

The hemostatic pathway in ischemic stroke

Clinical studies of genetic variation and
plasma protein measurements

Ellen Hanson
2012



UNIVERSITY OF GOTHENBURG

Department of Clinical Neuroscience and Rehabilitation
Institute of Neuroscience and Physiology
The Sahlgrenska Academy at the University of Gothenburg
Gothenburg, Sweden

Cover: Ischemic stroke, illustration by Matthew Holt, matthew@bodyrender.com

© Ellen Hanson 2012

ISBN 978-91-628-8518-2

Published articles have been reprinted with permission of the copyright holder.

Printed by Kompendiet /Aidla Trading AB, Gothenburg, Sweden, 2012

To my family

ABSTRACT

Although stroke is a common cause of death and disability in adults, there are few studies on stroke compared to other common diseases. A stroke could be either ischemic or hemorrhagic, but even within these two groups, disease etiology shows heterogeneity. In ischemic stroke, the different etiologic subtypes represent different underlying pathophysiologic mechanisms. However, the formation of a thrombus is a key mechanistic event in the majority of ischemic stroke events. Therefore, the aim of the present thesis was to test the hypothesis that hemostatic gene polymorphisms and/or plasma levels of hemostatic proteins are associated with ischemic stroke. A second aim was to investigate whether the associations differ between the etiologic subtypes of ischemic stroke.

The studies were based on the Sahlgrenska Academy Study on Ischemic Stroke (SAHLSIS), which includes 844 patients with ischemic stroke and 668 controls, all younger than 70 years of age. Patients were classified into the major etiologic subtypes of ischemic stroke, *i.e.* large-vessel disease, small-vessel disease, cardioembolic stroke and cryptogenic stroke. Genotyping was carried out using both low- and high-throughput methods. Plasma levels of hemostatic proteins were determined by immunological methods.

The initial studies in this thesis focused von Willebrand factor (VWF) as well as the VWF-cleaving protease ADAMTS13. We found that ADAMTS13 gene variation was associated with overall ischemic stroke and with the etiologic subtype of cryptogenic stroke. Regarding VWF, the plasma levels were increased in overall ischemic stroke, as well as in all four major etiologic subtypes, as compared to the controls. There were also significant differences in VWF levels between the subtypes, highlighting the importance of considering etiologic subtypes in ischemic stroke studies. ABO blood group strongly influences VWF plasma levels, but we found no association between ABO and ischemic stroke. We then went on by analyzing plasma levels and gene variants of the newly discovered factor VII-activating protease (FSAP). FSAP gene variation influenced the plasma levels, but was not associated with ischemic stroke. Plasma FSAP on the other hand, was independently associated with overall ischemic stroke and with all major etiologic subtypes, indicating that FSAP is involved in ischemic stroke independent of the underlying etiology. We also observed an association between coagulation factor XI (FXI) gene variants and overall ischemic stroke up to 70 years of age, suggesting that FXI might be involved in ischemic stroke with a relatively young age of onset.

In conclusion, these results support a role for prothrombotic mechanisms in the pathophysiology of ischemic stroke. These mechanisms appear to be of importance for all four major etiologic subtypes of ischemic stroke, while we also show that there are subtype-specific differences.

Keywords: ischemic stroke, etiologic subtypes of ischemic stroke, genetics, SNP, hemostasis, prothrombotic, ADAMTS13, VWF, ABO blood group, FSAP, FXI

