leurs 2005]. The use of poorly characterized assays in the majority of the early studies may have contributed to this discrepancy [Leurs 2005]. As for tPA, it is not the total amount of TAFI in plasma that influences its activity. The protein circulates in an inactive form and upon activation the activation peptide (AP) is released from the catalytic domain (TAFIa). The catalytic domain is then rapidly further degraded [Marx 2002]. It has therefore been concluded that it is the amount of activated TAFI that plays a crucial role in retarding fibrinolysis [Leurs 2004, Walker 2004], and assays that measure TAFIa functionality have been developed. The 325Ile allele of the TAFI Thr325Ile SNP has been shown to produce a protein with longer half-life and increased antifibrinolytic properties compared with the Thr325 allele [Schneider 2002]. Furthermore, early studies reported associations between several TAFI SNPs and circulating levels of TAFI antigen [Tregouet 2001]. However, it was demonstrated that some assays used for TAFI antigen determination had different assay sensitivity between isoforms, leading to overestimations of the genetic effects [Gils 2003, Guimaraes 2004]. Still, results from more recent studies using genotype-independent assays have confirmed a genetic influence on circulating TAFI levels [Frère 2005, Morange 2005, Frère 2006]. So far no association between TAFI SNPs and stroke risk has been reported [Leebeek 2005].

The hemostatic systems in hemorrhagic stroke

The hemostatic system has received far less attention in hemorrhagic stroke compared with ischemic stroke. There are a few studies that have reported increased thrombin-antithrombin III (TAT) and fibrin degradation products (D-dimer) levels in patients with ruptured aneurysms [Itoyama 1994, Peltonen 1997, Morga 2007], suggesting an activation of both coagulation and fibrinolysis in the acute stage of SAH. Furthermore, patients with elevated TAT and D-dimer were shown to be at increased risk of vasospasm and delayed ischemic neurological deficit after SAH [Peltonen 1997, Nina 2001] and antifibrinolytic drugs may offer protection against such secondary events [Hillman 2002]. However, prospective data on the impact of the hemostatic system on the risk of development and rupture of an intracerebral aneurysm are lacking.

To the best of my knowledge, only one gene implicated in the fibrinolytic pathway has been studied in relation to hemorrhagic stroke so far, i.e. PAI-1. One Dutch study on 44 candidate genes for development of intracranial aneurysms showed an association to a PAI-1 SNP; dbSNP ID: rs6956010 [Ruigrok 2006], but another study investigating the 4G>5G SNP in relation to ICH failed to show association [Catto 1997].

There are a few studies that have investigated coagulation factor genes, such as coagulation factor XIII (FXIII), in relation to hemorrhagic stroke. A common variant in FXIII, Val34Leu, has been shown to affect the structure of the fibrin clot [Ariens 2002]. The Leu34 variant is activated more rapidly compared with the Val34 variant, and produces clots with thinner cross-linked fibrin fibres and smaller pores [Lim 2003]. This SNP, and a few other FXIII SNPs, have been investigated in relation to hemorrhagic stroke. Some studies have reported associations [Reiner 2001, Catto 1998], but others have not been able to replicate these associations [Corral 2001, Endler 2003].

Inflammation in stroke

Evidence has accumulated that inflammation plays an important role in stroke, both in the development and destabilization of atherosclerotic plaques and during the ischemic event [Ridker 1997, Rost 2001, Chamorro 2004, Muir 2007]. Furthermore, several observations have suggested that infection is a trigger for acute ischemic stroke, possibly mediated by the prothrombotic effects of the inflammatory response [Grau 1997, Lindsberg 2003]. In line with this, a number of acute phase markers, such as C-reactive protein (CRP), the most commonly investigated marker of inflammation, have been reported as potential risk factors for ischemic stroke [Ridker 1997, Kuo 2005, Muir 2007]. An increase in risk for ischemic stroke has been observed in prospective studies, even at modestly elevated CRP concentrations, and the term “low grade chronic inflammation” has been coined. The utility of measuring CRP to establish risk for ischemic stroke is, however, questioned [Lowe 2005, Di Napoli 2005] and there are uncertainties regarding whether CRP is just a marker of underlying vascular disease predisposing to stroke, or if it plays an active role in processes that trigger an acute event [Bassuk 2004, Jialal 2004]. Because inflammation is tightly linked to atherosclerotic processes, it can be hypothesised that it may be more important in LVD, and to some extent in SVD, than in other forms of stroke. Thus, heterogeneity may provide a clue to the discrepant results on the utility of CRP as an independent risk factor for stroke. The only study that has investigated the risk of high CRP levels in relation to subtypes of stroke showed that elevated CRP in middle adulthood, and in men with healthy risk factor profiles, may be an important risk factor for thromboembolic stroke (a combination of atherothrombotic infarction and embolic events) [Curb 2003]. It is of note that studies on the association between acute CRP levels and subtypes of stroke are hampered by the acute inflammatory response following the brain injury and mainly reflect stroke severity [Muir 1999, Eikelboom 2003].

A number of studies have demonstrated that CRP levels are influenced by genetic variation [Kivimäki 2007, Hage 2007]. In the CRP gene a number of SNPs have been investigated and a trinucleotide variant, −286C>T>A (also denoted -390C>T>A), appears to be functional [Szalai 2005, Hage 2007]. Limited data is available regarding associations of CRP SNPs and stroke. Morita reported an association with the rare 1,059G>C SNP and ischemic stroke (excluding CE stroke) [Morita 2006]. However, Miller failed to detect any association in a group of 264 thromboembolic stroke patients from the Physicians´ Health Study [Miller 2005] and the Rotterdam Scan Study investigated several SNPs in relation to SVD, but failed to detect any association [Reitz 2007]. Still, if the risk increase associated with a particular genetic variant of CRP can be expected to be in the same range as the OR's reported for some other stroke susceptibility SNPs, all these studies have been underpowered and as recently stated, "this area requires further study" [Muir 2007].

Apart from CRP SNPs, polymorphisms in several genes that encode proteins involved in inflammatory mechanisms have been investigated. These include PDE4D and ALOX5AP which, as mentioned above, initially were identified as positional candidate genes. Some other genes that have been investigated are those encoding interleukin 1, interleukin 6, interleukin 18, TNF α, toll-like receptor 4, P-selectin and E-selectin [Um 2005]. In most cases, findings have been either negative or have not been replicated in subsequent studies [Dichgans 2007].

SUBJECTS AND METHODS

The Sahlgrenska Academy Study on Ischemic Stroke - SAHLSIS

Patients

Ischemic stroke patients below the age of 70 were consecutively recruited at four Stroke Units in Western Sweden, i.e. the Stroke Units at the Sahlgrenska University Hospital (SU), SU/Östra, Skaraborg and Södra Älvsborg hospitals. Inclusion was initiated in August 1998 and the goal of recruiting 600 patients was achieved in December 2003. Patients were included if they fulfilled the following criteria: (1) acute onset of clinical symptoms suggestive of stroke, (2) no hemorrhage on CT scan or MRI of the brain. Patients were excluded if (1) they were older than 69 years, (2) the following evaluation showed another etiology than ischemic stroke, and if (3) they had a diagnosis of cancer at advanced stage, infectious hepatitis or HIV. Patients were included regardless of previous cardiovascular or cerebrovascular disease. The upper age limit was chosen based on studies indicating that the genetic contribution is greater in patients suffering a stroke at younger age [Floßmann 2004, Schulz 2004]. Of 645 eligible stroke patients, 29 were unwilling to participate and 16 died before the patient or next-of-kin could give detailed informed consent.

Cases were examined during the acute stage (day 1-10 after the event) and at follow-up after 3 months. Examinations were carried out by a physician trained in stroke medicine. The protocol included questionnaires, ECG, anthropometrics and standardized blood and plasma sampling between 8:30 and 10:30 AM after an overnight fast of at least 8 hours. Blood pressure was measured after 10 minutes at rest in the supine position. All patients without contraindications at Sahlgrenska were investigated by MRI of the brain (89% of Sahlgrenska cases). Other Sahlgrenska patients and patients at other units were investigated by CT. Extracranial carotid and vertebral duplex ultrasound (82% of cases), MR angiography (31%), cerebral angiography (7%) transcranial Doppler ultrasound (24%), transthoracic and/or transesophageal echo-cardiography (78%) were performed when clinically indicated. Based on the clinical presentation patients were classified according to the OCSP classification [Bamford 1991] into the clinical subtypes TACI, PACI, POCI and LACI. Adjudication of etiological subtype, using the information from clinical, radiological, cardiac and ultrasound tests, was performed by two neurologists who were blinded to genotypes according to TOAST criteria [Adams 1993]. As to minimize interrater variability, the original TOAST were refined in a protocol [Gordon 1993] and risk factors, other than atrial fibrillation and carotid stenosis, were not included in the protocol. Patients were classified into the etiological categories LVD, SVD, CE stroke, other determined etiology, cryptogenic stroke, and undetermined stroke. Stroke of other determined etiologies included those with arterial dissection, vasculitis, hematologic disorders, and complications of cardiovascular procedures. Cryptogenic stroke was defined when no cause was identified despite

an extensive evaluation and undetermined stroke included cases in whom more than one etiological subtype was identified, or in whom the evaluation was cursory. Outcome was assessed after 3 months using the modified Rankin scale (mRS) [van Swieten 1988].

Controls

For each case, one control without a history of atherothrombotic disease was recruited. Each control was matched for age (+/- 1 year), sex and geographical residence area. The controls were randomly selected from participants in a population-based health survey [Wilhelmsen 1997] (Göteborg residents) or the Swedish Population Register (Skaraborg and Älvsborg residents, and controls younger than 30 years). If a selected control did not respond, refused to participate or had a history of atherothrombotic disease, a second and then a third matched control was invited. Of the 1107 selected controls, 208 did not respond, 191 were unwilling to participate, and 108 were excluded because of a history of stroke, coronary or peripheral artery disease, or signs of ischemic heart disease on resting-ECG according to the Minnesota code (1982). Controls were examined once by a research nurse, using the same questionnaires and following the same protocol as for cases.

The study was approved by the Ethics Committee of Göteborg University and data handling procedures were approved by the National Computer Data Inspection Board. All participants gave their written informed consent. Next-of-kin consented for those participants who were unable to communicate.

Case-control study on aneurysmal subarachnoid hemorrhage

Patients

Patients admitted to the Neurointensive Care Unit (NICU) at Sahlgrenska University Hospital with symptoms suggestive of SAH, combined with subarachnoid blood on CT, were considered for inclusion in this study. Inclusion started in October 2000 and in June 2004, 253 consecutive patients had been considered of whom 183 patients were included in the study. Patients were included if they fulfilled the following criteria: (1) aneurysmal origin of the hemorrhage proven by intra-arterial angiography, and (2) admission to the NICU within two days of the acute event. Patients were excluded if (1) failure to include the patient in the study prior to day 2, or (2) failure to receive informed consent.

The diagnostic CT scan was performed at the “first” hospital and was re-examined by a neuroradiologist. A venous blood sample was obtained after admission to the NICU. One year after the aSAH, clinical examinations combined with a structured face-to-face interview performed according to Wilson and co-workers [Wilson 1998] were used to categorize outcome in patients according to the extended Glasgow Outcome Scale (GOSE). Patients

who were unable to visit the outpatient clinic for practical reasons were visited at their nursing home.

Controls

For each case, two controls without a history of atherothrombotic disease were recruited. Each control was matched for age (+/- 2 years) and sex. The controls were randomly selected from participants in a population-based health survey [Wilhelmsen 1997] or the Swedish Population Register. If a selected control did not respond, refused to participate or had a history of atherothrombotic disease, a second and then a third matched control was invited. Controls were examined once, using the same questionnaires and following the same protocol as in SAHLSIS.

The study was approved by the Ethics Committee of Göteborg University. All participants gave informed consent. When patients were unable to communicate, his or her next-of-kin gave informed consent.

Biochemical analysis of plasma proteins

Blood was drawn without stasis into tubes containing 1/10 0.45 M sodium citrate buffer, pH 4.3 (Biopool Stabilyte®, Biopool International, Sweden) for determination of tPA and PAI-1, into Vacuette®Serum tubes (Greiner Bio-one, Austria) or Vacuette® EDTA K2 Tubes, containing 1.8 mg EDTA per 1ml blood (Greiner Bio-one, Austria), for determination of hsCRP and into tubes containing 1/10 vol. of 0.13 M sodium citrate for determination of intact TAFI and released AP. Plasma was isolated within two hours by centrifugation at 4°C and 2000g for 20 minutes. Plasma aliquots were immediately frozen and stored at -70˚C.

Venous plasma levels of total tPA and total PAI-1 antigen were determined at our lab by enzyme-linked immunosorbent assays (TintElize® tPA, Biopool International, Sweden and COALIZA® PAI-1 Chromogenix, Haemochrom Diagnostica, Sweden), which detect free and complexed forms of the respective proteins with equal efficiency [Rånby 1989, Meijer 1994]. Plasma tPA activity, i.e. the uncomplexed fraction of tPA, was measured at our lab by a biofunctional immunosorbent assay (BIA, Chromolize™ tPA, Biopool International). For comparison with tPA antigen, tPA activity was expressed in µg/L using the specific activity of 600 IU/µg [Jern 2004]. The two samples from each case and the corresponding matched control sample were assayed in duplicate on the same microtiter plate. The intra-assay coefficients of variation were on the average 2.2% for both tPA antigen and activity and 2.9% for PAI-1 antigen.

Plasma levels of TAFI were measured at prof Paul Declerck's lab in Belgium by sandwich-type ELISAs using monoclonal antibodies (MA) MA-T12D11/MA-T30E5-HRP for intact TAFI and MA-T12D11/MA-T18A8-HRP for released AP [Ceresa 2006]. Values were expressed relative to pooled human plasma. Intra-assay coefficients of variation were 6.2% for intact TAFI and 3.1% for AP.

Serum levels of high sensitivity CRP (hsCRP) was analyzed by our group in collaboration with Diagnostic Products Corporation, Mölndal, by a solid-phase chemiluminescent immunometric assay on IMMULITE 2000 (Diagnostic Products Corporation, USA) using the manufacturers reagents as directed. The intra-assay variation coefficient was on the average 3.4%.

Genetic variation

DNA extraction

DNA was extracted from venous blood samples using QIAamp® 96DNA Blood Kit (QIAGEN, Germany) using the manufacturer's reagents as directed. DNA concentration was quantified with either PicoGreen (Moleular probes, Netherlands) on a fluorometer (Fluostar Galaxy, BMG LABTECH, Germany) or with a ND-1000 spectrophotometer (NanoDrop Technologies, USA) and diluted in TE buffer to 10 ng/μl.

Selection of SNPs

The selection of SNPs for the genetic analysis were based on the groups previous resequencing projects, reports from the literature and data from public databases, including dbSNP (www.ncbi.nlm.nih.gov/projects/SNP), SeattleSNP (http://pga.mbt.washington.edu), and HapMap (www.hapmap.org). The tPA -7,351C>T was first described by our group in 2000 after resequencing of the tPA exons, promoter and enhancer [Ladenvall 2000]. The other SNPs that were genotyped in papers II, IV and V were selected based on reports from the literature. To select SNPs for paper III, we used information from HapMap to select tagSNPs that would capture unmeasured variation over the TAFI gene with an r^2>0.80. Using the "solid spine of LD" setting in the publicly available software Haploview version 3.2 (www.broad.mit.edu/personal/jcbarret/haploview), 7 SNPs were selected by TAGGER [de Bakker 2005] to capture genetic variation with MAF>0.05 over a 52.4 kb single haplotype block. In addition we chose to include 3 SNPs that in previous works had been studied in relation to TAFI levels, and 1 synonymous SNP in exon 6.

Genotyping

All SNPs were genotyped using 5´ nuclease (TaqMan) chemistry [Holland 1991, Lee 1993, Livak 1995]. It is based on fluorogenic-labeled oligonucleotide probes and uses the 5´→3´ exonuclease activity of *Taq* DNA polymerase (the ability to digest DNA ahead of it on its template) during PCR amplification. The set up of the reaction is very similar to a conventional PCR, but in addition two probes are included in the reaction, one for each allelic variant of the SNP. The probes are single-stranded oligonucleotides, complementary to the genomic region surrounding the SNP, and located between the two primers. A reporter fluorescent dye, or fluorophore, and quencher dye are covalently attached to the 5' and 3' ends of the probe, respectively. While the probe is intact, the close

proximity between quencher and fluorophore greatly reduces the fluorescence emitted by the reporter dye via fluorescence resonance energy transfer through space. During PCR, as DNA synthesis commences, the 5′→3′ exonuclease activity of the *Taq* polymerase degrades the probe that has annealed to the template as the primer is extended. This releases the reporter dye and breaks the close proximity to the quencher, thus relieving the quenching effect and allowing fluorescence of the fluorophore. Removal of the probe from the target strand allows primer extension to continue to the end of the template. The fluorescence detected at the end of the PCR is directly proportional to the amount of fluorophore released from each probe. Hence, the assay is able to discriminate between the different homozygote and the heterozygote individuals (Figure 4).

Advantages of the TaqMan chemistry, compared with other techniques, is that the whole assay is performed in a closed tube system and does not require post-PCR handling, a relatively high throughput, that the design of probes is fairly flexible (also, many pre-designed assays are available) and that it can be multiplexed (several SNPs genotyped simultaneously). Disadvantages are that multiple samples must be genotyped simultaneously to be able to discriminate between genotypes, the cost, and the fact that the actual sequence surrounding the SNP is not revealed. Compared with more recently developed techniques, the TaqMan chemistry also provides little possibilities of multiplexing.

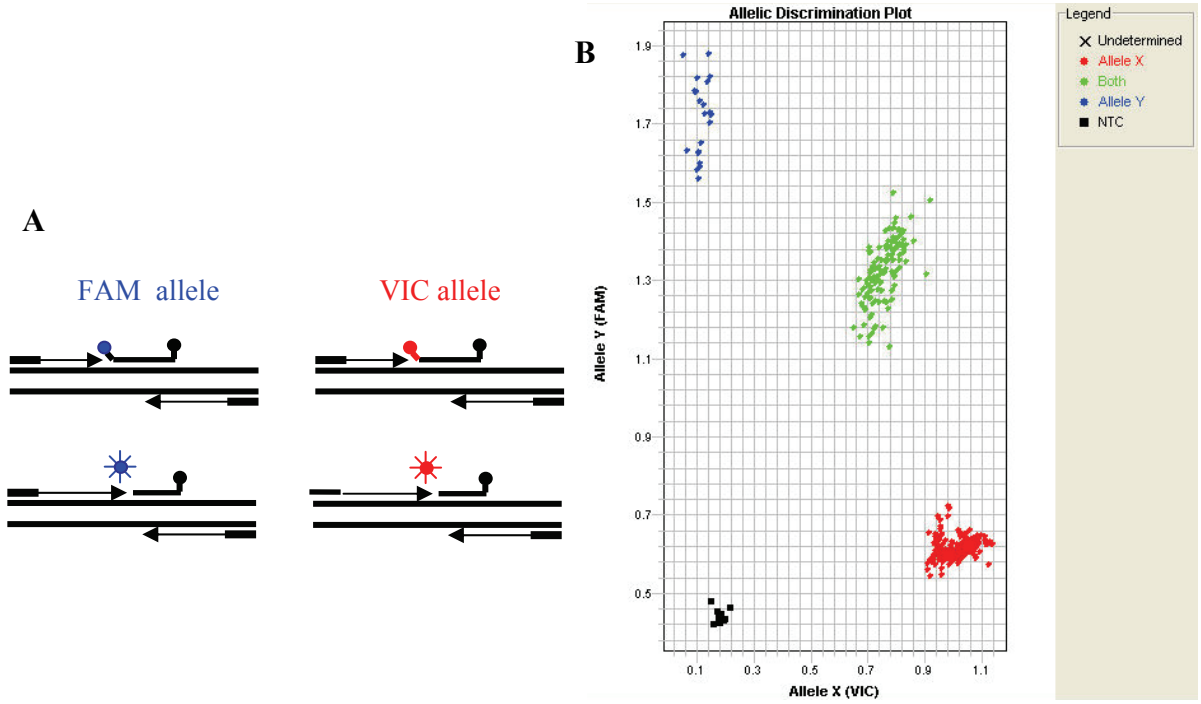

Figure 4. The TaqMan assay.
A. The probe that anneals with a perfect match to the template strand is degraded during PCR, releasing the corresponding fluorophore. B. Example of an allelic discrimination plot

All 5´nuclease assays used in papers II, IV and V were custom designed (Table 2) using sequences from our group's previous re-sequencing projects or from the NCBI Reference Sequence (RefSeq). The assay design for one SNP in paper IV (i.e the CRP -286 C>T>A, rs3091244) was originally published by Carlson et al. [Carlson 2005]. Designs were made using the Primer Express 1.5 software (Applied Biosystems, USA). Probe lengths were adjusted to give a calculated melting temperature (Tm) of 65-67˚C and primer pairs were selected with a Tm of 58-60˚C. When the genomic region surrounding the SNP was AT rich, or had neighbouring polymorphisms or repeats, a minor groove binder (MGB) was used to shorten the length of the probe. Both primer and probe concentrations were optimized, aiming to get good amplification with similar fluorescent signals from both fluorescent dyes. Some of the 5´nuclease assays in paper III were purchased as Assay-on-Demand (assays available from Applied Biosystems) (i.e the rs2181617 (Catalogue number C_15843088_10), rs7336399 (C_1872246_10), rs7337140 (C_9884940_10) and rs2404965 (C_1872240_10)). Assays were not readily available for rs9526136, rs1022953, rs17067700 and rs2146881 and these were purchased as Assay-by-Design (assays designed by Applied Biosystems after request). Applied Biosystems failed to design an assay for SNP rs1926447. Consequently, we made a design of our own (Table 2). The final 2 SNPs in paper III (rs940 and rs3742264) were analyzed using our group's own designs [Tjärnlund 2003].