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. **Ellen Hanson**, Katarina Jood, Staffan Nilsson, Christian Blomstrand, Christina Jern. Association between genetic variation at the ADAMTS13 locus and ischemic stroke. *Journal of Thrombosis and Haemostasis* 2009 Dec;7(12):2147-49.
- II. **Ellen Hanson**, Katarina Jood, Sara Karlsson, Staffan Nilsson, Christian Blomstrand, Christina Jern. Plasma von Willebrand factor in the etiologic subtypes of ischemic stroke. *Journal of Thrombosis and Haemostasis* 2011 Feb;9(2):275-81.
- III. **Ellen Hanson**,* Sara Karlsson,* Katarina Jood, Staffan Nilsson, Christian Blomstrand, Christina Jern. No evidence for an association between ABO blood group and overall ischemic stroke or any of the etiologic subtypes. *Thrombosis Research*, 2012 Apr 4. *In press*
- IV. **Ellen Hanson**,* Sandip M Kanse,* Amit Joshi, Staffan Nilsson, Katarina Jood, Christian Blomstrand, Christina Jern. Plasma factor VII-activating protease antigen levels and activity are increased in ischemic stroke. *Journal of Thrombosis and Haemostasis* 2012 May;10(5):848-56.
- V. **Ellen Hanson**, Staffan Nilsson, Katarina Jood, Bo Norrving, Gunnar Engström, Christian Blomstrand, Arne Lindgren, Olle Melander, Christina Jern. Genetic variants of coagulation factor XI show association with ischemic stroke up to 70 years of age. *In manuscript*

*Both authors contributed equally to this work.

CONTENTS

ABBREVIATIONS	10
INTRODUCTION	12
Ischemic stroke	12
Etiologic subtypes of ischemic stroke	14
Risk factors for ischemic stroke	15
Genetics	16
Single-nucleotide polymorphisms (SNPs) and haplotypes	17
Monogenic and complex inheritance	18
Strategies for the study of multifactorial diseases	19
Hemostasis	19
Platelet activation	20
Coagulation cascade	21
von Willebrand factor (VWF)	22
ADAMTS13	23
Factor VII-activating protease (FSAP)	24
Coagulation factor XI (FXI)	26
The ABO blood group system	27
AIMS OF THE THESIS	29
SUBJECTS AND METHODS	30
The Sahlgrenska Academy Study on Ischemic Stroke (SAHLSIS)	30
Patients	30
Controls	32
The Lund Stroke Register (LSR) and the Malmö Diet and Cancer study (MDC)	33
Studies of hemostatic protein levels and gene variants	35
Biochemical analysis of plasma proteins	35
Genetic variation	39
DNA extraction and quantification	39
Selection of gene variants	39
Genotyping	40
Haplotype phasing	45
Statistical concepts in genetic association studies	45
Power and effect measure	45
Correction for multiple testing	45
Replication	46
Hardy-Weinberg equilibrium (HWE)	46
RESULTS AND DISCUSSION	47
Characteristics of SAHLSIS	47
ADAMTS13 gene variation in ischemic stroke (Paper I)	48

VWF plasma levels in the etiologic subtypes of ischemic stroke (Paper II)	50
ABO blood group in ischemic stroke (Paper III)	54
Determinants of VWF plasma levels (Papers I-III)	55
FSAP plasma levels and gene variants in ischemic stroke (Paper IV)	57
FXI gene variants in ischemic stroke up to 70 years of age (Paper V)	61
Future perspectives	63
<u>CONCLUSIONS</u>	<u>66</u>
<u>POPULÄRVETENSKAPLIG SAMMANFATTNING</u>	<u>68</u>
<u>ACKNOWLEDGMENTS</u>	<u>70</u>
<u>REFERENCES</u>	<u>72</u>
<u>PAPERS I-V</u>	

ABBREVIATIONS

A	adenine
ADAMTS13	ADAM metallopeptidase with thrombospondin type 1 motif, 13
C	cytosine
CAD	coronary artery disease
CE stroke	cardioembolic stroke
CHD	coronary heart disease
CI	confidence interval
CT	computed tomography
CV	coefficient of variation
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
FSAP	factor VII-activating protease
FVII	factor VII
FXI	factor XI
FXII	factor XII
G	guanine
GWAS	genome-wide association study
GP	glycoprotein
HABP2	hyaluronan-binding protein 2
HMWK	high-molecular weight kininogen
hsCRP	high sensitive C-reactive protein
HWE	Hardy-Weinberg equilibrium
LACI	lacunar infarct
LD	linkage disequilibrium
LSR	Lund Stroke Register
LVD	large-vessel disease
MAF	minor allele frequency
MDC	Malmö Diet and Cancer study
MI-SNP	Marburg I SNP
MRI	magnetic resonance imaging
mRS	modified Rankin Scale
OCSP	Oxfordshire Community Stroke Project
OR	odds ratio
PACI	partial anterior circulation infarct
PAD	peripheral artery disease
PCR	polymerase chain reaction
POCI	posterior circulation infarct
PDGF-BB	platelet-derived growth factor BB
RFLP	restriction fragment length polymorphism