Table 2. 5´ nuclease assay designs

Oligonucleotide (5´→3´)	**Conc.**
tPA -7,351C/T, rs2020918	
FP: AGTGATCTCATTGCCGAGGTG	300 nM
RP: CCCAGAGTCCCAGGCCA	300 nM
C-probe (VIC)AAAGGAGCC**C**GCCCCAGACA(TAMRA)	100 nM
T-probe (FAM)CCAAAGGAGCC**T**GCCCCAGAC(TAMRA)	100 nM
PAI-1 -675 4G>5G, rs1799889	
FP: TCTTTCCCTCATCCCTGCC	900 nM
RP: CCAACCTCAGCCAGACAAGG	900 nM
4C-probe: (VIC)ACACGGCTGACT**CCCC**ACGT(TAMRA)	200 nM
5C-probe: (FAM)ACGGCTGACT**CCCCC**ACGT(TAMRA)	200 nM
TAFI Ala147Thr, rs3742264	
FP: GCTTATTGTTACTGTTTTTGCTACTTTTG	300 nM
RP: TGGCATGGATTCCACAGTCA	300 nM
Ala-probe:(VIC)CTGGAAAAGAACAA**G**CAGCCAAAAATG-(TAMRA)	100 nM
Thr-probe: (FAM)TGGAAAAGAACAA**A**CAGCCAAAAATGC-(TAMRA)	200 nM
TAFI Thr325Ile, rs1926447	
FP: AGCTCAAAGTTCTCTAAGATCATAAGAAGA	300 nM
RP: AGTCTCTAGTAGCCAGTGAAGCAGTTC	300 nM
Thr-probe: (VIC)-TTTACTA**G**TTTTCTCAATAGCA-(MGB)	100 nM
Ile-probe: (FAM)-TTTTACTA**A**TTTTCTCAATAGCA-(MGB)	100 nM

Table 2 (continued). 5′ nuclease assays

Oligonucleotide (5′→3′)	Conc.
TAFI 1,542 C>G, rs940	
FP: ACCTACTTTTTCTTTGATTTTCGACG	300 nM
RP: AGCGTGAGATGATCTTTGATTAAACTT	300 nM
C-probe: (VIC)CAAGCAA**C**TTTCGACG-(MGB)	100 nM
G-probe: (FAM)CAAGCAA**G**TTTCGACG-(MGB)	100 nM
CRP -732T>C, rs2794521	
FP: TGTGTCCAAGTATTCTCATTGTTCAA	300 nM
RP: CATTTAGTGCCAAGATGTCTAGAGAGTT	300 nM
T-probe: (VIC)-TATGAGTGAGAACA**T**GCGGTGTTTGGTT-(TAMRA)	100 nM
C-probe: (FAM)-AGTGAGAACA**C**GCGGTGTTTGGTTTT-(TAMRA)	100 nM
CRP 1,059 G>C, rs1800947	
FP: CGGTGGGAACTTTGAAGGAA	300 nM
RP: CGCCAGTTCAGGACATTAGGA	300 nM
G-probe: (VIC)-TGTGGGACTTTGTGCT**G**TCACCAGAT -(TAMRA)	100 nM
C-probe: (FAM)-TGGGACTTTGTGCT**C**TCACCAGATGA-(TAMRA)	100 nM
CRP 1,444 C>T, rs1130864	
FP: AGAAATTATCTCCAAGATCTGTCCAACT	300 nM
RP: GTCTGGTCTGGGAGCTCGTTA	300 nM
C-probe: (VIC)-TTTGGACC**G**TTTCC-(MGB)	100 nM
T--probe: (FAM)-TGGACC**A**TTTCCCA-(MGB)	100 nM
CRP -286 C>T>A, rs3091244*	
FP: TGTTGGAGAGGCAGCTACCA	1000 nM
RP: TCCTGCGAAAATAATGGGAAA	1000 nM
C-probe: (FAM)- TGGCCACT**C**GTTTAA-(MGB)	200 nM
T-probe: (VIC)-ATGGCCACT**T**GTTTAA-(MGB)	200 nM
A-probe: (NED)-ATGGCCACT**A**GTTTAA-(MGB)	200 nM
FXIII Val34Leu, rs5985	
FP: CAATAACTCTAATGCAGCGGAAGA	300 nM
RP: TGCTCATACCTTGCAGGTTGAC	300 nM
Val-probe: (VIC)TTCAGGGC**G**TGGTGCCCC(TAMRA)	100 nM
Leu-probe: (FAM)TCAGGGC**T**TGGTGCCCCG(TAMRA)	100 nM
FXIII Tyr204Phe, rs3024477	
FP: GATGATGCTGTGTATCTGGACAATG	300 nM
RP: CCAGCTTCTGGTCTTGATGTCA	300 nM
Tyr-probe: (VIC)-AAGAGT**A**TGTCCTGAATG-(MGB)	100 nM
Phe-probe: (FAM)-AGAGT**T**TGTCCTGAATG-(MGB)	100 nM
FXIII Pro564Leu, rs5982	
FP: TCACGTCGAACGTCTCCTTCT	300 nM
RP: AACCGTTACACCATCACAGCTTATC	300 nM
Pro564: (VIC)- ATTCTGCCTTC**G**GGAC-(MGB)	100 nM
Leu564: (FAM)- TCTGCCTTC**A**GGACC-(MGB)	100 nM

Thermal cycling conditions were: Two initial holds (50˚C for 2 min and 95˚C for 10 min) followed by a 40-cycle two-step PCR (95˚C for 15 s and annealing for 1 min). The annealing temperature was 60˚C for all assays except for PAI-1, 62˚C. FP denotes forward primer; RP, reverse primer; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxy-N,N,N',N'-tetramethyl-rhodamine; MGB, minor groove binder. VIC and NED are trademark products from Applied Biosystems. *the assay included 1 M betaine (Sigma-Aldrich), as proposed in the original design [Carlsson 2005].

PCR amplifications were carried out on Dual 96-Well GeneAmp® PCR System 9700 (Applied Biosystems, USA) and fluorescence was read on either an ABI PRISM® 7900HT Sequence Detector System (Applied Biosystems, USA) (papers III, IV and V) or on an ABI PRISM® 7700 Sequence Detector System (Applied Biosystems, USA) (paper II). In each assay we used 20 ng of genomic DNA, and primers, probes and PCR master mix from Applied Biosystems. The fluorescent signals were read directly after PCR. Each 96-well (ABI PRISM® 7700) and 384-well (ABI PRISM® 7900HT) plate included several samples without DNA as negative non-template controls that were spread over the plate. Duplicate samples of the three genotypes were used as positive controls in papers II and IV. In papers III and V, 12 control individuals were included in each separate run, to ensure consistency in genotyping between runs. To exclude the possibility of false genotype calls, 10% of the genotypes in papers II and IV were determined twice in separate runs. Because no errors were detected, we did not pursue this quality check in papers III and V. Determination of individual genotype was performed blinded to case/control status.

Haplotype inference

Because humans have 2 pairs of chromosomes, and because most people are heterozygous for several markers, it is a laborious task to determine with certainty what haplotypes an individual carries. Current genotyping and sequencing techniques are not capable of distinguishing what marker alleles that cosegregate on the same chromosome, thus the data produced by these techniques is "un-phased". "Phased" genotype data can be obtained by more elaborate techniques, for instance by genotyping sperm cell lines, or by a number of other recently developed methods [Andrés 2007]. However, these methods are low-throughput and costly. To circumvent this problem, a number of algorithms for inferring haplotypes from unphased genotype data have been developed [Niu 2004, Andrés 2007]. The earliest algorithm for haplotype reconstruction from genotype data was developed by Clark in 1990 [Clark 1990]. However Clarks´ algorithm requires homozygous or single-site heterozygous individuals to initiate, and has some other disadvantages [Niu 2004]. Subsequently the expectation-maximization (EM) algorithm was developed [Excoffier 1995]. It estimates haplotype probabilities based on maximum likelihood, finding the values of the haplotype probabilities which optimize the probability of the observed data. A disadvantage with the iterative nature of the EM algorithm is that it is sensitive to the initial value of haplotype frequencies. If there are local maxima, the iteration may lead to locally optimal maximum likelihood estimates. To avoid convergence to local maxima and saddle points, a variant of the EM algorithm, the stochastic-EM (SEM) algorithm was developed [Tregouet 2004]. As implemented in the THESIAS software, it can handle some missing genotype data and allows for simultaneous estimation of covariate adjusted haplotype-phenotype association parameters. In papers III and IV, THESIAS was used to infer haplotypes and to test associations between haplotypes and overall ischemic stroke and subtypes of stroke.

A number of other algorithms for haplotype reconstruction from genotype data have been developed [Niu 2004, Andrés 2007]. In a test of five of these algorithms on HapMap and simulated data, PHASE was the most accurate [Marchini 2006]. Consequently, PHASE [Stephens 2001, Stephens 2003] was used to reconstruct FXIII haplotypes in paper V.

Statistical analysis

Differences in categorical variables between groups were examined using the χ^2 test for proportions and with Student t test for continuous variables. When tests of normality indicated a skewed distribution of plasma variables, nonparametric tests were used or the plasma variables were logarithmically transformed. In papers III and IV, ANOVA and Tukey´s post-hoc test were used to test for differences in logarithmically transformed plasma variables by TOAST subtype.

In paper I conditional logistic regression was used to calculate multivariate ORs and 95% CIs for family history variables and risk factors in cases versus controls. In subsequent papers, we compared the results from conditional logistic regression with binary logistic regression when the association between plasma variable and overall ischemic group was considered. Because results were essentially the same, and because we chose to include the whole control group in etiological subtype regression analyses, only binary logistic regression results were reported for papers II-V. In papers III and IV, the reported ORs were scaled to estimate the OR associated with an increase of 1 SD in respective plasma variable. Associations between single SNPs and ischemic stroke were similarly investigated using binary logistic regression adjusted for traditional risk factors; please see each original paper for details.

In papers III and IV, THESIAS was used for haplotype association analyses and to estimate haplotype frequencies, pairwise LD coefficients, deviations from the Hardy-Weinberg equilibrium (HWE) and geometric means of the plasma variable associated with one dose of each haplotype. Thus, the mean plasma level of an individual then correspond to the sum of the mean levels associated with his/her two haplotypes. Deviations from genotype frequencies estimated by the HWE were tested using the χ^2 test in paper II, and in an online version of the DeFinetti program, available at http://ihg.gsf.de/cgi-bin/hw/hwa1.pl, in paper V. In paper V, PHASE was used for haplotype inference. The individual likelihood for each haplotype, as estimated by PHASE, was used in regression analyses [Schaid 2004, Kraft 2007].

In paper I, differences between risk factor subtype associations were assessed through analysis of the generalized logits for the subtypes versus controls. In papers I, III and IV, associations between functional outcome and relevant variables were examined in cases.

Adjustment for multiple testing was not made. Data were analyzed using SPSS 12.0 (papers I-IV), 15.0 (paper V) and SAS 8.2 (paper I). Statistical testing was performed at a 2-tailed $p<0.05$ level.

Power calculations

The PS Power and Sample Size Calculations software was used for power calculations [Dupont 1997]. In SAHLSIS, it was estimated that for SNPs with MAF ≥ 0.3, the study has a statistical power of 80% to detect ORs >1.4 at the 5% level. For etiological subtypes, the corresponding ORs were 1.7 to 2.3. For SNPs with MAF≥ 0.1, the study had sufficient power to detect ORs >1.65 in overall ischemic stroke and 2.1 to 2.9 in subtypes.

The case control study on SAH had 80% power to detect ORs >1.7 at the 5% level for SNPs with MAF≥0.3. For less common SNPs, with MAF≥0.1, the study was sufficiently powered to detect ORs > 2.1.

RESULTS

Family history of ischemic stroke before 70 years of age

Paper I

The first report from SAHLSIS investigated associations between ischemic stroke subtypes and various vascular risk factors, including family history of stroke and MI. The distribution of clinical subtypes (OCSP criteria) were: 62 (10%) TACI, 176 (29%) PACI, 150 (25%) POCI and 206 (34%) LACI. The distribution of TOAST subtypes are presented in Figure 5. Among the 600 patients, 108 (18%) had had a previous stroke. The corresponding number for the TOAST subtypes were: 19 (26%) in LVD, 24 (19%) in SVD, 21 (21%) in CE stroke and 18 (11%) in cryptogenic stroke.

Demographic and risk factor profiles are presented in Table 3. Hypertension, smoking, family history of stroke and occupation classified as lower education were all more common in stroke patients than in controls. There were no significant differences in BMI or number of siblings between cases and controls, or between ischemic stroke subtypes. Among patients, 121 (20%) did not display any of the established risk factors, i.e. hypertension, smoking or diabetes. The corresponding figures for subtypes were: 7 (10%) in LVD, 16 (13%) in SVD, 26 (26%) in CE stroke and 40 (25%) in cryptogenic stroke. Among controls, 288 (48%) were free of the established risk factors.

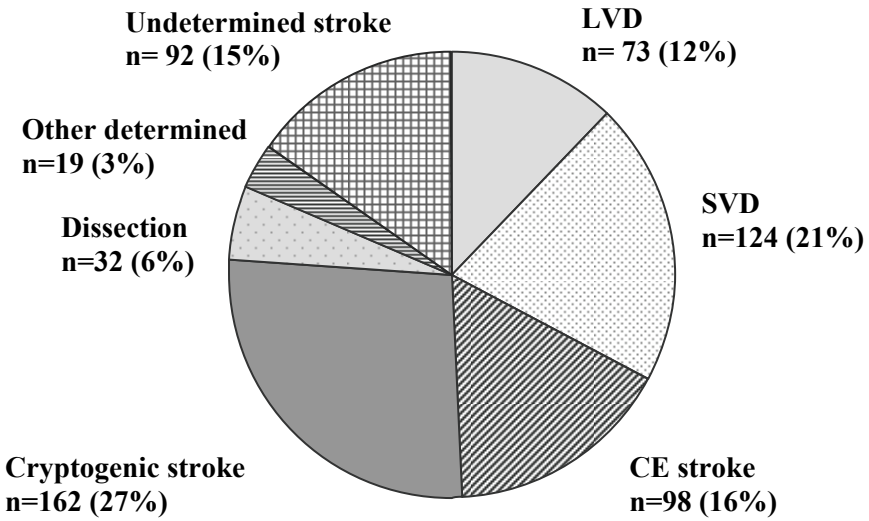

Figure 5. Distribution of ischemic stroke subtypes.

Multiple adjusted ORs for overall ischemic stroke and the major stroke subtypes according to vascular risk factors and family history variables are presented in Figure 6. Established risk factors showed association with overall ischemic stroke and with all major subtypes. Hyperlipidemia showed association with overall ischemic stroke, but not with any of the major subtypes. Lower occupational class was not associated with overall ischemic stroke or any subtype (data not shown). Analysis of the generalized logits for the subtypes versus controls revealed that the risk factor associations differed by subtype ($p<0.001$ for hypertension, diabetes and smoking, $p<0.01$ for family history of MI and $p<0.05$ for family history of stroke). Hypertension was strongly associated with SVD and smoking and diabetes displayed a strong association with LVD. Family history of stroke was associated with LVD, SVD and cryptogenic stroke, but not with CE stroke. Family history of MI was strongly associated with LVD, but no significant independent association was observed for the other subtypes.

Family history of stroke was significantly associated to ischemic stroke in both sexes (multiple adjusted OR 1.80; 95% CI 1.26-2.89 for women and OR 1.66; 95% CI 1.18-2.32 for men). The multivariate ORs for history of stroke in a sibling, mother or father were OR 2.20; 95% CI 1.03-4.71, OR 2.11; 95% CI 1.45-3.07 and OR 1.36; 95% CI 0.92-2.00, respectively.

Of the 600 cases included in SAHLSIS, 7 died before 3-month follow-up. Functional outcome at 3-month follow-up was dependent on stroke subtype. Death or dependency (mRS score ≥3) occurred in 22% of patients with ischemic stroke, 27% in LVD, 9% in SVD, 31% in CE stroke and 20% in cryptogenic stroke. Patients reporting a positive family history of stroke had a lower risk of an unfavourable outcome (multivariate OR 0.53; 95% CI 0.34-0.84; $p<0.01$). Family history of MI and other vascular risk factors were not significantly associated with functional outcome (data not shown).

Table 3. Risk factors and family history variables in cases, controls and the major stroke subtypes

	Control	Ischemic stroke	LVD	SVD	CE stroke	Cryptogenic stroke
	n=600	n=600	n=73	n=124	n=98	n=162
Mean age, y (SD)	56 (10)	56 (10)	59 (8)*	58 (7)*	57 (10)	53 (12)**
Male sex, n (%)	385 (64)	385 (64)	54 (74)	77 (62)	66 (67)	95 (59)
No. of siblings, mean (SD)	2.1 (1.9)	2.3 (2.0)	2.3 (1.8)	2.3 (2.2)	2.1 (1.6)	2.3 (2.0)
Hypertension, n (%)	224 (37)	354 (59)***	44 (60)***	89 (72)***	50 (51)**	87 (54)***
Diabetes, n (%)	33 (6)	114 (19)***	25 (34)***	26 (21)***	19 (19)***	23 (14)***
Hyperlipidemia, n (%)	403 (67)	413 (76)**	53 (82)*	77 (71)	73 (82)**	107 (71)
Current smoking, n (%)	109 (18)	233 (39)***	39 (53)***	54 (44)***	34 (35)***	60 (37)***
BMI, mean (SD)	26.5 (4.0)	26.5 (4.5)	26.7 (4.6)	26.8 (4.3)	26.8 (4.8)	26.1 (3.9)
Occupation, lower education, n %	282 (52)	324 (62)***	43 (67)*	64 (57)	50 (60)	94 (65)***
Family history of stroke, n (%)	162 (27)	229 (41)***	31 (47)**	52 (43)***	30 (34)	61 (40)**
Family history of MI, n (%)	202 (34)	215 (38)	41 (62)***	35 (29)	42 (47)*	52 (34)
Personal history of CAD and/or PAD	-	109 (18)	21 (29)	10 (8)	40 (41)	16 (10)

CAD indicates coronary artery disease and PAD peripheral artery disease. Differences between cases and controls were examined with the χ^2 test for proportions and with Student's *t* test for continuous variables. *p<0.05; **p<0.01; ***p<0.001.

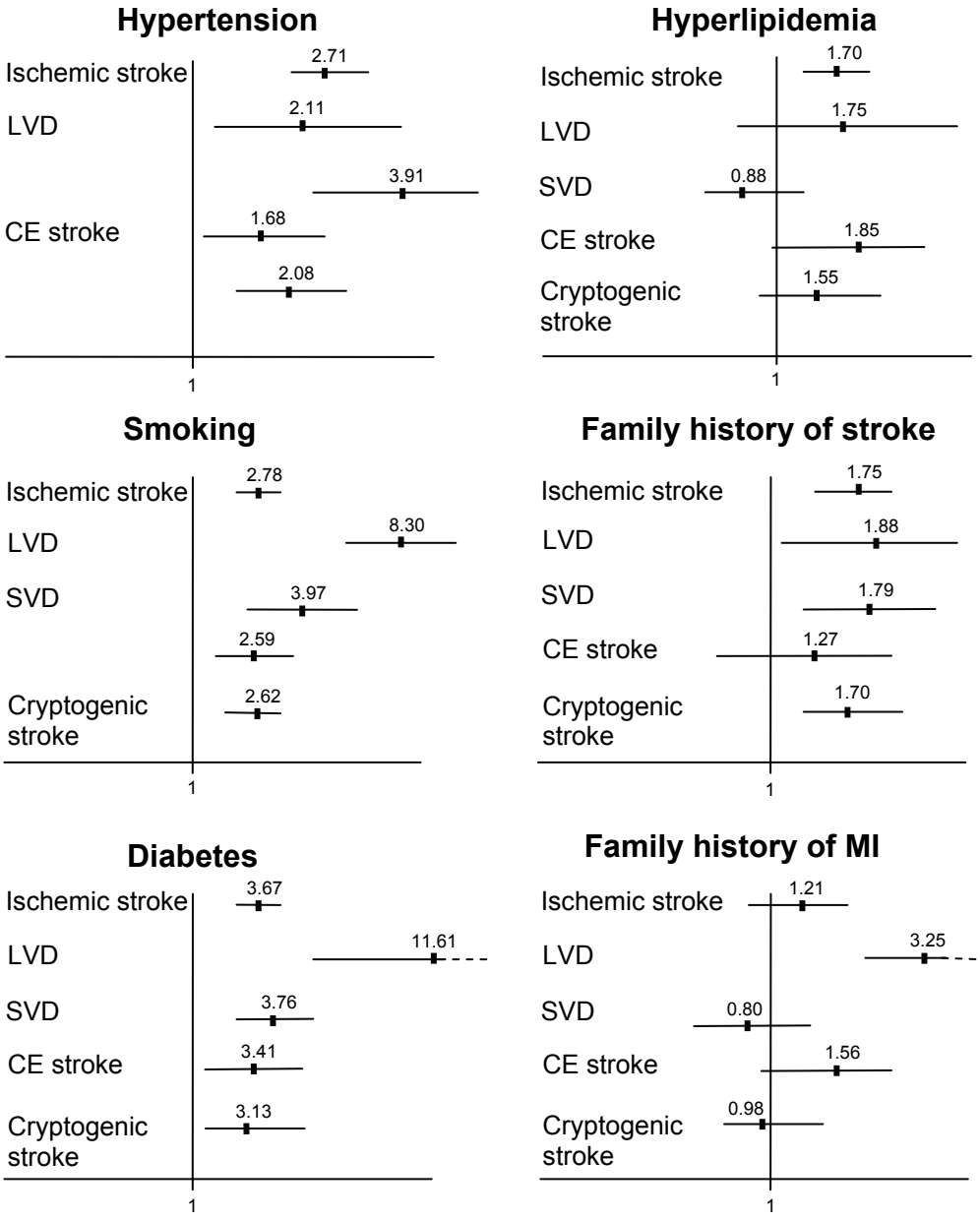

Figure 6. Multivariate ORs for risk factors and family history variables in ischemic stroke and the major subtypes. Variables included in the logistic regression were age, sex, hypertension, diabetes, smoking, hyperlipidemia, occupational class, family history of stroke, and family history of MI. Ischemic stroke 600 cases / 600 controls, LVD 73 cases / 600 controls, SVD 124 cases / 600 controls, CE stroke 98 cases / 600 controls, and cryptogenic stroke 162 cases / 600 controls.

Fibrinolytic gene polymorphisms and ischemic stroke

Paper II

In this first candidate gene association based on SAHLSIS, the possible association between ischemic stroke and the tPA -7,351C>T and PAI-1 4G>5G SNPs were investigated. Association between ischemic stroke and genotype combinations were also considered, as were plasma levels of the respective proteins.

In the acute phase, tPA antigen and activity as well as PAI-1 antigen plasma levels were significantly higher in cases than in controls (Figure 7). At 3-month follow-up, plasma tPA and PAI-1 antigen remained elevated, whilst tPA activity was similar, in overall ischemic stroke compared with controls. Among cases, PAI-1 antigen was lower in the acute phase compared with 3-month follow-up ($p<0.001$), and tPA antigen and activity were higher in the acute phase ($p<0.05$ and $p<0.01$, respectively). For each plasma measure, there was a strong correlation in plasma levels between the two time-points (Spearmans´ $\rho>0.54$). Plasma levels of tPA and PAI-1 antigen at 3-month follow-up were significantly associated with overall ischemic stroke (ORs per µg/L increase 1.10 95% CI 1.06-1.14 and 1.01 95% CI 1.01-1.01, respectively).

Differences among TOAST subtypes were observed for plasma levels of tPA antigen at both time points, and for tPA activity during the acute phase, ($p<0.001$, $p<0.001$ and $p<0.05$, respectively), whereas no subtype differences were present for PAI-1 antigen or for tPA activity at follow-up. These differences were explained by high tPA antigen levels among those with LVD and CE stroke (Figure 8), and by a lack of increase in tPA activity for SVD in the acute phase (data not shown). There were no evidence of plasma tPA or PAI-1 levels being influenced by genotype ($p>0.26$).

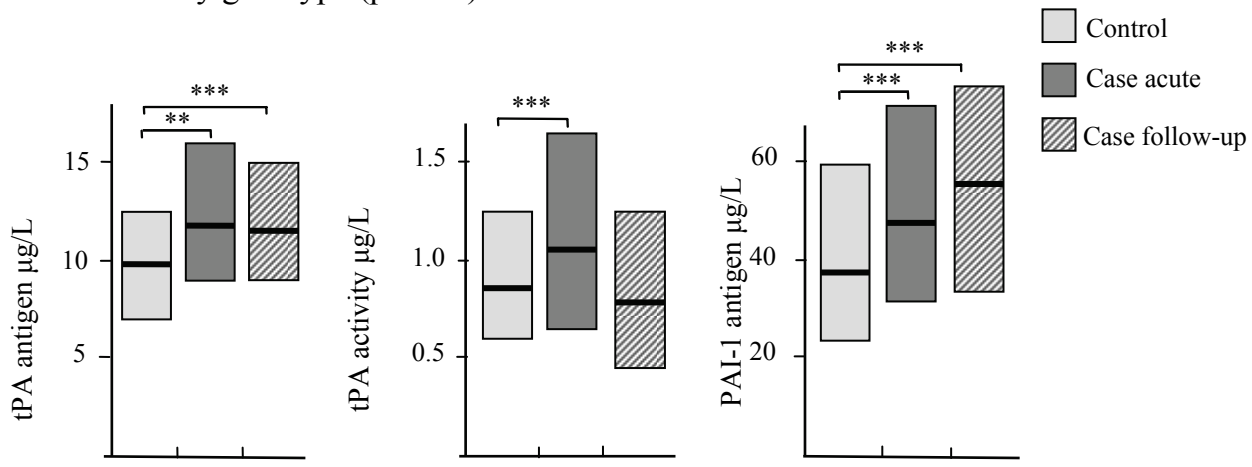

Figure 7. Median and inter-quartile range for plasma levels of tPA antigen, tPA activity and PAI-1 antigen. Control versus overall ischemic stroke (Mann Whitney U test) **p<0.01; ***p<0.001.

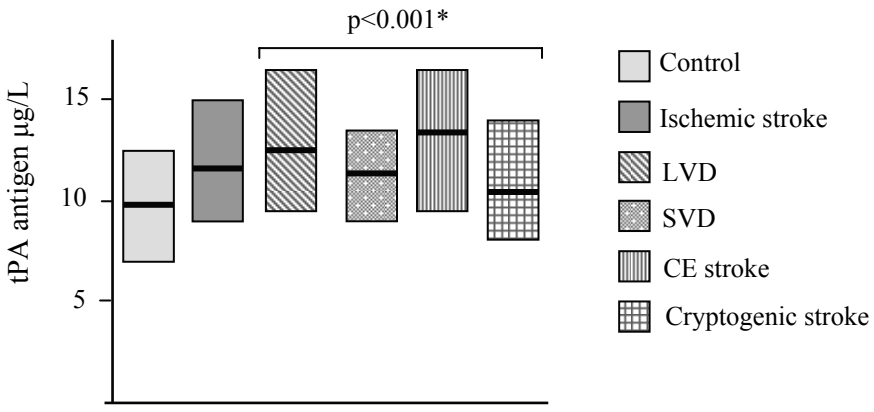

Figure 8. Median and inter-quartile range for plasma levels of tPA antigen at 3-month follow-up. *Kruskal Wallis test between subtypes.

No associations were detected between individual genotypes and ischemic stroke (Figure 9). When genotype combinations were considered, a protective effect was observed for the tPA CC/PAI-1 4G4G genotype combination, compared to the tPA CC/PAI-1 5G carrier reference genotype combination (Figure 10). When restricting the analysis to those with first-ever strokes, the results were essentially the same as for the whole group (multivariate OR 1.09; 95% CI 0.84-1.42 for tPA T allele carriers versus CC; OR 0.85; 95% CI 0.64-1.14 for PAI-1 4G4G versus 5G allele carriers and OR 0.61; 95% CI 0.40-0.95 for the tPA CC/PAI-1 4G4G genotype combination versus tPA CC/PAI-1 5G carriers).

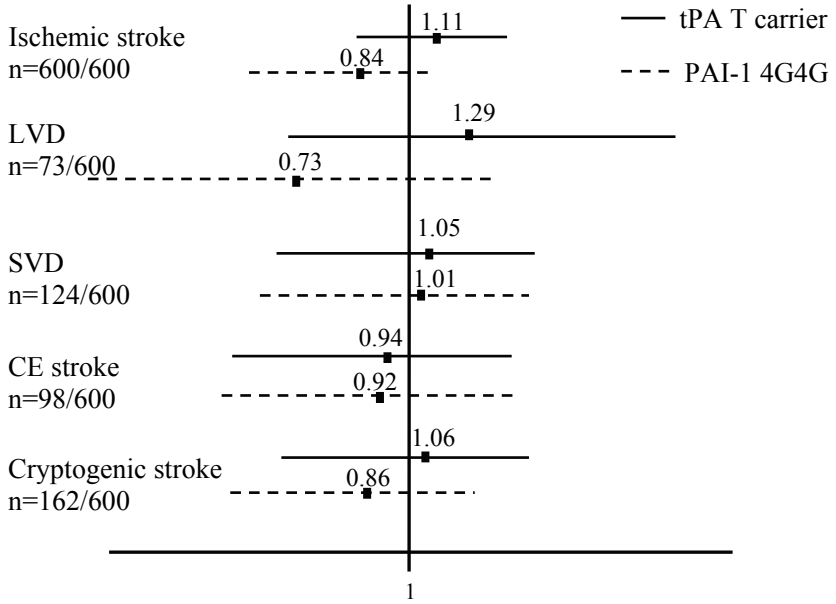

Figure 9. Multivariate ORs and 95% CI of ischemic stroke and TOAST subtypes for tPA T allele carriers versus CC genotype and for PAI-1 4G4G genotype versus 5G allele carriers. Variables included in the logistic regression models were age, sex, hypertension, diabetes, smoking, the tPA -7,351C>T SNP, the PAI-1 -675 4G>5G SNP, and plasma tPA antigen at 3-month follow-up.

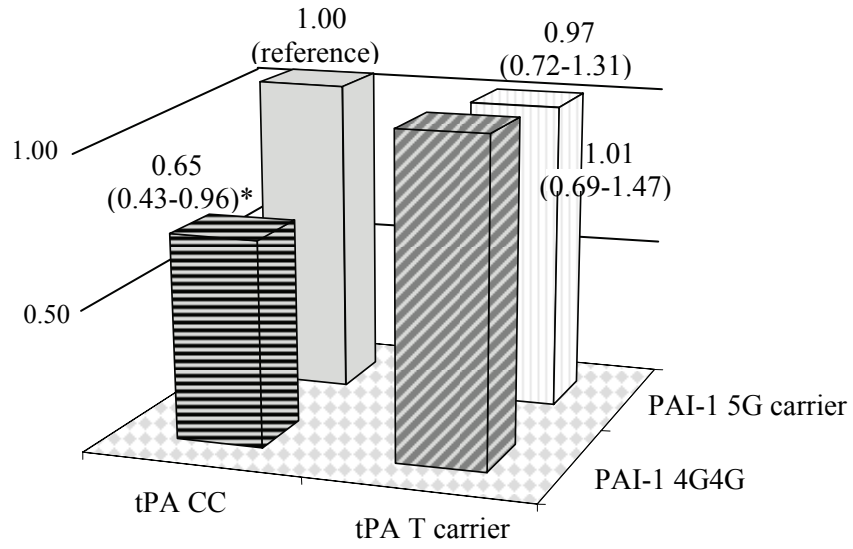

Figure 10. Multivariate ORs and 95% CI of ischemic stroke for combination of the genetic variants. Number of cases/controls for the different genotype combinations were: 195/190 for tPA CC/PAI-1 5G carrier; 64/88 for tPA CC/PAI-1 4G4G; 243/224 for tPA T carrier/PAI-1 4G4G and 96/98 for tPA T carriers /PAI-1 4G4G. *$p<0.05$.

Thrombin activatable fibrinolysis inhibitor activation peptide shows association with all major subtypes of ischemic stroke and with TAFI gene variation

Paper III

In this study genetic variation over the TAFI locus was investigated, and two recently developed assays for TAFI determination were used to investigate plasma levels of TAFI in SAHLSIS. Compared with controls, levels of both released AP and intact TAFI were significantly higher in cases, both in the acute phase and at 3-month follow-up (Figure 11).

In patients, released AP was higher at follow-up compared with the acute phase ($p<0.05$), whilst no differences between time-points were observed for intact TAFI. With respect to released AP, levels were increased in all TOAST subtypes, compared with controls, both in the acute phase and at 3-month follow-up ($p<0.001$ throughout). Acute phase intact TAFI levels were elevated in LVD ($p<0.001$), SVD ($p<0.001$) and cryptogenic stroke ($p<0.01$). At follow-up, levels remained higher for LVD ($p<0.001$) and for cryptogenic stroke ($p<0.05$), compared with controls. Intact TAFI and released AP levels were significantly correlated (Spearmans´ ρ 0.42-0.48) and were not influenced by median day of sampling in the acute phase ($p>0.20$ for both measurements). In a subgroup of controls who did not receive any medication for hypertension, diabetes or

hyperlipidemia (n=485), established risk factors explained 10.1% and 16.7% of the variation in intact TAFI and released AP, respectively. In this subgroup 89 (18%) were classified as smokers, 138 (28%) as hypertensives and 11 (2%) as having diabetes.

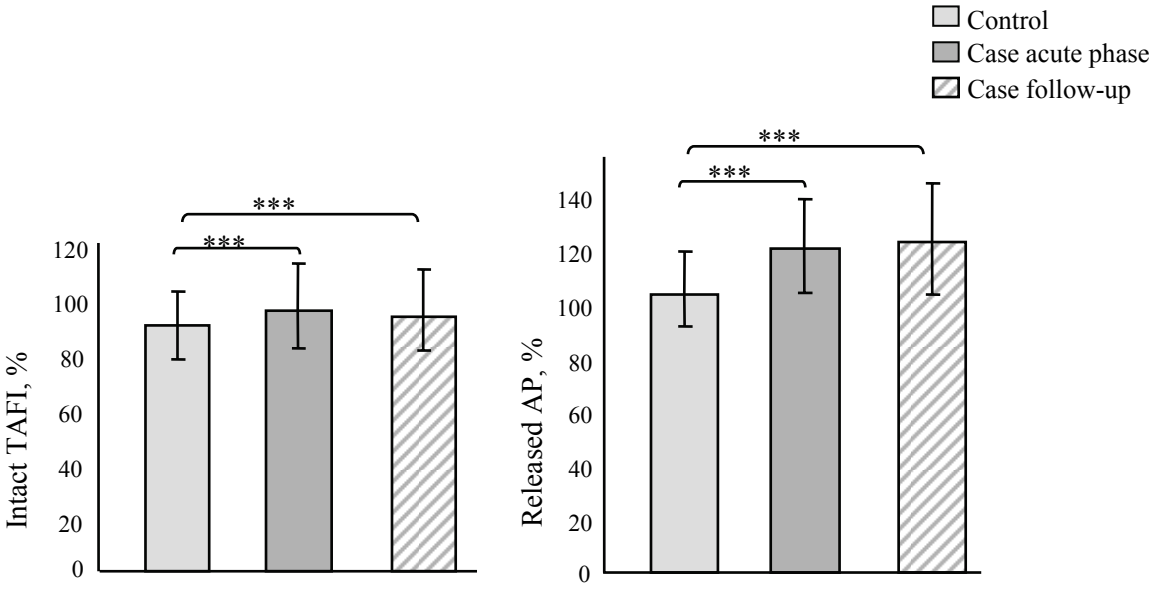

Figure 11. Median and inter-quartile range of intact TAFI and released AP in controls and in overall ischemic stroke. Control versus overall ischemic stroke (Mann Whitney U test) ***p<0.001.

Multivariate ORs for one standard deviation (SD) increase in intact TAFI and released AP are presented in Figure 12. Independent associations were observed for all groups for released AP. With regard to intact TAFI, independent associations were observed for overall ischemic stroke and all subtypes apart from CE stroke in the acute phase and for overall ischemic stroke, LVD and cryptogenic stroke at 3-month follow-up (Figure 12).

There were no differences in TAFI levels by clinical subtypes (OCSP criteria, data not shown). Levels in the acute phase were not related to outcome, but at 3-month follow-up, TAFI levels were significantly higher in cases with an unfavourable outcome (mRS score ≥3) (adjusted ORs for death or dependency 1.31; 95% CI 1.02-1.68, p=0.04, and 1.39; 95% CI 1.06-1.83, p=0.02 for intact TAFI and for released AP, respectively).