RNA	ribonucleic acid
SAHLSIS	the Sahlgrenska Academy Study on Ischemic Stroke
scu-PA	single-chain urokinase-type plasminogen activator
SD	standard deviation
SHP	standard human plasma
SNP	single-nucleotide polymorphism
SVD	small-vessel disease
T	thymine
TACI	total anterior circulation infarct
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TOAST	Trial of Org 10172 in Acute Stroke Treatment
t-PA	tissue-type plasminogen activator
TTP	thrombotic thrombocytopenic purpura
VWD	von Willebrand disease
VWF	von Willebrand factor
WP bodies	Weibel-Palade bodies

INTRODUCTION

Stroke is one of the leading causes of death and disability in adults worldwide.¹ Even so, there are gaps of knowledge regarding risk factors, treatment and long-term outcome, as compared to other common diseases.² One reason for this may be that stroke is a syndrome rather than a single disease, and this heterogeneity complicates the design of clinical studies. Stroke is a collective term for the condition with sudden symptoms that occurs when the blood supply to the brain is interrupted, and the subsequent shortage of oxygen and nutrients cause damage to the brain tissue. This can be caused by blockage of a blood vessel (ischemic stroke) or a ruptured blood vessel (hemorrhagic stroke). The effects of a stroke depend on which part of the brain is injured and how severely it is affected. Typical symptoms include sudden weakness, loss of sensation, or difficulty with speaking or seeing. The clinical presentation gives some clues, but to be able to clearly differentiate between an ischemic and a hemorrhagic stroke, neuroimaging is required. Since the present studies include patients with ischemic stroke, the following sections in this thesis will only focus on stroke of this pathology.

Although there are several established risk factors for ischemic stroke, such as increasing age and hypertension, much is still unexplained. Genetic factors might account for some of this unexplained risk, as is evident in twin, family and animal studies of ischemic stroke.³⁻⁵ Hemostatic factors constitute another plausible risk factor since the formation of a blood clot, thrombus, is a key mechanistic event in ischemic stroke, and disturbance of the hemostatic balance is central to the pathogenesis of thrombosis.⁶ Furthermore, both environmental and genetic factors influence hemostatic disturbances, and it is likely that gene-environment interactions are of importance for thrombotic diseases, e.g. ischemic stroke.^{7,8} Interestingly, both genetic and hemostatic markers seem to have a more pronounced influence in younger ischemic stroke sufferers, than in older individuals.^{3,9}

Against this background, the purpose of this thesis was to study the hemostatic pathway in ischemic stroke. To this end, we investigated variation in candidate genes and performed plasma protein measurements in a clinical sample of relatively young patients with ischemic stroke and healthy controls.

Ischemic stroke

About 85% of all strokes are ischemic and occur when an artery supplying the brain with blood becomes obstructed, suddenly decreasing or stopping blood flow and

ultimately causing a brain infarction. Artery occlusion is most commonly caused by a thrombus, which can cause blockage in two ways. The thrombus can either form locally at the site of occlusion and partially or completely interrupt the blood flow, or form in another part of the circulation and then follow the blood stream until it obstructs arteries in the brain. This free-roaming clot is called an embolus and often forms in the heart or on atherosclerotic plaques in large arteries, e.g. cervical arteries or aortic arch.