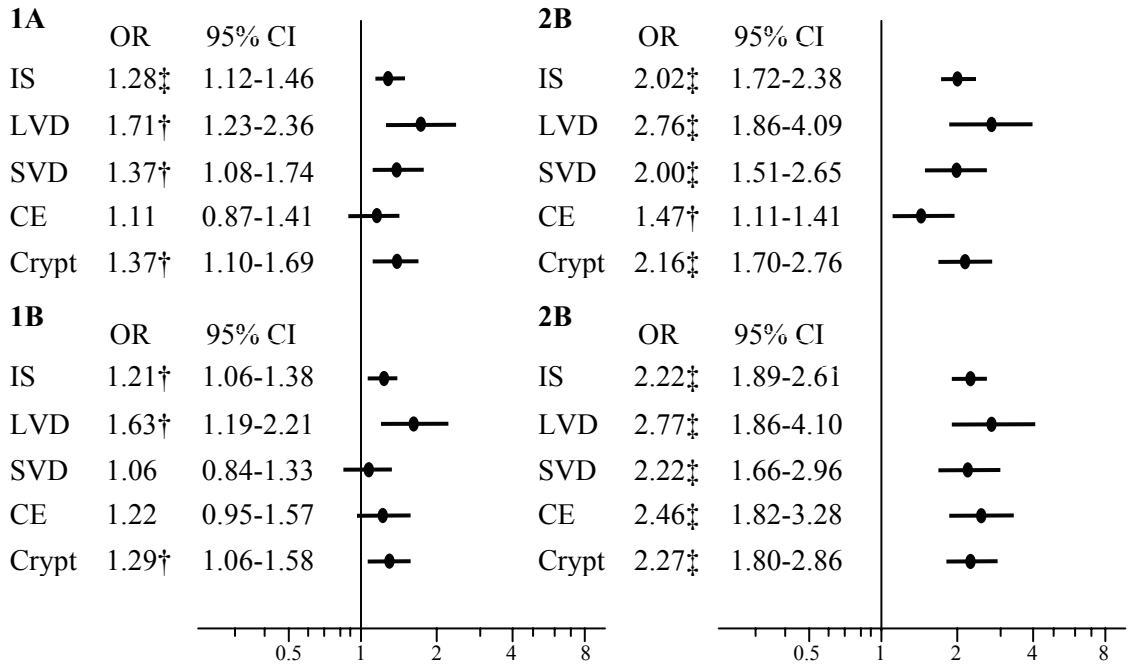

Figure 12. Multivariate ORs and 95% CI of ischemic stroke and TOAST subtypes per 1-SD increase in intact TAFI level (1A and 1B) and released activation peptide (2A and 2B). 1A and 2A, Acute phase; 1B and 2B, 3-month follow-up. Variables included in the logistic regression models were age, sex, hypertension, diabetes, smoking, hyperlipidemia and waist-hip ratio. *p<0.05; †p<0.01; ‡p<0.001.

The tagSNPs were distributed into 2 haplotypes blocks. Four common (frequency>1%) haplotypes in Block 1 accounted for 96% of the chromosomes and in Block 2, 7 common haplotypes accounted for 96% of the chromosomes. Intact TAFI differed for 9 and released AP for 8 of the 11 SNPs (p<0.01 for all). The SNPs that showed strongest associations were rs9526136 and rs17067700. Including both these variants in a multivariate linear model on data from the control group showed that they explained 16.7% and 13.7% of the variance in released AP and intact TAFI, respectively. Haplotype analysis revealed that the most common haplotype in block 1 (H1A) was associated with a higher expected mean of both released AP and intact TAFI compared to all other H1 haplotypes (p<0.001) (Figure 13). In block 2, H2B and H2E showed higher levels of both TAFI measurements (p<0.01) (Figure 13). Furthermore, H2D and H2F were associated with lower levels (p<0.05) compared to H2A.

No significant association was detected between any SNP and overall ischemic stroke in single SNP models. Similarly, no haplotype from either block showed a significant association to overall ischemic stroke (p>0.06). However, subtype analysis revealed an association between H2B and cryptogenic stroke, with an increased risk for H2B carriers (adjusted OR 1.57; 95% CI 1.03-2.40, p=0.03). Furthermore, the H1B haplotype was associated with a decreased (adjusted OR 0.55; 95% CI 0.35-0.87, p=0.01), and H2D as well as H2E an increased (adjusted ORs 1.99; 95% CI 1.16-3.40, p=0.01 and 2.48; 95% CI 1.38-4.44, p<0.01, respectively) risk of SVD. No significant association was observed for any other subtype.

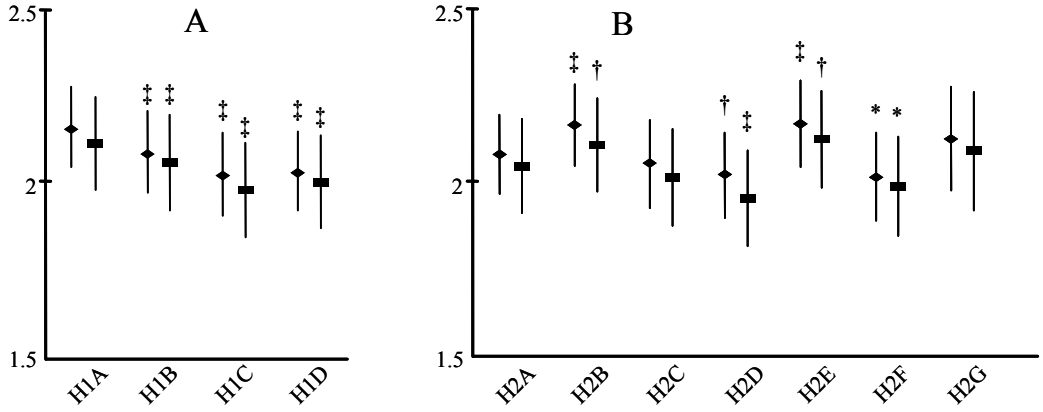

Figure 13. Expected adjusted means associated with one dose of haplotype over (A) block 1 and (B) block 2 in controls under the assumption of additive haplotype effects. Significant differences in haplotype mean, compared with the most common haplotype in each block, are shown. *p<0.05; †p<0.01; ‡p<0.001. ◆ ln [intact TAFI], ■ ln [released AP].

Serum C-reactive protein concentration and genotype in relation to ischemic stroke subtype

Paper IV

In this paper levels of the acute-phase reactant CRP and genetic variants at the CRP locus were investigated in SAHLSIS. Compared with controls, levels were higher both in the acute phase and at 3-month follow-up, in overall ischemic stroke (Figure 14) and in all TOAST subtypes. Serum CRP concentrations differed by etiologic subtype. In the acute phase, CRP levels were highest in CE stroke (data not shown), while the LVD group showed higher CRP levels at follow-up compared with all other subtypes (Figure 15). Multivariate logistic regression, adjusting for traditional risk factors, revealed independent associations for all subtypes in the acute phase, and for overall ischemic stroke and LVD at 3-month follow-up (Figure 16).

Cases with a clinical presentation indicating an extensive infarct (TACI, OCSP criteria) had significantly higher CRP levels in the acute phase than other OCSP subtypes ($p<0.001$). At 3-month follow-up, there were no differences in CRP levels between OCSP groups. Patients with an unfavourable outcome after 3 months (mRS score ≥3) had higher CRP levels both in the acute phase ($p<0.001$) and at 3-month follow-up ($p<0.01$) compared with those that had a favourable outcome (mRS<3). Exclusion of those who died before follow-up did not alter these results.

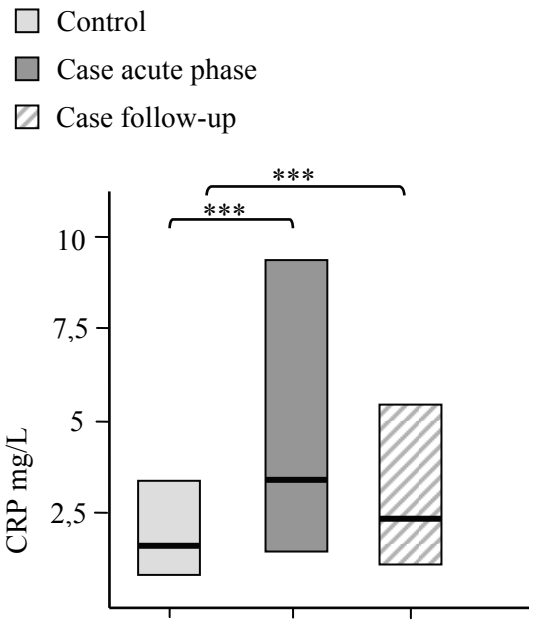

Figure 14. Median and inter-quartile range of serum CRP levels in controls and in overall ischemic stroke. Control versus overall ischemic stroke (Mann Whitney U test) ***p<0.001.

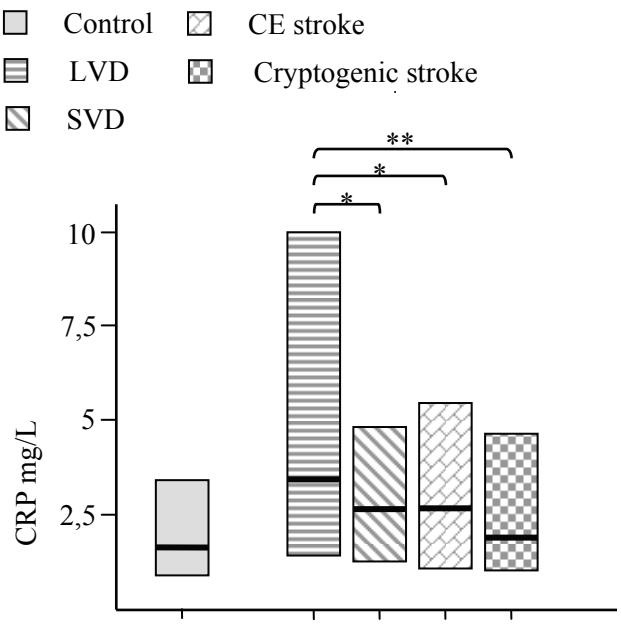

Figure 15. Median and inter-quartile range of serum CRP levels at 3-month follow-up in controls and in the different etiologic subtypes. LVD versus other subtypes (Mann Whitney U test) *p<0.05, **p<0.01.

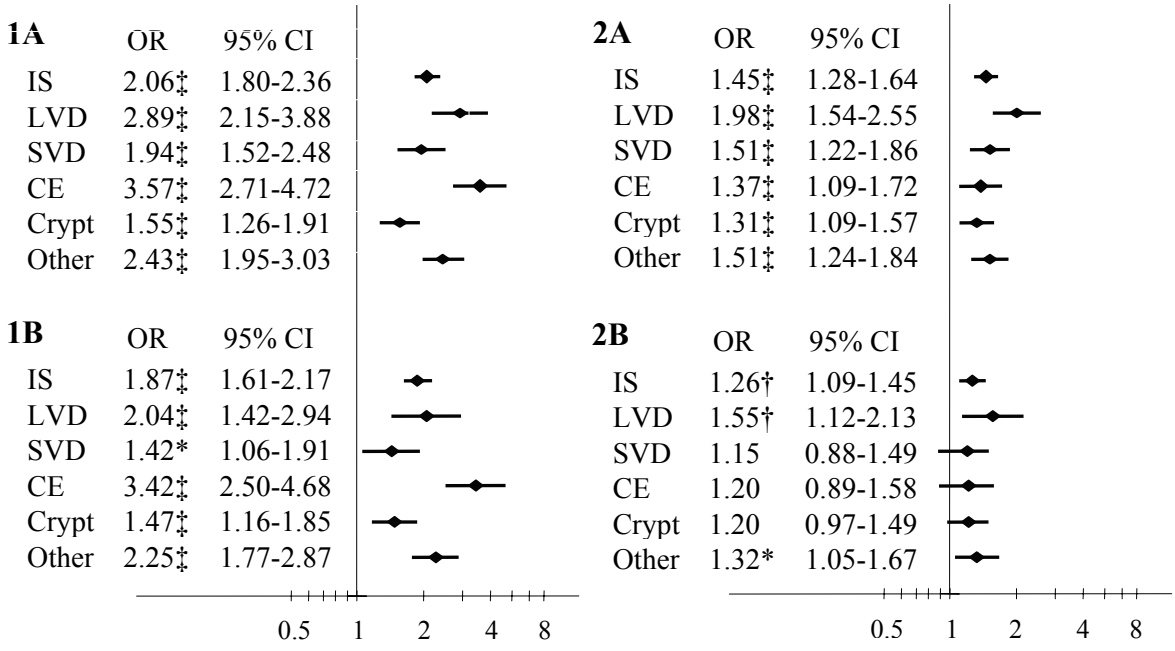

Figure 16. ORs and 95% CI of ischemic stroke and TOAST subtypes per 1-SD increase in serum CRP level in the acute phase (1A and 1B) and at 3-month follow-up (2A and 2B). A univariate analysis and B multivariate analysis, adjusted for age, sex, diabetes, smoking, hypertension, hyperlipidemia and waist-hip ratio. IS denotes overall ischemic stroke. *p<0.05; †p<0.01; ‡p<0.001.

No significant association was detected between any SNP and overall ischemic stroke (data not shown). Etiological subtype analyses revealed an association between the 1,059 G>C SNP and CE stroke, with an increased risk for C allele carriers (OR 2.2; 95% CI 1.26-3.97, p<0.01). This association remained after inclusion of CRP in the model (multivariate OR 1.31; 95% CI 1.01-1.70 for CRP and OR 2.5; 95% CI 1.41-4.63 for the 1,059C allele). Three of the four SNPs were associated with serum CRP concentration, the strongest association was observed for CRP -286C>T>A. In controls, the associations for these SNPs were: CRP -286C>T>A (CC vs. other allelic combinations, ΔCRP=0.75mg/L (95%CI 0.64-0.88), p<0.001), 1,059G>C (GG vs. other allelic combinations, ΔCRP=1.38mg/L (95%CI 1.09-1.75), p<0.05) and 1,444C>T (CC vs. other allelic combinations, ΔCRP=0.80mg/L (95%CI 0.69-0.94), p<0.01).

There was a high degree of LD between all pairs of markers (|D´|>0.9). Haplotype analysis revealed that 5 common haplotypes (frequency>1%) accounted for 99% of the chromosomes. The frequency distributions of these haplotypes were similar in cases and controls, and in consequence there was no association between ischemic stroke and CRP haplotypes (data not shown). In parallel to the results from the individual SNP analysis, CRP levels differed by haplotype. Compared with the most common haplotype, the H2 haplotype (harbouring the T allele of -286 C>T>A and the T allele of 1444 C>T) was associated with high and the H4 haplotype (harbouring the C allele of 1,059 G>C) with low CRP levels (Figure 17). Acute phase CRP levels in cases were significantly higher for the H5 haplotype (harbouring the A allele of -286 C>T>A), a finding not observed in controls or in cases at follow-up (Figure 17).

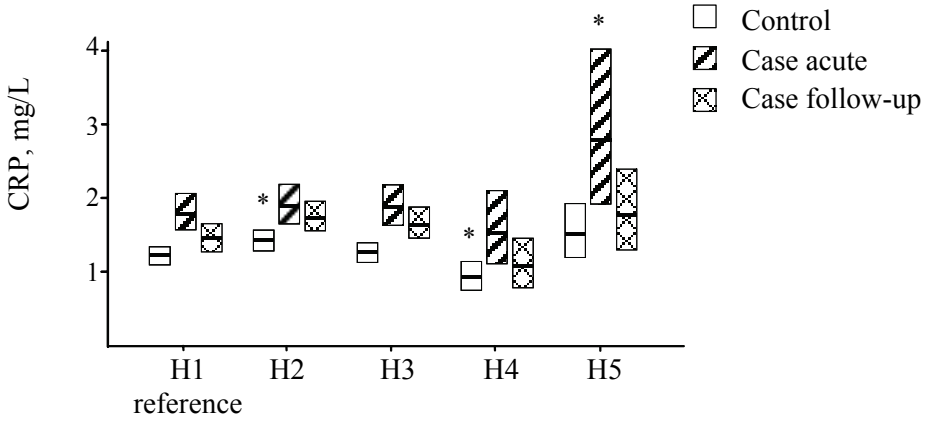

Figure 17. Unadjusted expected geometric mean and 95% CI of CRP according to estimated haplotypes in controls and cases, in the acute phase and at 3-month follow up. CRP levels in H2-H5 were compared with H1 in the corresponding group (controls, acute phase and 3-month follow-up). *p<0.05.

Association between factor XIII single nucleotide polymorphisms and aneurysmal subarachnoid hemorrhage

Paper V

In this study, genetic variations in four genes of importance for fibrinolysis were investigated in a study on aneurysmal SAH (aSAH). Demographic and risk factor profiles are presented in Table 4. A majority of subjects were female. More than twice as many SAH patients compared to controls were smokers. The OR for aSAH in smokers compared to non-smokers was 4.9 (95% CI 3.3–7.2, $p<0.001$). There was no statistically significant difference in the frequency of treatment for hypertension between patients and controls. After one year 126 (70%) of the patients had a favourable outcome (GOSE 5-8) (Table 4), while 23 (13%) had died. Those with a favourable outcome were younger (54 versus 57 years, $p<0.05$) and were less likely to have had hypertensive treatment prior to the aSAH ($p<0.05$). Gender and smoking status were not related to outcome.

Table 4. Baseline characteristics. Unfavourable outcome was defined as GOSE 1-4. ‡ $p<0.001$, χ^2 test

	Control n=366	**SAH n=183**	**Total n=549**
Mean age, y (SD)	55 (10)	55 (11)	55 (10)
Female sex, n (%)	270 (74)	135 (74)	405 (74)
Current smoking, n (%)	74 (20)	98 (54)‡	170 (32)
Hypertension, n (%)	68 (19)	42 (23)	110 (20)
Unfavorable outcome, n (%)		56 (30)	

No significant association was detected between aSAH and the tPA -7,351C>T, PAI-1 -675 4G>5G or TAFI Ala147Thr SNPs. There was, however, a significant association between aSAH and the FXIII Val34Leu SNP. Carriers of the Leu34 allele showed an increased risk of aSAH compared to subjects homozygous for the Val34 allele (OR=1.48 95%CI: 1.03-2.12, p=0.03). After inclusion of smoking and hypertension in a multivariate model the association attenuated, OR=1.44 95%CI: 0.98-2.12, p=0.07.

Because another study [Reiner 2001] had reported an association between FXIII SNPs and hemorrhagic stroke, including SAH, we went on to type two other FXIII variants, i.e. the FXIII Tyr204Phe and Pro564Leu SNPs. We did not detect any association between aSAH and the FXIII Tyr204Phe SNP. There was a trend for an increased risk of aSAH in subjects homozygous for the FXIII Leu564 allele compared with subjects homozygous for the Pro564 allele (multivariate OR=2.65 95%CI: 0.91-7.74, p=0.07).

The Reiner study was based on young women. Consequently we performed a gender-specific subanalysis for the FXIII SNPs. Women homozygous for the Leu564 allele showed an increased risk of aSAH compared to women homozygous for the Pro564 allele (multivariate OR=4.87 95%CI: 1.35-17.5, p=0.02). This association was not detected in men (multivariate OR=0.34

95%CI: 0.03-4.05, p=0.39). Furthermore, an increased risk of aSAH for carriers of the Leu34 allele was detected in women (multivariate OR=1.59 95%CI: 1.01-2.50, p=0.05) but not in men (multivariate OR=1.06 95%CI: 0.49-2.29, p=0.88). However, the difference in risk between sexes was not significant for any of the SNPs.

Both the H2 and the H3 haplotypes were more frequent in aSAH compared to controls (Figure 18). The association between aSAH and haplotype H2 remained statistically significant after adjustment for smoking and hypertension (Figure 18). A gender-stratified subanalysis revealed that the associations between aSAH and the H2 and H3 haplotypes were statistically significant in women (multivariate OR=1.58 95%CI: 1.03-2.43, p=0.04 and OR=1.95 95%CI: 1.18-3.40, p=0.02, respectively), but not in men (data not shown).

No single SNP or FXIII haplotype showed association to outcome one year after the event. However, the tPA -7,351 CC/PAI-1 -675 4G4G genotype combination was significantly more common in patients with an unfavourable outcome, compared with patients with a favourable outcome. The multivariate OR for an unfavourable outcome (GOSE 1-4) for this genotype combination compared with the reference (i.e. tPA -7,351 CC / PAI-1 -675 5G-carriers) was 3.88; 95% CI: 1.34-11.26, p=0.013.

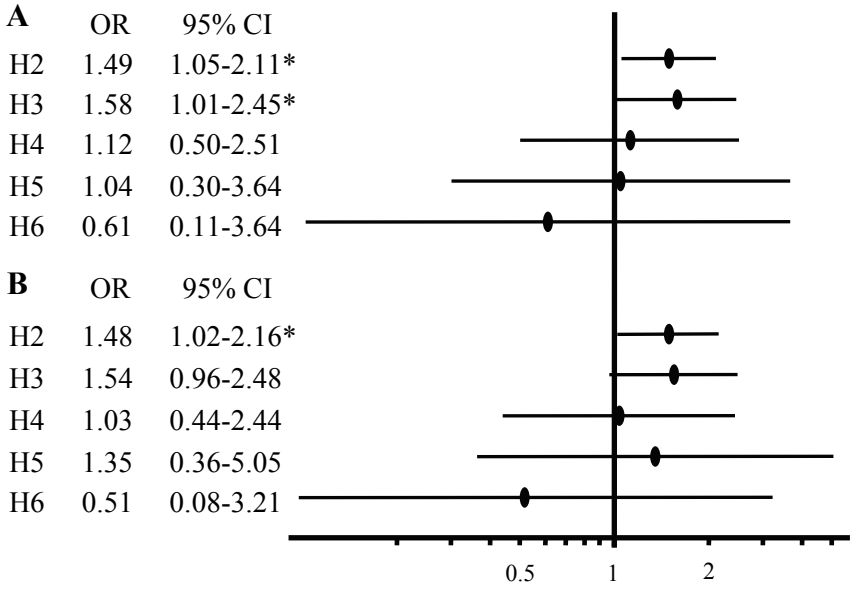

Figure 18. ORs and 95% confidence intervals for aSAH for FXIII haplotypes compared with the reference haplotype, H1. (A) Univariate analysis and (B) multivariate analysis, adjusted for smoking and hypertension. *p<0.05.

DISCUSSION

The focus of this thesis has been on the hereditary component of stroke, in particular the role of fibrinolytic and inflammatory candidate genes. By investigating genetic variation in relation to ischemic stroke, its etiological subtypes and subarachnoid hemorrhage, the role of these variants was illuminated from different angles. The effect of susceptibility genes on stroke may differ by age, sex, ethnicity and stroke subtype. Accordingly, inclusion in SAHLSIS was restricted to a younger white population from Western Sweden and great effort was put on the classification of stroke subtype and collection of risk factor information. Similarly, only patients with an identified aneurysm were included in the study on SAH. The controls were selected from the same population, matched for sex and age and investigated with regard to risk factors. With these designs, our aim was to avoid some of the pit falls that have hampered genetic association studies in stroke.

The fibrinolytic system has been the focus of our group's research for some time and because the tPA -7,351 C>T was associated with an increased risk for MI [Ladenvall 2002], it was an obvious choice for the first candidate gene study, once inclusion in SAHLSIS was complete. The first study also covered the main inhibitor of tPA, i.e. PAI-1. TAFI has been shown to downregulate fibrinolysis, and was investigated in the third study. Because inflammation has been shown to influence fibrinolysis and thrombotic mechanisms, the forth study investigated CRP. Finally in the last study, SNPs were selected from 4 genes with a role in fibrinolysis and investigated in relation to aSAH. In general our results suggest that the fibrinolytic system may influence the risk of developing all main subtypes of ischemic stroke, but may have less impact on aSAH. Inflammation seems to have largest effect on stroke with an atherosclerotic origin, i.e. LVD.

Characteristics of the SAHLSIS

SAHLSIS provides the first report on the distribution of ischemic stroke subtypes in the age-group between 18-69 years. The distribution was "intermediate", compared with studies including all age groups [Grau 2001, Polychronopoulos 2002, Jerrard-Dunne 2003, Schulz 2003] and those including very young patients (<45-55 years) [Adams 1995, Nedeltchev 2005, Rasura 2006, Telman 2007]. The proportions of LVD, cryptogenic stroke and stroke due to other determined etiology were higher compared to elderly patients, and lower compared to the very young.

We also assessed clinical stroke subtype according to OCSP. Among the 208 patients presenting with LACI, 118 were classified as SVD according to TOAST criteria. Because of an incomplete evaluation, it was not possible to assign TOAST subtype in 18 patients with LACI. In another 72 LACI cases, presence of symptoms indicating another determined etiology disqualified a diagnosis of

SVD. Thus, in 38% of the patients presenting with a LACI who underwent a complete evaluation, the underlying cause was not SVD. This finding is similar to what has previously been reported [Bamford 1987, Boiten 1991, Lindgren 1994, Tei 1999].

In line with previous studies [Grau 2001], functional outcome after 3 months was dependant on etiologic subtype, with the best outcome in SVD and the worst in CE stroke. However, it should be noted that the favourable outcome in SVD is restricted to short-term outcome. In the longer perspective the risk of recurrent stroke is similar to that for other subtypes, and there is a risk of death from cardiovascular diseases as well as cognitive decline and dementia [Norrving 2003].

Family history and other risk factors in ischemic stroke

A significant association between family history of stroke and risk of overall ischemic stroke was found in SAHLSIS. This is in line with results from a few previous smaller studies on ischemic stroke with younger age at onset [Matias-Guiu 1990 Shintani 1993, Vitullo 1996, Kubota 1997] and with a meta-analysis of prospective studies that concluded that a positive family history of stroke is associated with an increased risk of stroke (OR 1.3 95% CI 1.2-1.5) [Floßmann 2004]. In addition, a stronger genetic influence in younger age groups has been noted [Floßmann 2004, Schulz 2004].

In line with our hypothesis, we found that the association between family history and stroke differed by stroke ischemic subtype. A positive family history was associated with LVD, SVD and cryptogenic stroke, but not with CE stroke. Family history of stroke has not been studied in detail before in this age group. However, our results are in line with a few earlier studies, in which adult patients were included regardless of age and classified according to TOAST. In a large case-control study of ischemic stroke in the United Kingdom, Jerrard-Dunne et al [2003] found association with LVD and SVD, but not with CE or undetermined stroke. Similar results have also been reported from a Greek case-control study [Polychronopoulos 2002]. Furthermore, a lower frequency of family history in CE stroke as compared with the other subtypes has been reported from the Oxfordshire population-based case-case study [Schulz 2004]. However, in contrast to these studies and for the first time, we also found an association between family history of stroke and cryptogenic stroke. This finding is particularly interesting, as the cryptogenic group represents a group of stroke in whom other factors than those related to atherosclerosis, small vessel disease and heart disease are of importance.

In contrast to family history of stroke, family history of MI was not associated with overall ischemic stroke. However, a strong association between family history of MI and LVD was found. This is consistent with previous results

[Jerrard-Dunne 2003, Schulz 2004] and may reflect a shared genetic susceptibility for atherosclerotic disease in coronary and precerebral vessels.

With regard to other risk factors, hypertension, smoking, diabetes and hyperlipidemia were all associated with overall ischemic stroke. When investigated according to ischemic stroke subtype, variations in the strength of risk factor associations were found. LVD was strongly associated with smoking and diabetes and SVD with hypertension. Similar patterns have previously been reported [Grau 2001, Jerrard-Dunne 2003, Schulz 2004]. However, the strong association between LVD and diabetes has not been reported before. This may indicate a more important role for diabetes in ischemic stroke due to LVD in younger age groups. Concerning hyperlipidemia, the results should be treated with some caution. The definition of hyperlipidemia included lipid lowering treatment (mainly statins). In some patients, statins may have been prescribed to patients with diabetes and/or atherothrombotic disease independently of lipid level.

Another novel finding is that family history of stroke was associated with a favourable outcome after 3 months. The association was independent of subtype and vascular risk factors. Thus, our results indicates a stronger genetic susceptibility for less severe stroke, or, alternatively, better neuroprotective capacity and recovery for genetically influenced ischemic stroke cases. In line with our results, influence of family history on non-fatal, but not fatal stroke [Wannamethee 1996] and on subclinical but not clinical stroke [Morrison 2000] has been reported.

Our findings on differences between subtypes as regards family history of stroke and MI may have implications for the design of genetic studies. Part of the inconsistency of results from candidate gene studies in ischemic stroke may be explained by the etiologic heterogeneity of the disease. Because stroke subtyping requires considerable work-up, few studies have had sufficient power for analysis of the different subtypes. One way to proceed would be to design genetic studies that target specific etiologic subtypes of stroke. Our results suggest that such studies may preferably include ischemic stroke cases due to LVD, SVD and cryptogenic stroke.

TPA, PAI-1 and ischemic stroke

Plasma levels of tPA and PAI-1 antigen were elevated in SAHLSIS cases at both time points, compared with controls. This is consistent with previous data from both prospective and case-control studies [Catto 1997, Ridker 1994, Johansson 2000]. Plasma tPA activity was higher in cases than in controls in the acute phase, but not at 3-month follow-up. This suggests a general fibrinolytic activation in response to the ischemic event. In line with this notion, we found that cases with clinical presentation indicating an extensive infarct (TACI) displayed highest tPA activity during the acute phase (1.8 μg/L, as compared to

1.2, 1.4 and 1.2 μg/L in LACI, POCI and PACI, respectively, Kruskal Walllis test, $p<0.001$).

Likewise, tPA activity in the acute phase differed by etiological subtype, with CE stroke displaying the highest and SVD the lowest levels. At 3-month follow-up, there was no significant difference in tPA activity between OCSP or TOAST subtypes. This further supports a correlation between tPA activity during the acute phase and infarct volume.

In contrast to a smaller study investigating tPA and PAI-1 levels in the acute phase [Zunker 1999], we found significant differences in tPA antigen between etiologic subtypes at both time points. Multivariate analysis showed that tPA antigen at 3-month follow-up was independently associated with overall ischemic stroke, LVD and CE stroke. As tPA antigen is a marker of atherosclerotic disease, this may indicate that atherosclerosis is less frequent in the other subtypes.

The PAI-1 -675 4G>5G SNP was not associated with plasma PAI-1 antigen, while there was a tendency for a protective effect of the PAI-1 4G4G genotype on ischemic stroke risk. This contrasts the increased risk of MI reported for this genotype [Eriksson 1995], as well as the observed association between stroke and high plasma PAI-1 levels. However, this somewhat contradictory finding has been reported before [Roest 2003, Casas 2004, Attia 2007] and may reflect the complex role of the fibrinolytic system in the brain.

The tPA -7,351 C>T was unrelated to tPA antigen or activity and we found no association with overall ischemic stroke. The lack of association with ischemic stroke is in line with a recent meta-analysis [Attia 2007] and with preliminary data from a population-based prospective study within northern Sweden [Johansson 2002]. This contrast with what has been reported for MI [Ladenvall 2002], and may be a consequence of the more complex role of tPA in the brain [Mizoi 1993, Benchenane 2004] as compared to the heart.

When we analyzed combinations of the two SNPs, a reduced risk of ischemic stroke was observed for subjects homozygous for both the tPA C and the PAI-1 4G alleles (the genetic variant conferring high tPA release with high PAI-1 expression). This suggests a complex interplay between these proteins in plasma and in the brain tissue. However, this subgroup analysis should be interpreted with caution, as the power is low, and as multiple testing may contribute to false associations.

TAFI and ischemic stroke

Both intact TAFI and released AP, as measured by two novel genotype-independent ELISAs, were elevated in SAHLSIS cases as compared to controls. The association between plasma TAFI and ischemic stroke was independent of

established risk factors, and it was stronger for released AP than for intact TAFI. Released AP showed independent associations to all ischemic stroke subtypes, whereas intact TAFI showed association with LVD, cryptogenic stroke, and acute-phase SVD. The finding of increased plasma TAFI in ischemic stroke is in agreement with three smaller studies, despite somewhat different study designs [Montaner 2003, Santamaria 2003, Leebeek 2005]. In agreement with the hypothesis of Leebeek [2005], we could not detect any difference in TAFI levels between OCSP groups, suggesting that TAFI levels do not reflect an acute-phase response. To further support this hypothesis, the correlation with hsCRP levels was very weak and 3-month follow-up levels of released AP were higher compared with the acute phase levels in cases.

This study also provides the first data on TAFI levels by TOAST subtype. In the acute phase, LVD had higher plasma levels of both TAFI measurements than CE stroke, while no differences between other subtypes were detected. At 3-month follow-up there were no significant differences between subtypes for any of the TAFI measurements. Results from multivariate analysis showed an independent association between released AP and all four major ischemic stroke subtypes, indicating that TAFI activation may contribute to the development of ischemic stroke irrespective of the underlying etiology. Indeed, the adjusted ORs were similar for all subtypes.

The study also included a comprehensive genetic analysis of the TAFI locus. To the best of our knowledge, this is the first study of the TAFI gene based on a selection of tagging SNPs from the HapMap project. Traditional risk factors explained only 10-15% of the variance in both released AP and intact TAFI levels. The combined effect of 2 intronic SNPs, rs9526136 and rs17067700, explained an additional 10% to 15% of the variance in both measurements. This is in line with other recent studies investigating the contribution of TAFI gene variation on TAFI levels [Frère 2006]. A comparison of our data with the 3 suggested quantitative trait loci put forth by Frère indicates that the intronic rs17067700 may be in partial LD with the 5´ loci and that the rs9526136 may be a marker of the 3´ locus. Analysis of HapMap data support this last relationship (r^2=0.96).

We could not detect any associations between TAFI gene variants and ischemic stroke. Similar results were reported in a smaller study investigating 3 TAFI SNPs [Leebeek 2005]. However, analysis by etiologic subtype showed an increased risk of cryptogenic stroke in the H2B group, which displays increased TAFI levels and harbours the rs9526136 G allele, which is in strong LD with the putative 3' quantitative trait nucleotide. We also detected associations between TAFI haplotypes and SVD. A decreased risk was observed for H1B and an increased risk for H2D and H2E. In contrast to the finding for cryptogenic stroke, this is difficult to reconcile from a mechanistic point of view because TAFI H2D shows lower and H2E higher plasma levels of both TAFI measurements. However, given the relatively weak associations and multiple testing, these may

be chance findings and it follows that TAFI gene variants in subtypes of ischemic stroke need to be investigated in future studies.

CRP and ischemic stroke

Serum CRP levels in SAHLSIS were higher at both time points in cases compared with controls. This is in line with a recent meta-analysis on the association between CRP level and stroke in the general population [Kuo 2005]. All 4 main TOAST subtypes showed a significant association with CRP levels at the 3-month follow-up. However, after adjustment for traditional risk factors, this association remained for LVD only. This result is in line with studies showing increased CRP levels in patients with MI [Ridker 1997], as well as with results from a small study showing that follow-up CRP levels predict future ischemic events in transient ischemic attack and stroke patients with intracranial large-artery occlusive disease [Arenillas 2003]. The lack of association for SVD is in agreement with recent results from the Rotterdam Scan Study [Reitz 2007]. Taken together, these results suggest that CRP may be of more importance for stroke of an atherosclerotic cause.

With regard to CRP levels in the acute phase, highest levels by TOAST subtype were observed for CE stroke. Analysis by OCSP group showed the highest levels in the TACI group, suggesting that a more intense inflammatory response is related to infarct size. In accordance with our results, an association between acute CRP and infarct size has been reported [Beamer 1995, Muir 1999]. Because CE stroke was most prevalent in the TACI group, it is possible that not only infarct size, but also the cardiac condition resulting in an embolic episode, contributes to an increased CRP level.

An elevated CRP value in the acute phase was associated with functional outcome at 3 months. This is in line with other studies and has raised suggestions that CRP may be used as an independent risk marker of future ischemic stroke [Rost 2001, Di Napoli 2001]. However, in a statement from the CRP pooling project members, more evidence was requested before recommending measurement of CRP in the routine evaluation of cerebrovascular disease risk in primary prevention [Di Naploi 2005]. With regard to secondary prevention, the authors conclude that CRP adds to existing prognostic markers, but there are uncertainties regarding what effect different therapies may have and more studies are requested [Di Naploi 2005]. In SAHLSIS, we also detected an association between CRP levels at follow-up and unfavourable outcome after 3 months. At this time point, CRP levels were unrelated to OCSP type, supporting the idea that CRP levels may help identifying those at increased risk of unfavourable outcome after ischemic stroke.

This study also investigated the effect of 4 different polymorphisms on CRP levels. Associations were observed for the -286C>T>A, 1,059G>C, and 1,444C>T SNPs, which is in line with other studies [Carlsson 2005, Szalai 2005].

Haplotype analysis showed that the clade of haplotypes associated with low CRP levels corresponded to the C variant of the triallelic -286C>T>A, and the haplotype carrying both the -286C and the 1,059C allele was associated with the lowest levels of CRP. Moreover, the haplotype harbouring the -286T and 1,444T alleles was associated with high CRP levels. Similar results have been reported in other recent studies investigating the effect of CRP gene variation on CRP levels. [Carlssson 2005, Kivimäki 2007]. Recent data suggest that the CRP -286C>T>A may be a functional variant [Szalai 2005] and that variation in the CRP gene may account for approximately 5% of the variation in circulating CRP levels [Kivimäki 2007].

We did not detect any association between CRP gene variation and overall ischemic stroke, in line with recent results from the Physicians´ Health Study [Miller 2005]. Analysis by etiologic subtype revealed a significant and independent association for the 1,059 G>C SNP and CE stroke. However, the increased risk was observed for the allele that is associated with low CRP levels (1,059C), and the result is based on a subgroup analysis with limited power. This finding may be a chance finding and needs to be confirmed in independent replication studies.

Fibrinolytic gene variation and aSAH

This is the first genetic association study based on the case-control aSAH material recruited at the NICU at Sahlgrenska University Hospital. Four genes involved in fibrinolysis were investigated. In line with other studies on SAH [Feigin 2005], the majority of cases were female, and there was a strong association between smoking and aSAH. However, in contrast to other studies [van Gijn 2001, Feigin 2005] we could not detect any significant association between hypertension and aSAH. It is of note that the classification of hypertension in this case-control study was based entirely on prior treatment. It can thus be speculated that several individuals with hypertension were misclassified, and that the effect of hypertension on aSAH in our study is underestimated.

No associations between aSAH and SNPs in the tPA, PAI-1 and TAFI genes were detected. However, a weak association was detected for the FXIII Val34Leu in univariate analysis. Analogous findings for ICH have been reported before [Catto 1998], but results have been inconclusive and based on small studies and slightly different phenotypes [Reiner 2001, Corral 2001]. There was also a tendency for an association between the FXIII Pro564Leu SNP and aSAH. Investigation of FXIII haplotypes suggests that the less common allele of the Val34Leu and Pro564Leu SNPs may serve as markers of haplotypes H2, H3 and, in combination, H4. Compared with the results from analyzing single SNPs, the association parameters between H2 and aSAH are similar to when analyzing Val34Leu, while the estimated effect size of H3 on aSAH is somewhat higher compared to when analyzing Pro564Leu alone. In multivariate analysis, the

effect estimates of haplotypes H2 and H3 are of similar magnitude as in univariate analysis, but the confidence intervals are broadened and significance is lost for H3.

When stratified by gender, these FXIII associations were detected in women, but not in men. However, there were no significant gender-specific differences in the associations to aSAH. It should be noted that this subanalysis had very little power to detect an association in the male subgroup, and even less to identify a possible sex-by-genotype interaction. Still, one might speculate that a lack of, or weaker, association in men may have contributed to the discrepancy in data from studies on FXIII gene variation and hemorrhagic stroke. As more women than men suffer a SAH, the possible gender-specific effect of genetic variation in FXIII is interesting and could be tested in future studies.

Future perspectives

The development of techniques and algorithms, and increase in publicly available data, is transforming the field of genetic epidemiology at a tremendous pace. When the results of the human genome project were initially released in 2001, state-of-the-art technology was shotgun sequencing and genotyping technologies that analyzed one genotype at a time. The rather laborious restriction fragment length polymorphism (RFLP) technique, using different restriction endonucleases was losing ground to more recent techniques for SNP genotyping, based on clever use of enzymatic and detection methods. These novel techniques relied on four general mechanisms for allelic discrimination: allele-specific hybridization, allele-specific primer extension, allele-specific oligonucleotide ligation, and allele-specific invasive cleavage [Kwok 2001].