As is briefly highlighted above, the disease etiology is heterogeneous even within the group of ischemic stroke. Based on the presumed pathophysiology of ischemic stroke, further classification into etiologic subtypes can be performed (Figure 1). A common classification system is the Trial of Org 10172 in Acute Stroke Treatment (TOAST),¹⁰ with which ischemic stroke is divided into the major etiologic subtypes; large-vessel disease (LVD), small-vessel disease (SVD), cardioembolic (CE) stroke, other determined etiology of stroke, and undetermined etiology of stroke. Another approach is to group ischemic strokes according to clinical presentation as in the Oxfordshire Community Stroke Project (OCSP) criteria,¹¹ which separates the subtypes; total anterior circulation infarct (TACI), partial anterior circulation infarct (PACI), posterior circulation infarct (POCI), and lacunar infarct (LACI). The TOAST classification system is based on clinical symptoms as well as thorough investigations of the underlying etiology and thus, reflects the underlying disease mechanism, whereas the OCSP classification relies only on the initial symptoms and provides a crude measure of the extent and location of the infarction.¹² In the papers included in this thesis, much emphasis was therefore put on the etiologic subtypes as determined by TOAST criteria.

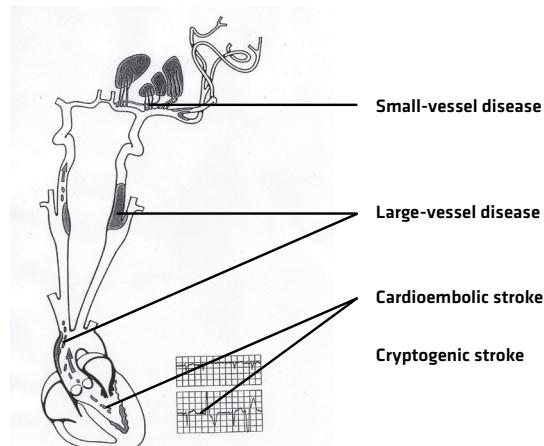


Figure 1. The four major etiologic subtypes of ischemic stroke.

Etiologic subtypes of ischemic stroke

Ischemic stroke caused by large-vessel disease (LVD)

LVD is considered to cause approximately 15-20% of ischemic strokes,¹³ but the proportion of LVD within a population varies depending on age, sex and ethnicity. The term LVD denotes significant stenosis or occlusion in large and medium sized precerebral and cerebral arteries, presumably due to atherosclerosis.¹⁰ Normally, the atherosclerotic plaques develop near branching points and places of confluence, e.g. the carotid bifurcation. Distal artery-to-artery embolization or hemodynamic mechanisms are the most common causes of ischemic stroke in these patients.¹⁴ Although the underlying pathology in LVD is atherosclerotic lesions, the mere presence of plaques does not imply causality and an accurate classification of LVD. Other clinical findings, including symptoms and location of the infarct, are also considered and should be consistent with the location of the atherosclerotic lesion for the classification of LVD. In addition, potential sources of cardiac embolism should be excluded.

Ischemic stroke caused by small-vessel disease (SVD)

The incidence of ischemic stroke due to SVD is considered to be about one fourth.¹⁵ SVD is an occlusion of the end-arteries supplying the deep white matter, basal ganglia, thalamus and brain stem. The pathogenesis of the vessel occlusions in SVD is poorly understood, but microatheroma and lipohyalinosis have been suggested to contribute.¹⁶ The infarcts are usually small (<15 mm in diameter). Typically, the clinical symptoms are related to size and location and manifest themselves as so called lacunar syndromes.¹⁷ These syndromes are characterized by the absence of cortical symptoms and visual field deficits, and include pure motor stroke, pure sensory stroke, ataxic hemiparesis and sensorimotor stroke.¹⁸ However, even though lacunar syndromes are frequently caused by SVD, not all patients with lacunar syndromes have SVD. Vasculitis, hematological diseases, monogenic disorders and other unusual forms of stroke may also cause small deep infarcts.¹⁹ Lacunar syndromes may also arise from artery-to-artery embolism or cardioembolism. Thus, potential sources of embolism (originating from stenosis in larger extracranial arteries or the heart) should be excluded.