Since then, driving down the genotyping cost and increasing throughput has meant increasing SNP content on arrays, and in general, a move to multiplex assays. Today, the most common platforms for whole-genome association studies, Illumina and Affymetrix, offer arrays that are capable of analyzing 1 million SNPs and copy-number variants using different techniques and populated with slightly different content. As regards sequencing, recently developed technology (such as the Illumina/Solexa 1G genome analyzer, Roche/454 Life Sciences Genome Sequencer FLX system, or the Applied Biosystems/APG SOLiD™ system) is capable of producing vast amount of sequence in few runs. It is perhaps just a question of time before platforms will be available that sequence the whole human genome in a single run. All these methods produce vast amounts of data and the development of algorithms, software and quality control systems are struggling to keep up. Certainly, there will be a high demand for skilled bioinformaticians to manipulate and keep track of statistical procedures to analyze this data.

In line with this development, there is a general tendency for association studies of complex diseases to move towards typing more SNPs on larger study

populations. More high ranked journals also start to require replication of associations in independent samples. In general this development is sound, will help to identify new pathophysiological pathways [Melander 2007] and strengthen genetic research. Regarding stroke, these advances will be of benefit not only for research aiming at the investigation of susceptibility genes for developing a first stroke, but also to identify individuals who are at increased risk of an unfavourable outcome, recurrent stroke and to understand variations in response to drugs (pharmacogenetics). However, there are a few concerns I think ought to be raised. First, even though the current SNP arrays tag a large proportion of variation in the genome, studies that target single candidate genes (of relevance to the disease) may get better coverage of a particular gene, and better statistical power to detect an association. The few susceptibility genes that have been reported for complex diseases typically have ORs in the range of 1.2-1.5, and it should be noted that the strength of a signal from an individual SNP does not increase by typing more SNPs. Thus, genetic variants that confer small disease risks may be missed in this "most significant SNPs/genes approach" after multiple testing. A way to circumvent the problem of multiple testing, in diseases such as stroke, could be to use pathway-based approaches for analysis of GWA studies [Wang 2007].

Second, most of the larger studies investigating stroke have not classified the patients according to etiological subtype. After some initial interesting findings, part of the research community is optimistic that large materials with less well characterized patients and controls are "good enough" to detect associations using GWA methodology [Wellcome Trust 2007]. Our results, however, indicate that there are differences in risk factor profiles and pathophysiological mechanisms leading to hemorrhagic and ischemic stroke, and the various subtypes. Furthermore, because of increased presence of several traditional risk factors in stroke, compared to control populations, these must be adjusted for when searching for associations. Good phenotyping is important in stroke [Gulcher 2005], and an advantage of studies such as SAHLSIS.

Third, there is a possibility that the journals requiring replication and costly GWA studies will hamper genetic research, particularly in countries where less money is put into research. Even though the cost per genotype is reduced when using the array techniques, the prize tag for running a study such as SAHLSIS on the Affymetrix 500K array would arise to some 30 million SEK. Furthermore, results from the Welcome Trust Case Control Consortium suggest that inclusion of at least 2000 cases will be required for the studies to be sufficiently powered [Wellcome Trust 2007, Chanock 2007]. Hopefully, this demand for larger studies will not refrain researchers from conducting genetic studies, but result in more collaboration between research groups and stimulate clinicians to participate in the collection of well characterized stroke patients. For the stroke community, such consortiums and collaborations might help improve both power and funding [Bhatia 2005, Pendlebury 2007].

CONCLUSIONS

There is an independent association between family history of stroke and ischemic stroke with onset before 70 years. The association differs by etiologic subtype and is present in LVD, SVD and cryptogenic stroke, but not in CE stroke. Family history of MI is associated with LVD only. This may reflect a shared genetic susceptibility for atherosclerotic disease in coronary and precerebral vessels. Taken together, our results support a hereditary contribution to ischemic stroke before 70 years of age that is dependent on stroke subtype (paper I).

We could not detect any association between variation in genes of importance for fibrinolysis and ischemic stroke. However, plasma levels of the fibrinolytic inhibitor TAFI is influenced by genetic variation, and there is a possibility that our studies were underpowered to detect a small effect of these genes on ischemic stroke. Plasma levels of tPA, PAI-1 and TAFI are increased in all TOAST subtypes, suggesting that fibrinolysis is of importance for all main etiologic subtypes of ischemic stroke (papers II and III).

Serum levels of CRP are influenced by genetic variation in the CRP gene, but we could not detect any association between these markers and ischemic stroke. In the acute phase, CRP levels are influenced by the extent of brain damage. At 3-month follow-up levels are associated with LVD, suggesting a larger impact of CRP in stroke with an atherosclerotic origin (paper IV).

There is an increased frequency of carriers of either the FXIII Leu34 allele or the FXIII Leu564 allele in patients admitted to a NICU following aneurysmal subarachnoid hemorrhage. No significant association was detected for variants in the tPA, PAI-1 or TAFI genes. Given the physiological role of the FXIII Leu34 allele, the finding on FXIII is biologically plausible (paper V).

POPULÄRVETENSKAPLIG SAMMANFATTNING

Stroke, eller slaganfall, är en av de folksjukdomar som orsakar flest dödsfall och handikapp hos vuxna runt om i världen. I Sverige drabbas varje år ca 25 000 personer av en första stroke och ca 9 000 som tidigare drabbats insjuknar på nytt. Stroke är en gemensam term för hjärninfarkt (ischemisk stroke) och hjärnblödning (hemorrhagisk stroke). Vanligast förekommande är hjärninfarkt och den orsakas i de flesta fall av blodproppsbildning i hjärnans kärl. Den nedsatta blodcirkulationen leder till hjärnskada. Det finns många olika bakomliggande orsaker till hjärninfarkt. Därför finns det ett klassificeringssystem med vars hjälp man grupperar individers infarkter utifrån hur de mest sannolikt har uppkommit. Tre grupper dominerar: storkärlssjuka innebär att blodproppen härrör från åderförkalkning i halskärlen, småkärlssjukdom att proppbildningen uppstått lokalt i hjärnans blodkärl och kardiell emboli att proppen bildats i hjärtat och transporterats via blodkärl till hjärnan där den sedan fastnat och orsakat syrebrist. På samma sätt kan man klassificera de olika typerna av hjärnblödning. Subaraknoidalblödning (hjärnhinneblödning) är en särskild typ av hjärnblödning som i regel beror på bristning av ett pulsåderbråck (aneurysm) i blodkärl mellan de hinnor som omger och skyddar hjärnan. Trots en ökad kunskap om riskfaktorer som bidrar till att stroke utvecklas, och trots betydande förbättringar avseende behandling och rehabilitering efter stroke under de senaste åren, så finns det vissa tecken på att insjuknandet i stroke ökar. Det är därför av stor betydelse att identifiera och kunna behandla de faktorer som leder till att folk insjuknar i stroke.

Det finns ingen enskild faktor som ensamt bidrar till att man utvecklar stroke. Stroke anses därför vara en multifaktoriell sjukdom, och risken att insjukna påverkas i ungefär lika delar av både arv och miljö. Tidigare studier har visat att de viktigaste riskfaktorerna, som alla är möjliga att påverka, är: högt blodtryck, diabetes, rökning och förmaksflimmer. En ganska stor andel av de personer som drabbas av stroke har dock inte någon av de etablerade riskfaktorerna. Av dem som saknar de etablerade riskfaktorerna är många yngre. Dessutom anses den ärftliga komponenten vara mer betydelsefull hos yngre. Det är dock inte klarlagt vilka genetiska varianter som bidrar till insjuknande i stroke eller om olika genetiska varianter kan påverka vilken typ av stroke som en individ mest sannolikt kan komma att utveckla.

Syftet med den aktuella avhandlingen var därför att undersöka om den ärftliga komponenten, och andra riskfaktorer, skiljer sig åt vid olika typer av hjärninfarkt. Vidare studerades betydelsen av genetisk variation i delar av det kroppsegna blodproppslösande skyddssystemet (fibrinolysen) vid olika typer av stroke. Slutligen studerades även om genetisk variation i ett protein som är en del av kroppens immunförsvar påverkar risken att drabbas av hjärninfarkt.

I delarbete I studerades sambanden mellan etablerade riskfaktorer, ärftlighet och hjärninfarkt i ”the Sahlgrenska Academy Study on Ischemic Stroke” (SAHLSIS). Det är en studie som omfattar 600 patienter, som drabbats av hjärninfarkt före 70 års ålder, och 600 friska kontrollpersoner. Ett djupgående arbete utfördes för att kartlägga patienternas bakomliggande orsaker till stroke och de klassificerades i

grupperna storkärlssjuka, småkärlssjuka och kardiell emboli. Hos 27% av patienterna gick det inte att identifiera någon bakomliggande orsak, trots en omfattande utredning (kryptogen stroke). Våra resultat visar att riskfaktorprofilen skiljer sig åt mellan olika typer av hjärninfarkt. Beträffande ärftliga faktorer visade det sig att de patienter som drabbats av storkärlssjukdom, småkärlssjukdom och kryptogen stroke i större utsträckning än kontrollpersonerna hade släktingar som tidigare drabbats av stroke. Detta samband saknades för kardiell emboli. Att ha släktingar som tidigare drabbats av hjärtinfarkt visade sig enbart ha betydelse för att insjukna i storkärlssjukdom.

I delarbete II och III studerades såväl genetiska varianter som nivåer i blodet av proteiner som är viktiga för kroppens förmåga att lösa blodproppar i relation till hjärninfarkt. Det visade sig att nivåerna av de tre proteiner som studerades genomgående var förhöjda hos patienterna jämfört med kontrollgruppen. Dock var skillnaden i nivåer mellan olika typer av hjärninfarkt inte så stor. När de genetiska markörerna analyserades var för sig fann vi inga statistiskt signifikanta samband mellan genetiska varianter och risken att insjukna i hjärninfarkt. Dock såg vi tecken på en skyddande effekt hos individer som hade en kombination av en genetisk markör för hög tPA-frisättning och en genetisk markör för hög produktion av dess huvudsakliga hämmare, PAI-1. Sammanfattningsvis tyder våra resultat på att kroppens blodproppslösande system är viktigt vid alla typer av ischemisk stroke.

Delarbete IV fokuserade på CRP, ett protein som ingår i kroppens immunförsvar och som snabbt ökar i koncentration i blodet när man drabbas av infektion eller inflammation. Såväl nivåer i blodet som genetiska varianter av CRP studerades i relation till hjärninfarkt. Vi såg ett samband mellan olika genetiska varianter och mängden CRP i blod, samt att CRP-nivåerna var förhöjda hos patienter jämfört med kontrollgruppen. Speciellt höga var nivåerna hos dem som drabbats av storkärlssjuka. Dock kunde vi inte se något samband mellan genetiska varianter och insjuknande i hjärninfarkt. Våra resultat tyder alltså på att inflammation framförallt har ett samband med hjärninfarkt som uppstått till följd av åderförkalkning.

I delarbete V studerades kroppens blodproppslösande system i relation till subaraknoidalblödning. Våra resultat visade att vissa genetiska varianter av FXIII, ett protein som är inblandat i att stabilisera blodproppar, var vanligare hos patienter som drabbats av subaraknoidalblödning än i kontrollgruppen. Eftersom det tidigare har visats att just dessa genetiska varianter påverkar funktionen hos FXIII är fyndet biologiskt trovärdigt, men det måste konfirmeras i ytterligare större studier.

Sammanfattningsvis talar våra resultat för att olika riskfaktorers betydelse skiljer sig åt mellan olika typer av stroke. Ärftlighet för stroke har störst betydelse för storkärlssjukdom, småkärlssjukdom och kryptogen stroke, medan kroppens blodproppslösande system har betydelse för alla typer av ischemisk stroke. Inflammation har framförallt ett samband med hjärninfarkt som uppstått till följd av åderförkalkning. Resultaten understryker vikten av noggrann kartläggning av vilka mekanismer som lett fram till att en patient utvecklat stroke.

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REFERENCES

Abdellah Z, Ahmadi A, Ahmed S, Aimable M, Ainscough R, Almeida J et al. Finishing the euchromatic sequence of the human genome. *Nature*. 2004;431:931-45.

Adams HP, Jr., Bendixen BH, Kappelle LJ, Biller J, Love BB, Gordon DL, Marsh EE, 3rd. Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10172 in Acute Stroke Treatment. *Stroke.* 1993;24:35-41.

Adams HP Jr, Kappelle LJ, Biller J, Gordon DL, Love BB, Gomez F, Heffner M. Ischemic stroke in young adults. Experience in 329 patients enrolled in the Iowa Registry of stroke in young adults. *Arch Neurol* 1995;52:491-5.

Andrés AM, Clark AG, Shimmin L, Boerwinkle E, Sing CF, Hixson JE. Understanding the accuracy of statistical haplotype inference with sequence data of known phase. *Genet Epidemiol* 2007;31**:**659-71.

Arboix A, Morcillo C, Garcia-Eroles L, Oliveres M, Massons J, Targa C. Different vascular risk factor profiles in ischemic stroke subtypes: a study from the "Sagrat Cor Hospital of Barcelona Stroke Registry". *Acta Neurol Scand* 2000;102:264-70.

Arboix A, Marti-Vilalta JL. New concepts in lacunar stroke etiology: the constellation of small-vessel arterial disease. *Cerebrovasc Dis* 2004;17 Suppl 1:58-62.

Arenillas JF, Alvarez-Sabin J, Molina CA, et al. C-reactive protein predicts further ischemic events in first-ever transient ischemic attack or stroke patients with intracranial large-artery occlusive disease. *Stroke* 2003;34**:**2463-8.

Ariens RA, Lai TS, Weisel JW, Greenberg CS, Grant PJ. Role of factor XIII in fibrin clot formation and effects of genetic polymorphisms. *Blood* 2002;100:743-54.

Ariyaratnam R, Casas JP, Whittaker J, Smeeth L, Hingorani AD, Sharma P. Genetics of ischaemic stroke among persons of non-European descent: a meta-analysis of eight genes involving approximately 32,500 individuals. *PLoS Med* 2007;4:e131.

Attia J, Thakkinstian A, Wang Y, et al. The PAI-1 4G/5G gene polymorphism and ischemic stroke: an association study and meta-analysis. *J Stroke Cerebrovasc Dis* 2007;16:173-9.

Bak S, Gaist D, Sindrup SH, Skytthe A, Christensen K. Genetic liability in stroke: a long-term follow-up study of Danish twins. *Stroke* 2002;33:769-74.

Bamford J, Sandercock P, Jones L, Warlow C. The natural history of lacunar infarction: the Oxfordshire Community Stroke Project. *Stroke* 1987;18**:**545-51.

Bamford J, Sandercock P, Dennis M, Burn J, Warlow C. Classification and natural history of clinically identifiable subtypes of cerebral infarction. *Lancet.* 1991;337:1521-26.

Bassuk SS, Rifai N, Ridker PM. High-sensitivity C-reactive protein: clinical importance. *Curr Probl Cardiol* 2004;29:439-93.

Beamer NB, Coull BM, Clark WM, Hazel JS, Silberger JR. Interleukin-6 and interleukin-1 receptor antagonist in acute stroke. *Ann Neurol* 1995;37**:**800-5.

Benchenane K, Lopez-Atalaya JP, Fernandez-Monreal M, Touzani O, Vivien D. Equivocal roles of tissue-type plasminogen activator in stroke-induced injury. *Trends Neurosci* 2004;27**:**155-60.

Berger K, Stogbauer F, Stoll M, et al. The glu298asp polymorphism in the nitric oxide synthase 3 gene is associated with the risk of ischemic stroke in two large independent case-control studies. *Hum Genet* 2007;121:169-78.

Bhatia M, Rothwell PM. A systematic comparison of the quality and volume of published data available on novel risk factors for stroke versus coronary heart disease. *Cerebrovasc Dis* 2005;20:180-6.

Boiten J, Lodder J. Lacunar infarcts. Pathogenesis and validity of the clinical syndromes. *Stroke* 1991;22**:**1374-8.

Brass LM, Isaacsohn JL, Merikangas KR, Robinette CD. A study of twins and stroke. *Stroke* 1992;23:221-3.

Brass LM, Alberts MJ. The genetics of cerebrovascular disease. *Baillieres Clin Neurol* 1995;4:221-45.

Bredt DS. Endogenous nitric oxide synthesis: biological functions and pathophysiology. *Free Radic Res* 1999;31:577-96.

Carlson CS, Eberle MA, Rieder MJ, Yi Q, Kruglyak L, Nickerson DA. Selecting a maximally informative set of single nucleotide polymorphisms for association analyses using linkage disequilibrium. *Am J Hum Genet*. 2004;74:106-20.

Carlson CS, Aldred SF, Lee PK, Tracy RP, Schwartz SM, Rieder M, Liu K, Williams OD, Iribarren C, Lewis EC, Fornage M, Boerwinkle E, Gross M, Jaquish C, Nickerson DA, Myers RM, Siscovick DS, Reiner AP. Polymorphisms within the C-reactive protein (CRP) promoter region are associated with plasma CRP levels. *Am J Hum Genet*. 2005;77:64-77.

Casas JP, Hingorani AD, Bautista LE, Sharma P. Meta-analysis of genetic studies in ischemic stroke: thirty-two genes involving approximately 18,000 cases and 58,000 controls. *Arch Neurol* 2004;61:1652-61.

Casas JP, Bautista LE, Smeeth L, Sharma P, Hingorani AD. Homocysteine and stroke: evidence on a causal link from mendelian randomisation. *Lancet* 2005;365:224-32.

Catto AJ, Carter AM, Stickland M, Bamford JM, Davies JA, Grant PJ. Plasminogen activator inhibitor-1 (PAI-1) 4G/5G promoter polymorphism and levels in subjects with cerebrovascular disease. *Thromb Haemost* 1997;77:730-4.

Catto AJ, Kohler HP, Bannan S, Stickland M, Carter A, Grant PJ. Factor XIII Val 34 Leu: a novel association with primary intracerebral hemorrhage. *Stroke* 1998;29:813-6.

Ceresa E, Brouwers E, Peeters M, Jern C, Declerck PJ, Gils A. Development of ELISAs measuring the extent of TAFI activation. *Arterioscler Thromb Vasc Biol* 2006;26:423-8.

Chamorro A. Role of inflammation in stroke and atherothrombosis. *Cerebrovasc Dis* 2004;17 Suppl 3:1-5.

Chanock SJ, Manolio T, Boehnke M, et al. Replicating genotype-phenotype associations. *Nature* 2007;447:655-60.

Churchill FB. William Johannsen and the genotype concept. *J Hist Biol* 1974;7:5-30.

Clark AG. Inference of haplotypes from PCR-amplified samples of diploid populations. *Mol Biol Evol* 1990;7**:**111-22.

Clarke JT. Narrative review: Fabry disease. *Ann Intern Med* 2007;146:425-33.

Corral J, Iniesta JA, Gonzalez-Conejero R, Villalon M, Vicente V. Polymorphisms of clotting factors modify the risk for primary intracranial hemorrhage. *Blood* 2001;97:2979-82.

Curb JD, Abbott RD, Rodriguez BL, et al. C-reactive protein and the future risk of thromboembolic stroke in healthy men. *Circulation* 2003;107:2016-20.

de Bakker PI, Yelensky R, Pe'er I, Gabriel SB, Daly MJ, Altshuler D. Efficiency and power in genetic association studies. *Nat Genet* 2005;37**:**1217-23.

de Faire U, Friberg L, Lundman T. Concordance for mortality with special reference to ischaemic heart disease and cerebrovascular disease. A study on the Swedish Twin Registry. *Prev Med* 1975;4:509-17.

de Jong G, Kessels F, Lodder J. Two types of lacunar infarcts: further arguments from a study on prognosis. *Stroke* 2002;33:2072-6.

Dichgans M, Markus HS. Genetic association studies in stroke: methodological issues and proposed standard criteria. *Stroke* 2005;36:2027-31.

Dichgans M. Genetics of ischaemic stroke. *Lancet Neurol* 2007;6:149-61.

Di Napoli M, Papa F, Bocola V. Prognostic influence of increased C-reactive protein and fibrinogen levels in ischemic stroke. *Stroke* 2001;32**:**133-8.

Di Napoli M, Schwaninger M, Cappelli R, et al. Evaluation of C-reactive protein measurement for assessing the risk and prognosis in ischemic stroke: a statement for health care professionals from the CRP Pooling Project members. *Stroke* 2005;36:1316-29.

Dupont WD and Plummer WD: PS power and sample size program available for free on the Internet. *Controlled Clin Trials* 1997;18:274.

Eichner JE, Dunn ST, Perveen G, Thompson DM, Stewart KE, Stroehla BC. Apolipoprotein E polymorphism and cardiovascular disease: a HuGE review. *Am J Epidemiol* 2002;155:487-95.

Eikelboom JW, Hankey GJ, Baker RI, et al. C-reactive protein in ischemic stroke and its etiologic subtypes. *J Stroke Cerebrovasc Dis* 2003;12:74-81.

Endler G, Funk M, Haering D, et al. Is the factor XIII 34Val/Leu polymorphism a protective factor for cerebrovascular disease? *Br J Haematol* 2003;120:310-4.

Eriksson P, Kallin B, van 't Hooft FM, Bavenholm P, Hamsten A. Allele-specific increase in basal transcription of the plasminogen-activator inhibitor 1 gene is associated with myocardial infarction. *Proc Natl Acad Sci U S A* 1995;92**:**1851-5.

European Carotid Surgery Trialists´ Collaborative group. Randomised trial of endarterectomy for recently symptomatic carotid stenosis: final results of the MRC European Carotid Surgery Trial (ECST). *Lancet* 1998;351:1379-87.

Excoffier L, Slatkin M. Maximum-likelihood estimation of molecular haplotype frequencies in a diploid population. *Mol Biol Evol* 1995;12**:**921-7.

Feigin VL, Lawes CM, Bennett DA, Anderson CS. Stroke epidemiology: a review of population-based studies of incidence, prevalence, and case-fatality in the late 20th century. *Lancet Neurol* 2003;2:43-53.

Feigin VL, Rinkel GJ, Lawes CM, et al. Risk factors for subarachnoid hemorrhage: an updated systematic review of epidemiological studies. *Stroke* 2005;36**:**2773-80.

Ferro JM. Vasculitis of the central nervous system. *J Neurol* 1998;245:766-76.

Ferro JM. Cardioembolic stroke: an update. *Lancet Neurol* 2003;2:177-88.

Floßmann E, Schultz UG, Rothwell PM. Systematic review of methods and results of studies of the genetic epidemiology of ischemic stroke. *Stroke* 2004;35:212-27.

Floßmann E, Schulz UG, Rothwell PM. Potential confounding by intermediate phenotypes in studies of the genetics of ischaemic stroke. *Cerebrovasc Dis* 2005;19:1-10.

Franco RF, Trip MD, ten Cate H, et al. The 20210 G-->A mutation in the 3'-untranslated region of the prothrombin gene and the risk for arterial thrombotic disease. *Br J Haematol* 1999;104:50-4.

Frère C, Morange PE, Saut N, et al. Quantification of thrombin activatable fibrinolysis inhibitor (TAFI) gene polymorphism effects on plasma levels of TAFI measured with assays insensitive to isoform-dependent artefact. *Thromb Haemost* 2005;94:373-9.

Frère C, Tregouet DA, Morange PE, et al. Fine mapping of quantitative trait nucleotides underlying thrombin-activatable fibrinolysis inhibitor antigen levels by a transethnic study. *Blood* 2006;108:1562-8.

Gaist D, Vaeth M, Tsiropoulos I, Christensen K, Corder E, Olsen J, Sorensen HT. Risk of subarachnoid haemorrhage in first degree relatives of patients with subarachnoid haemorrhage: follow up study based on national registries in Denmark. *BMJ.* 2000;320:141-5.

Gan R, Sacco RL, Kargman DE, Roberts JK, Boden-Albala B, Gu Q. Testing the validity of the lacunar hypothesis: the Northern Manhattan Stroke Study experience. *Neurology* 1997;48:1204-11.

Gils A, Alessi MC, Brouwers E, et al. Development of a genotype 325-specific proCPU/TAFI ELISA. *Arterioscler Thromb Vasc Biol* 2003;23:1122-7.

Goldstein LB, Adams R, Alberts MJ, Appel LJ, Brass LM, Bushnell CD, Culebras A, DeGraba TJ, Gorelick PB, Guyton JR, Hart RG, Howard G, Kelly-Hayes M, Nixon JV, Sacco RL. Primary prevention of ischemic stroke: A guideline from the American Heart Association/American Stroke Association Stroke Council: cosponsored by the Atherosclerotic Peripheral Vascular Disease Interdisciplinary Working Group; Cardiovascular Nursing Council; Clinical Cardiology Council; Nutrition, Physical Activity, and Metabolism Council; and the Quality of Care and Outcomes Research Interdisciplinary Working Group. *Circulation* 2006;113:e873-923.

Gordon DL, Bendixen BH, Adams HP, Jr., Clarke W, Kappelle LJ, Woolson RF. Interphysician agreement in the diagnosis of subtypes of acute ischemic stroke: implications for clinical trials. The TOAST Investigators. *Neurology* 1993;43**:**1021-7.

Grau AJ, Buggle F, Ziegler C, et al. Association between acute cerebrovascular ischemia and chronic and recurrent infection. *Stroke* 1997;28:1724-9.

Grau AJ, Weimar C, Buggle F, et al. Risk factors, outcome, and treatment in subtypes of ischemic stroke: the German stroke data bank. *Stroke* 2001;32**:**2559-66.

Gretarsdottir S, Sveinbjornsdottir S, Jonsson HH, et al. Localization of a susceptibility gene for common forms of stroke to 5q12. *Am J Hum Genet* 2002;70:593-603.

Gretarsdottir S, Thorleifsson G, Reynisdottir ST, et al. The gene encoding phosphodiesterase 4D confers risk of ischemic stroke. *Nat Genet* 2003;35:131-8.

Guimaraes AH, van Tilburg NH, Vos HL, Bertina RM, Rijken DC. Association between thrombin activatable fibrinolysis inhibitor genotype and levels in plasma: comparison of different assays. *Br J Haematol* 2004;124:659-65.

Gulcher JR, Gretarsdottir S, Helgadottir A, Stefansson K. Genes contributing to risk for common forms of stroke. *Trends Mol Med* 2005;11:217-24.

Hage FG, Szalai AJ. C-reactive protein gene polymorphisms, C-reactive protein blood levels, and cardiovascular disease risk. *J Am Coll Cardiol* 2007;50:1115-22.

Hankey GJ. Potential new risk factors for ischemic stroke: what is their potential? *Stroke* 2006;37:2181-8.

HapMap Consortium. The International HapMap Project. *Nature.* 2003;426:789-96.

HapMap Consortium. A haplotype map of the human genome. *Nature* 2005;437:1299-320.

Hassan A, Markus HS. Genetics and ischaemic stroke. *Brain* 2000;123:1784-812.

Hassan A, Sham PC, Markus HS. Planning genetic studies in human stroke: sample size estimates based on family history data. *Neurology* 2002;58:1483-8.

Hassan A, Gormley K, O'Sullivan M, et al. Endothelial nitric oxide gene haplotypes and risk of cerebral small-vessel disease. *Stroke* 2004;35:654-9.

Helgadottir A, Manolescu A, Thorleifsson G, et al. The gene encoding 5-lipoxygenase activating protein confers risk of myocardial infarction and stroke. *Nat Genet* 2004;36:233-9.

Helgadottir A, Gretarsdottir S, St Clair D, et al. Association between the gene encoding 5-lipoxygenase-activating protein and stroke replicated in a Scottish population. *Am J Hum Genet* 2005;76:505-9.

Hillman J, Fridriksson S, Nilsson O, Yu Z, Saveland H, Jakobsson KE. Immediate administration of tranexamic acid and reduced incidence of early rebleeding after aneurysmal subarachnoid hemorrhage: a prospective randomized study. *J Neurosurg* 2002;97**:**771-8.

Holland PM, Abramson RD, Watson R, Gelfand DH. Detection of specific polymerase chain reaction product by utilizing the 5'→3' exonuclease activity of Thermus aquaticus DNA polymerase. *Proc Natl Acad Sci U S A* 1991;88:7276-80.

Hrafnkelsdóttir T, Wall U, Jern C, Jern S. Impaired capacity for endogenous fibrinolysis in essential hypertension. *Lancet* 1998;352:1597-8.

Hrafnkelsdóttir T, Gudnason T, Wall U, Jern C, Jern S. Regulation of local availability of active tissue-type plasminogen activator in vivo in man. *J Thromb Haemost* 2004;2:1960-8.

Humphries SE, Morgan L. Genetic risk factors for stroke and carotid atherosclerosis: insights into pathophysiology from candidate gene approaches. *Lancet Neurol* 2004;3:227-35.

Itoyama Y, Fujioka S, Takaki S, Morioka M, Hide T, Ushio Y. Significance of elevated thrombin-antithrombin III complex and plasmin-alpha 2-plasmin inhibitor complex in the acute stage of nontraumatic subarachnoid hemorrhage. *Neurosurgery* 1994;35:1055-60.

Jackson C, Sudlow C. Comparing risks of death and recurrent vascular events between lacunar and non-lacunar infarction. *Brain* 2005;128:2507-17.

Jannes J, Hamilton-Bruce MA, Pilotto L, et al. Tissue plasminogen activator -7351C/T enhancer polymorphism is a risk factor for lacunar stroke. *Stroke* 2004;35:1090-4.

Jeffs B, Clark JS, Anderson NH, et al. Sensitivity to cerebral ischaemic insult in a rat model of stroke is determined by a single genetic locus. *Nat Genet* 1997;16:364-7.

Jern C, Selin L, Jern S. In vivo release of tissue-type plasminogen activator across the human forearm during mental stress. *Thromb Haemost* 1994;72:285-91.

Jern C, Ladenvall P, Wall U, Jern S. Gene polymorphism of t-PA is associated with forearm vascular release rate of t-PA. *Arterioscler Thromb Vasc Biol* 1999;19:454-9.

Jern C, Blomstrand C, Westerlind A. Evidence of a net release of tissue-type plasminogen activator across the human cerebral vasculature. *Thromb Haemost*. 2004;91:1019-25.

Jerrard-Dunne P, Cloud G, Hassan A, Markus HS. Evaluating the genetic component of ischemic stroke subtypes: a family history study. *Stroke* 2003;34:1364-9.

Jialal I, Devaraj S, Venugopal SK. C-reactive protein: risk marker or mediator in atherothrombosis? *Hypertension* 2004;44:6-11.

Johansson L, Jansson JH, Boman K, Nilsson TK, Stegmayr B, Hallmans G. Tissue plasminogen activator, plasminogen activator inhibitor-1, and tissue plasminogen activator/plasminogen activator inhibitor-1 complex as risk factors for the development of a first stroke. *Stroke* 2000;31:26-32.

Johansson L. Hemostatic factors in cardiovascular disease with special reference to stroke. Umeå University Dissertations, new series no 815, 2002.

Jood K, Jern C, Blomstrand C, Jern S, Hrafnkelsdottir T. Local tissue-type plasminogen activator release in patients with ischemic stroke. *J Thromb Haemost* 2007;5:1320-3.

Joutel A, Corpechot C, Ducros A, et al. Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. *Nature* 1996;383:707-10.

Joutel A, Favrole P, Labauge P, et al. Skin biopsy immunostaining with a Notch3 monoclonal antibody for CADASIL diagnosis. *Lancet* 2001;358:2049-51.

Kalimo H, Ruchoux MM, Viitanen M, Kalaria RN. CADASIL: a common form of hereditary arteriopathy causing brain infarcts and dementia. *Brain Pathol* 2002;12:371-84.

Kastelein JJ, Ordovas JM, Wittekoek ME, et al. Two common mutations (D9N, N291S) in lipoprotein lipase: a cumulative analysis of their influence on plasma lipids and lipoproteins in men and women. *Clin Genet* 1999;56:297-305.

Khurana VG, Sohni YR, Mangrum WI, et al. Endothelial nitric oxide synthase T-786C single nucleotide polymorphism: a putative genetic marker differentiating small versus large ruptured intracranial aneurysms. *Stroke* 2003;34:2555-9.

Khurana VG, Meissner I, Sohni YR, et al. The presence of tandem endothelial nitric oxide synthase gene polymorphisms identifying brain aneurysms more prone to rupture. *J Neurosurg* 2005;102:526-31.

Kissela BM, Sauerbeck L, Woo D, et al. Subarachnoid hemorrhage: a preventable disease with a heritable component. *Stroke* 2002;33:1321-6.

Kivimäki M, Lawlor DA, Smith GD, et al. Variants in the CRP gene as a measure of lifelong differences in average C-reactive protein levels: the Cardiovascular Risk in Young Finns Study, 1980-2001. *Am J Epidemiol* 2007;166**:**760-4.

Kolominsky-Rabas PL, Weber M, Gefeller O, Neundoerfer B, Heuschmann PU. Epidemiology of ischemic stroke subtypes according to TOAST criteria: incidence, recurrence, and long-term survival in ischemic stroke subtypes: a population-based study. *Stroke* 2001;32:2735-40.

Kraft P, Stram DO. Re: the use of inferred haplotypes in downstream analysis. *Am J Hum Genet.* 2007 Oct;81:863-5.

Krischek B, Inoue I. The genetics of intracranial aneurysms. *J Hum Genet* 2006;51:587-94.

Kubota M, Yamaura A, Ono J, et al. Is family history an independent risk factor for stroke? *J Neurol Neurosurg Psychiatry* 1997;62**:**66-70.

Kuller LH. Epidemiology and prevention of stroke, now and in the future. *Epidemiol Rev* 2000;22:14-7.

Kuo HK, Yen CJ, Chang CH, Kuo CK, Chen JH, Sorond F. Relation of C-reactive protein to stroke, cognitive disorders, and depression in the general population: systematic review and meta-analysis. *Lancet Neurol* 2005;4:371-80.

Kwok PY. Methods for genotyping single nucleotide polymorphisms. *Annu Rev Genomics Hum Genet* 2001;2:235-58.

Ladenvall P, Wall U, Jern S, Jern C. Identification of eight novel single-nucleotide polymorphisms at human tissue-type plasminogen activator (t-PA) locus: association with vascular t-PA release in vivo. *Thromb Haemost* 2000;84:150-5.

Ladenvall P, Johansson L, Jansson JH, Jern S, Nilsson TK, Tjarnlund A, Jern C, Boman K. Tissue-type plasminogen activator -7,351c/t enhancer polymorphism is associated with a first myocardial infarction. *Thromb Haemost* 2002;87:105-9.

Lee LG, Connell CR, Bloch W. Allelic discrimination by nick-translation PCR with fluorogenic probes. *Nucleic Acids Res* 1993;21:3761-6.

Leebeek FW, Goor MP, Guimaraes AH, et al. High functional levels of thrombin-activatable fibrinolysis inhibitor are associated with an increased risk of first ischemic stroke. *J Thromb Haemost* 2005;3:2211-8.

Leurs J, Nerme V, Sim Y, Hendriks D. Carboxypeptidase U (TAFIa) prevents lysis from proceeding into the propagation phase through a threshold-dependent mechanism. *J Thromb Haemost* 2004;2:416-23.

Leurs J, Hendriks D. Carboxypeptidase U (TAFIa): a metallocarboxypeptidase with a distinct role in haemostasis and a possible risk factor for thrombotic disease. *Thromb Haemost* 2005;94:471-87.

Levine JS, Branch DW, Rauch J. The antiphospholipid syndrome. *N Engl J Med* 2002;346:752-63

Leys D, Kwiecinski H, Bogousslavsky J, Bath P, Brainin M, Diener HC, Kaste M, Sivenius J, Hennerici MG, Hacke W. Prevention. European Stroke Initiative. *Cerebrovasc Dis* 2004;17 Suppl 2:15-29.

Li Z, Sun L, Zhang H, et al. Elevated plasma homocysteine was associated with hemorrhagic and ischemic stroke, but methylenetetrahydrofolate reductase gene C677T polymorphism was a risk factor for thrombotic stroke: a Multicenter Case-Control Study in China. *Stroke* 2003;34:2085-90.

Lim BC, Ariens RA, Carter AM, Weisel JW, Grant PJ. Genetic regulation of fibrin structure and function: complex gene-environment interactions may modulate vascular risk. *Lancet* 2003;361:1424-31.

Lindgren A, Norrving B, Rudling O, Johansson BB. Comparison of clinical and neuroradiological findings in first-ever stroke. A population-based study. *Stroke* 1994;25**:**1371-7.

Lindgren A, Lindoff C, Norrving B, Astedt B, Johansson BB. Tissue plasminogen activator and plasminogen activator inhibitor-1 in stroke patients. *Stroke* 1996;27:1066-71.

Lindgren A, Lovkvist H, Hallstrom B, et al. Prevalence of stroke and vascular risk factors among first-degree relatives of stroke patients and control subjects. A prospective consecutive study. *Cerebrovasc Dis* 2005;20:381-7.

Lindsberg PJ, Grau AJ. Inflammation and infections as risk factors for ischemic stroke. *Stroke* 2003;34:2518-32.

Livak KJ, Marmaro J, Todd JA. Towards fully automated genome-wide polymorphism screening. *Nat Genet* 1995;9:341-2.

Lohmussaar E, Gschwendtner A, Mueller JC, et al. ALOX5AP gene and the PDE4D gene in a central European population of stroke patients. *Stroke* 2005;36:731-6.

Loscalzo J, Welch G. Nitric oxide and its role in the cardiovascular system. *Prog Cardiovasc Dis* 1995;38:87-104.

Lowe GD. Circulating inflammatory markers and risks of cardiovascular and non-cardiovascular disease. *J Thromb Haemost* 2005;3:1618-27.

Marchini J, Cutler D, Patterson N, Stephens M, Eskin E, Haplerin E, Lin S, Qin ZS, Munro HM, Abecasis GR, Donnelly P; International HapMap Consortium. A comparison of phasing algorithms for trios and unrelated individuals. *Am J Hum Genet* 2006;78:437-50.

Markus HS, Martin RJ, Simpson MA, Dong YB, Ali N, Crosby AH, Powell JF. Diagnostic strategies in CADASIL. *Neurology* 2002;59:1134-8.

Martinez-Fernandez E, Gil-Peralta A, Garcia-Lozano R, et al. Mitochondrial disease and stroke. *Stroke* 2001;32:2507-10.

Marx PF, Dawson PE, Bouma BN, Meijers JC. Plasmin-mediated activation and inactivation of thrombin-activatable fibrinolysis inhibitor. *Biochemistry* 2002;41:6688-96.

Matias-Guiu J, Alvarez J, Insa R, et al. Ischemic stroke in young adults. II. Analysis of risk factors in the etiological subgroups. *Acta Neurol Scand* 1990;81**:**314-7.

Melander O. Are minor gene effects of clinical importance? *J Hypertens* 2007;25**:**505-7.

Medin J, Nordlund A, Ekberg K. Increasing stroke incidence in Sweden between 1989 and 2000 among persons aged 30 to 65 years: evidence from the Swedish Hospital Discharge Register. *Stroke* 2004;35:1047-51.

Meijer P, Pollet DE, Wauters J, et al. Specificity of antigen assays of plasminogen activator inhibitor in plasma: Innotest PAI-1 immunoassay evaluated. Clin Chem 1994;40:110-5.

Meschia JF, Brown RD, Jr., Brott TG, Chukwudelunzu FE, Hardy J, Rich SS. The Siblings With Ischemic Stroke Study (SWISS) protocol. *BMC Med Genet* 2002;3:1.

Meschia JF, Brott TG, Brown RD, Jr., et al. Phosphodiesterase 4D and 5-lipoxygenase activating protein in ischemic stroke. *Ann Neurol* 2005;58:351-61.

Meschia JF, Kissela BM, Brott TG, et al. The Siblings With Ischemic Stroke Study (SWISS): a progress report. *Clin Med Res* 2006;4:12-21.