Ischemic stroke caused by cardioembolism (CE stroke)

CE stroke is responsible for about one fourth of all first-ever strokes.¹³ This type of stroke is caused by an embolus originating from the heart that occludes cerebral arteries. Atrial fibrillation is a frequent source of cardiac emboli and these infarctions tend to be large, severely disabling, and prone to recurrence.¹⁴ Other high risk sources for cardioembolism are a recent myocardial infarction, left ventricular thrombosis, prosthetic valves, and endocarditis, among others.²⁰ Patent foramen ovale has also been suggested as a cardiac source of emboli as this

condition has been reported to be overrepresented, especially in young ischemic stroke sufferers without known causes of their stroke. However, as patent foramen ovale is rather prevalent in the normal population and there is great uncertainty about the mechanism by which this condition causes stroke, it remains a controversial cause of CE stroke.²¹ Anticoagulant medication has proven to be a successful therapy in CE stroke, in contrast to the other major etiologic subtypes of ischemic stroke.

Cryptogenic stroke

Ischemic strokes that remain without a definite cause even after extensive work-up are classified as cryptogenic. This subtype constitute about 30% of all ischemic strokes,²² but the proportion varies considerably between studies depending on the extent of the investigation. Patients presenting with cryptogenic stroke are commonly younger, as compared to the other etiologic subtypes. It is expected that cryptogenic stroke is itself a heterogeneous entity.²³

Other causes of ischemic stroke

In addition to the major classifications of ischemic stroke described above, there are also several rare causes. These include arterial dissection, vasculitis, migraine, hypercoagulable states, hematologic disorders and rare monogenic disorders.²⁴⁻²⁷

Risk factors for ischemic stroke

High age, male sex, certain ethnicities, and a family history of stroke have all been identified as risk factors for ischemic stroke.^{13,28} These factors are not modifiable but can help in the discrimination of individuals with increased risk of stroke, in whom preventive therapies can be initiated. Hypertension is a major risk factor for stroke and pharmacological treatment of hypertension has proven an effective tool to significantly reduce the risk of stroke.²⁹ Other modifiable risk factors include atrial fibrillation, diabetes mellitus, cigarette smoking, alcohol consumption, unhealthy diet, obesity, dyslipidemia, and physical inactivity.^{28,30,31} In recent years, psychosocial stress, inflammatory markers, hemostatic markers, and genetic polymorphisms have emerged as novel risk factors, but the causal role of these factors remains to be determined.³² Moreover, due to the variety of mechanisms underlying the pathophysiology of ischemic stroke, it can be expected that the etiologic subtypes of ischemic stroke also have different risk factor profiles. Therefore, it is important to investigate potential risk factors in the etiologic subtypes separately.

Genetics

The field of modern genetics stems from the work of Gregor Mendel (1822-1884), who provided a theory for the model of inheritance by studying selective cross-breeding in pea plants. He concluded that for each characteristic that he studied, each individual carried two ‘factors’, and that these factors were passed on from each parent to their offspring. To describe these factors or units of hereditary information, the term *gene* was used by Wilhelm Johannsen (1857-1927) in the early 1900s. He is also credited with introducing the concepts of *phenotype*, the observed characteristics of an organism, and *genotype*, the hereditary information that an organism carries.³³ Much of the genetic research during the 20th century was focused on ascertaining the physical nature of genes, and the cellular mechanisms by which they work. Early molecular genetic studies showed that genes generally encode proteins, and that genes are made of deoxyribonucleic acid (*DNA*). In 1953, the structure of DNA was resolved by James Watson (1928-) and Francis Crick (1916-2004).³⁴ DNA is a double-helix polymer composed of two intertwining *nucleotide* chains held together by complementary pairing of adenine (A) with thymine (T), and cytosine (C) with guanine (G) (Figure 2). The sequence of nucleotides, or *bases*, along a strand of DNA determines the genetic code for all living species. In humans, DNA is tightly coiled within 23 pairs of *chromosomes* located in the cell nucleus. A smaller chromosome is also found in the mitochondria of the cell, and together with the nuclear DNA, it makes up the human *genome*. Since almost all of the genetic information in humans reside in the nuclear DNA, and since the genetic studies in this thesis are only concerned with this type of DNA, the term genome will henceforth be used to describe all nuclear DNA.