Miller DT, Zee RY, Suk Danik J, et al. Association of common CRP gene variants with CRP levels and cardiovascular events. *Ann Hum Genet* 2005;69:623-38.

Mizoi K, Yoshimoto T, Takahashi A, Fujiwara S, Koshu K, Sugawara T. Prospective study on the prevention of cerebral vasospasm by intrathecal fibrinolytic therapy with tissue-type plasminogen activator. *J Neurosurg* 1993;78**:**430-7.

Montaner J, Ribo M, Monasterio J, Molina CA, Alvarez-Sabin J. Thrombin-activable fibrinolysis inhibitor levels in the acute phase of ischemic stroke. *Stroke* 2003;34:1038-40.

Morange PE, Tregouet DA, Frere C, et al. TAFI gene haplotypes, TAFI plasma levels and future risk of coronary heart disease: the PRIME Study. *J Thromb Haemost* 2005;3:1503-10.

Morga R, Czepko R, Dembinska-Kiec A, Danilewicz B. Assessment of the haemostatic system in patients surgically treated for ruptured cerebral aneurysm. *Neurol Neurochir Pol* 2007;41:296-305.

Morita A, Nakayama T, Soma M. Association study between C-reactive protein genes and ischemic stroke in Japanese subjects. *Am J Hypertens* 2006;19:593-600.

Morrison AC, Fornage M, Liao D, Boerwinkle E. Parental history of stroke predicts subclinical but not clinical stroke: the Atherosclerosis Risk in Communities Study. *Stroke* 2000;31:2098–102.

Muir KW, Weir CJ, Alwan W, Squire IB, Lees KR. C-reactive protein and outcome after ischemic stroke. *Stroke* 1999;30:981-5.

Muir KW, Tyrrell P, Sattar N, Warburton E. Inflammation and ischaemic stroke. *Curr Opin Neurol* 2007;20:334-42.

Murray CJ, Lopez AD. Mortality by cause for eight regions of the world: Global Burden of Disease Study. *Lancet* 1997;349:1269-76.

Nahed BV, Bydon M, Ozturk AK, Bilguvar K, Bayrakli F, Gunel M. Genetics of intracranial aneurysms. *Neurosurgery* 2007;60:213-25.

Natowicz M, Kelley RI. Mendelian etiologies of stroke. *Ann Neurol* 1987;22(2):175-92.

Nedeltchev K, der Maur TA, Georgiadis D, et al. Ischaemic stroke in young adults: predictors of outcome and recurrence. *J Neurol Neurosurg Psychiatry* 2005;76**:**191-5.

Newby DE, Wright RA, Labinjoh C, et al. Endothelial dysfunction, impaired endogenous fibrinolysis, and cigarette smoking: a mechanism for arterial thrombosis and myocardial infarction. *Circulation* 1999;99:1411-5.

Nilsson-Ardnor S, Janunger T, Wiklund PG, Lackovic K, Nilsson AK, Lindgren P, Escher SA, Stegmayr B, Asplund K, Holmberg D. Genome-wide linkage scan of common stroke in families from northern Sweden. *Stroke* 2007;38:34-40.

Nina P, Schisano G, Chiappetta F, Luisa Papa M, Maddaloni E, Brunori A, Capasso F, Corpetti MG, Demurtas F. A study of blood coagulation and fibrinolytic system in spontaneous subarachnoid hemorrhage. Correlation with hunt-hess grade and outcome. *Surg Neurol*. 2001;55:197-203.

Niu T. Algorithms for inferring haplotypes. *Genet Epidemiol* 2004;27:334-47.

Norrving B. Long-term prognosis after lacunar infarction. *Lancet Neurol* 2003;2**:**238-45.

Norrving, B. Förekomst i befolkningen. Vårdalinstitutets Tematiska rum : Stroke – vård, omsorg och rehabilitering. http://www.vardalinstitutet.net. 2007, in Swedish.

Opherk C, Peters N, Herzog J, Luedtke R, Dichgans M. Long-term prognosis and causes of death in CADASIL: a retrospective study in 411 patients. *Brain* 2004;127:2533-9.

Pearson H. What is a gene? *Nature* 2006;441:398-401.

Peltonen S, Juvela S, Kaste M, Lassila R. Hemostasis and fibrinolysis activation after subarachnoid hemorrhage. *J Neurosurg* 1997;87:207-14.

Pendlebury ST. (2007) Worldwide under-funding of stroke research. *International Journal of Stroke* 2007;2:80–4.

Peters N, Opherk C, Danek A, Ballard C, Herzog J, Dichgans M. The pattern of cognitive performance in CADASIL: a monogenic condition leading to subcortical ischemic vascular dementia. *Am J Psychiat* 2005;162:2078-85.

Petty GW, Brown RD, Jr., Whisnant JP, Sicks JD, O'Fallon WM, Wiebers DO. Ischemic stroke subtypes: a population-based study of incidence and risk factors. *Stroke* 1999;30:2513-6.

Polychronopoulos P, Gioldasis G, Ellul J, et al. Family history of stroke in stroke types and subtypes. *J Neurol Sci* 2002;195:117-22.

Rasura M, Spalloni A, Ferrari M, et al. A case series of young stroke in Rome. *Eur J Neurol* 2006;13**:**146-52.

Razvi SS, Davidson R, Bone I, Muir KW. The prevalence of cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy (CADASIL) in the west of Scotland. *J Neurol Neurosurg Psychiatry* 2005;76:739-41.

Reiner AP, Schwartz SM, Frank MB, et al. Polymorphisms of coagulation factor XIII subunit A and risk of nonfatal hemorrhagic stroke in young white women. *Stroke* 2001;32**:**2580-6.

Reitz C, Berger K, de Maat MP, et al. CRP gene haplotypes, serum CRP, and cerebral small-vessel disease: the Rotterdam Scan Study and the MEMO Study. *Stroke* 2007;38:2356-9.

Ridker PM, Hennekens CH, Stampfer MJ, Manson JE, Vaughan DE. Prospective study of endogenous tissue plasminogen activator and risk of stroke. *Lancet* 1994;343:940-3.

Ridker PM, Cushman M, Stampfer MJ, Tracy RP, Hennekens CH. Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. *N Engl J Med* 1997;336:973-9.

Roest M, Banga JD. Editorial comment-- genetic make-up for increased PAI-1 expression protects against stroke. *Stroke* 2003;34**:**2828-9.

Rolfs A, Bottcher T, Zschiesche M, et al. Prevalence of Fabry disease in patients with cryptogenic stroke: a prospective study. *Lancet* 2005;366:1794-6.

Rosand J, Bayley N, Rost N, de Bakker PI. Many hypotheses but no replication for the association between PDE4D and stroke. *Nat Genet* 2006;38:1091-2.

Rosenberg RD, Aird WC. Vascular-bed--specific hemostasis and hypercoagulable states. *N Engl J Med* 1999;340:1555-64.

Rost NS, Wolf PA, Kase CS, et al. Plasma concentration of C-reactive protein and risk of ischemic stroke and transient ischemic attack: the Framingham study. *Stroke* 2001;32:2575-9.

Rothwell PM. The high cost of not funding stroke research: a comparison with heart disease and cancer. *Lancet* 2001;357:1612-6.

Rovira A, Grive E, Alvarez-Sabin J. Distribution territories and causative mechanisms of ischemic stroke. *Eur Radiol* 2005;15:416-26.

Ruigrok YM, Rinkel GJ, Wijmenga C. Genetics of intracranial aneurysms. *Lancet Neurol* 2005;4:179-89.

Ruigrok YM, Rinkel GJ, van't Slot R, Wolfs M, Tang S, Wijmenga C. Evidence in favor of the contribution of genes involved in the maintenance of the extracellular matrix of the arterial wall to the development of intracranial aneurysms. *Hum Mol Genet* 2006;15:3361-8.

Rånby M, Nguyen G, Scarabin PY, et al. Immunoreactivity of tissue plasminogen activator and of its inhibitor complexes. *Thromb Haemost* 1989;61:409-14.

Saito K, Nakayama T, Sato N, et al. Haplotypes of the plasminogen activator gene associated with ischemic stroke. *Thromb Haemost* 2006;96:331-6.

Santamaria A, Oliver A, Borrell M, et al. Risk of ischemic stroke associated with functional thrombin-activatable fibrinolysis inhibitor plasma levels. *Stroke* 2003;34**:**2387-91.

Sarti C, Rastenyte D, Cepaitis Z, Tuomilehto J. International trends in mortality from stroke, 1968 to 1994. *Stroke* 2000;31:1588-601.

Saxena R, Koudstaal P. Anticoagulants versus antiplatelet therapy for preventing stroke in patients with nonrheumatic atrial fibrillation and a history of stroke or transient ischemic attack. *Cochrane Database Syst Rev* 2004**:**CD000187.

Sayed-Tabatabaei FA, Oostra BA, Isaacs A, van Duijn CM, Witteman JC. ACE polymorphisms. *Circ Res* 2006;98:1123-33.

Schaid DJ. Evaluating associations of haplotypes with traits. *Genet Epidemiol* 2004;27**:**348-64.

Schievink WI. Spontaneous dissection of the carotid and vertebral arteries. *N Engl J Med* 2001;344:898-906.

Schneider M, Boffa M, Stewart R, Rahman M, Koschinsky M, Nesheim M. Two naturally occurring variants of TAFI (Thr-325 and Ile-325) differ substantially with respect to thermal stability and antifibrinolytic activity of the enzyme. *J Biol Chem* 2002;277:1021-30.

Schulz UG, Rothwell PM. Differences in vascular risk factors between etiological subtypes of ischemic stroke: importance of population-based studies. *Stroke* 2003;34:2050-9.

Schulz UG, Floßmann E, Rothwell PM. Heritability of ischemic stroke in relation to age, vascular risk factors, and subtypes of incident stroke in population-based studies. Stroke 2004;35:819-24.

Sebastiani P, Ramoni MF, Nolan V, Baldwin CT, Steinberg MH. Genetic dissection and prognostic modeling of overt stroke in sickle cell anemia. *Nat Genet* 2005;37:435-40.

Sharma P. Meta-analysis of the ACE gene in ischaemic stroke. *J Neurol Neurosurg Psychiatry* 1998;64:227-30.

Shintani S, Kikuchi S, Hamaguchi H, Shiigai T. High serum lipoprotein(a) levels are an independent risk factor for cerebral infarction. *Stroke* 1993;24**:**965-9.

Socialstyrelsen. Socialstyrelsens riktlinjer för strokesjukvård 2005 – medicinskt och hälsoekonomiskt faktadokument. Socialstyrelsen 2005, in Swedish.

Souto JC, Blanco-Vaca F, Soria JM, et al. A genomewide exploration suggests a new candidate gene at chromosome 11q23 as the major determinant of plasma homocysteine levels: results from the GAIT project. *Am J Hum Genet* 2005;76:925-33.

Steinberg MH, Adewoye AH. Modifier genes and sickle cell anemia. *Curr Opin Hematol* 2006;13:131-6.

Stegmayr B, Asplund K, Wester PO. Trends in incidence, case-fatality rate, and severity of stroke in northern Sweden, 1985-1991. *Stroke* 1994;25:1738-45.

Stephens M, Smith NJ, Donnelly P. A new statistical method for haplotype reconstruction from population data. *Am J Hum Genet* 2001;68**:**978-89.

Stephens M, Donnelly P. A comparison of bayesian methods for haplotype reconstruction from population genotype data. *Am J Hum Genet* 2003;73**:**1162-9.

Strachan T & Read AP. Human Molecular Genetics, 3rd edition. ISBN 0-8153-4182-1. Garland Publishing 2004a; pp 316.

Strachan T & Read AP. Human Molecular Genetics, 3rd edition. ISBN 0-8153-4182-1. Garland Publishing 2004b; pp 102.

Stram DO. Tag SNP selection for association studies. *Genet Epidemiol* 2004;27:365-74.

Sudlow C, Martinez Gonzalez NA, Kim J, Clark C. Does apolipoprotein E genotype influence the risk of ischemic stroke, intracerebral hemorrhage, or subarachnoid hemorrhage? Systematic review and meta-analyses of 31 studies among 5961 cases and 17,965 controls. *Stroke* 2006;37:364-70.

Switzer JA, Hess DC, Nichols FT, Adams RJ. Pathophysiology and treatment of stroke in sickle-cell disease: present and future. *Lancet Neurol* 2006;5:501-12.

Szalai AJ, Wu J, Lange EM, et al. Single-nucleotide polymorphisms in the C-reactive protein (CRP) gene promoter that affect transcription factor binding, alter transcriptional activity, and associate with differences in baseline serum CRP level. *J Mol Med* 2005;83:440-7.

Tatlisumak T, Fisher M. Hematologic disorders associated with ischemic stroke. *J Neurol Sci* 1996;140:1-11.

Teasdale GM, Wardlaw JM, White PM, Murray G, Teasdale EM, Easton V. The familial risk of subarachnoid haemorrhage. *Brain* 2005;128:1677-85.

Tei H, Uchiyama S, Koshimizu K, Kobayashi M, Ohara K. Correlation between symptomatic, radiological and etiological diagnosis in acute ischemic stroke. *Acta Neurol Scand* 1999;99**:**192-5.

Telman G, Kouperberg E, Sprecher E, Yarnitsky D. Distribution of etiologies in patients above and below age 45 with first-ever ischemic stroke. *Acta Neurol Scand* 2007. Epub ahead of print, Nov 27.

Teunissen LL, Rinkel GJ, Algra A, van Gijn J. Risk factors for subarachnoid hemorrhage: a systematic review. *Stroke* 1996;27:544-9.

Thambisetty M, Newman NJ. Diagnosis and management of MELAS. *Expert Rev Mol Diagn* 2004;4:631-44.

Timsit SG, Sacco RL, Mohr JP, et al. Brain infarction severity differs according to cardiac or arterial embolic source. *Neurology* 1993;43:728-33.

Tiret L, Rigat B, Visvikis S, et al. Evidence, from combined segregation and linkage analysis, that a variant of the angiotensin I-converting enzyme (ACE) gene controls plasma ACE levels. *Am J Hum Genet* 1992;51:197-205.

Tjärnlund A, Andersson M, Jood K, Ladenvall P, Jern C. Rapid genotyping of haemostatic gene polymorphisms using the 5' nuclease assay. *Thromb Haemost*. 2003;89:936-42.

Tjärnlund-Wolf A, Olsson L, Medcalf RL, Jern C. Regulation of endogenous tissue-type plasminogen activator expression is modulated by the -7,351C>T enhancer polymorphism. *J Thromb Haemost* 2006;4:1414-18.

Touzé E, Rothwell PM. Sex differences in heritability of ischemic stroke. A systematic review and meta-analysis. *Stroke* 2007. Epub ahead of print, Nov 21.

Tregouet DA, Aubert H, Henry M, et al. Combined segregation-linkage analysis of plasma thrombin activatable fibrinolysis inhibitor (TAFI) antigen levels with TAFI gene polymorphisms. *Hum Genet* 2001;109:191-7.

Tregouet DA, Escolano S, Tiret L, Mallet A, Golmard JL. A new algorithm for haplotype-based association analysis: the Stochastic-EM algorithm. *Ann Hum Genet* 2004;68**:**165-77.

Truelsen T, Mahonen M, Tolonen H, Asplund K, Bonita R, Vanuzzo D. Trends in stroke and coronary heart disease in the WHO MONICA Project. *Stroke* 2003;34:1346-52.

Tsantes AE, Nikolopoulos GK, Bagos PG, et al. Plasminogen activator inhibitor-1 4G/5G polymorphism and risk of ischemic stroke: a meta-analysis. *Blood Coagul Fibrinolysis* 2007;18:497-504.

Um JY, Jeong HJ, Park RK, Hong SH, Kim HM. Aspects of gene polymorphisms in cerebral infarction: inflammatory cytokines. *Cell Mol Life Sci* 2005;62:824-33.

van Gijn J, Rinkel GJ. Subarachnoid haemorrhage: diagnosis, causes and management. *Brain* 2001;124:249-78.

van Swieten JC, Koudstaal PJ, Visser MC, Schouten HJ, van Gijn J. Interobserver agreement for the assessment of handicap in stroke patients. *Stroke.* 1988;19:604-7.

Vitullo F, Marchioli R, Di Mascio R, Cavasinni L, Pasquale AD, Tognoni G. Family history and socioeconomic factors as predictors of myocardial infarction, unstable angina and stroke in an Italian population. PROGETTO 3A Investigators. *Eur J Epidemiol* 1996;12**:**177-85.

Voetsch B, Loscalzo J. Genetic determinants of arterial thrombosis. *Arterioscler Thromb Vasc Biol* 2004;24:216-29.

Wald DS, Law M, Morris JK. Homocysteine and cardiovascular disease: evidence on causality from a meta-analysis. *BMJ* 2002;325:1202-07.

Walker JB, Bajzar L. The intrinsic threshold of the fibrinolytic system is modulated by basic carboxypeptidases, but the magnitude of the antifibrinolytic effect of activated thrombin-activable fibrinolysis inhibitor is masked by its instability. *J Biol Chem* 2004;279:27896-904.

Wall U, Jern C, Bergbrant A, Jern S. Enhanced levels of tissue-type plasminogen activator in borderline hypertension. *Hypertension* 1995;26:796-800.

Wang K, Li M, Bucan M. Pathway-based approaches for analysis of genomewide association studies. *Am J Hum Genet* 2007;81:1278–83.

Wannamethee SG, Shaper AG, Ebrahim S. History of parental death from stroke or heart trouble and the risk of stroke in middle-aged men. *Stroke* 1996;27:1492-8.

Watson JD, Crick FH. Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature* 1953;171:737-8.

Warlow C, Sudlow C, Dennis M, Wardlaw J, Sandercock P. Stroke. *Lancet* 2003;362:1211-24.

Wellcome Trust Case Control Consortium. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature* 2007;447:661-78.

WHO. The world health report 2004. Annex Table 2: Deaths by cause, sex and mortality stratum in WHO regions, estimates for 2002. Geneva: World Health Organization.

WHO MONICA Project Principal Investigators. The World Health Organization MONICA Project (monitoring trends and determinants in cardiovascular disease): a major international collaboration. *J Clin Epidemiol* 1988;41:105-14.

Wilcox WR, Banikazemi M, Guffon N, et al. Long-term safety and efficacy of enzyme replacement therapy for Fabry disease. *Am J Hum Genet* 2004;75:65-74.

Wilhelmsen L, Johansson S, Rosengren A, Wallin I, Dotevall A, Lappas G. Risk factors for cardiovascular disease during the period 1985-1995 in Goteborg, Sweden. The GOT-MONICA Project. *J Intern Med* 1997;242:199-211.

Wilson JT, Pettigrew LE, Teasdale GM. Structured interviews for the Glasgow Outcome Scale and the extended Glasgow Outcome Scale: guidelines for their use. *J Neurotrauma* 1998;15:573-85.

Wittrup HH, Tybjaerg-Hansen A, Nordestgaard BG. Lipoprotein lipase mutations, plasma lipids and lipoproteins, and risk of ischemic heart disease. A meta-analysis. *Circulation* 1999;99:2901-7.

Wolf AT, Medcalf RL, Jern C. The t-pa -7351c>t enhancer polymorphism decreases sp1 and sp3 protein binding affinity and transcriptional responsiveness to retinoic acid. *Blood* 2005;105:1060-7.

Woo D, Kaushal R, Kissela B, et al. Association of Phosphodiesterase 4D with ischemic stroke: a population-based case-control study. *Stroke* 2006;37:371-6.

Zee RY, Cheng S, Hegener HH, Erlich HA, Ridker PM. Genetic variants of arachidonate 5-lipoxygenase-activating protein, and risk of incident myocardial infarction and ischemic stroke: a nested case-control approach. *Stroke* 2006;37:2007-11.

Zunker P, Schick A, Padro T, Kienast J, Phillips A, Ringelstein EB. Tissue plasminogen activator and plasminogen activator inhibitor in patients with acute ischemic stroke: relation to stroke etiology. *Neurol Res* 1999;21**:**727-32.