Figure 2. The level of DNA organization within the human cell nucleus.

The work of Francis Crick exceeds that of determining the structure of DNA. In 1958 he postulated the *central dogma* of molecular biology, which states that the genetic information within cells are sequentially transferred from DNA to ribonucleic acid (RNA) to protein, and cannot be transferred back from protein to nucleic acid. Although this hypothesis remains largely true, there are some important exceptions, as an example, RNA has been ascribed more diverse roles than simply acting as a messenger for DNA. The protein-coding parts of the genome, *i.e.* genes, constitute about 1.2% of the genome, and are distributed on all chromosomes.³⁵ Genes are separated into *exons* that carry the instructions for making proteins, and *introns*, which are non-coding regions in between the exons. The definition of a gene has changed considerably since Johannsen first introduced the term to describe units of inheritance. The working model for many scientists has been the concept of a gene as a protein-coding DNA sequence, one gene encodes one protein. Today, this definition seems far too simplified and many observations have highlighted the complexity of genes. For instance, two neighboring protein-coding DNA sequences can be transcribed simultaneously creating a fused RNA, some DNA sequences can transcribe more than one protein, while some DNA sequences are transcribed into RNA that does not encode a protein.³⁶

Single-nucleotide polymorphisms (SNPs) and haplotypes

During his voyage around the world, Charles Darwin (1809-1882) understood that variation is central to biology. Through his thorough work he could present compelling evidence that all species of life have evolved from a common origin and that species are constantly evolving through the process of natural selection. Today, Darwin's theory, together with Mendel's laws of inheritance, is the cornerstone of modern evolutionary science.

In our everyday life, we observe that there are considerable differences in phenotypes among humans. Despite this, the genetic sequences of different people are astonishingly similar. When comparing DNA sequences between humans they are identical for long stretches of nucleotides, but occasionally, there will be differences. The main source of genetic variation is *mutations*, random alterations of the nucleotide sequence. Mutations can range in size from a single nucleotide to a large chromosomal segment and they can arise potentially anywhere in the genome. Sometimes mutations affect the phenotype, usually if they are located within or near a gene, but often they pass unnoticed. When mutations are inherited and accumulate within a population they are referred to as *polymorphisms*, *i.e.* they occur in at least 1% of the population. The most common variation in the human genome is called a single-nucleotide polymorphism (SNP). For example, at a particular location in the genome, called a *locus*, one individual can have an

A while another individual has a C. The different variants of the chromosomal region that this polymorphism gives rise to are called *alleles*. Besides being used to describe the total genetic makeup of an individual, *genotype* can denote a pair of alleles at a specific locus.

Loci located in close proximity on a chromosome tend to be inherited together, they are said to be *linked*. Genetic linkage arises because when two loci are close, the likelihood of them being separated by a *chromosomal crossover* (exchange of DNA between homologous chromosomes that results in recombination of genetic material) is small. Blocks or regions of linked alleles on a chromosomal segment are called *haplotypes*. Over generations, many chromosomal crossovers will occur, which could separate an allele from its original haplotype. Alleles at nearby loci will, however, often remain together for many generations. This non-random association of alleles is known as *linkage disequilibrium* (LD). LD is measured by r^2 or the absolute value of D' that both range between 0 and 1. A D' of 1 indicates that two alleles have not been separated by genetic recombination. The r^2 value is the square of a correlation coefficient between pairs of loci and indicates how predictable one gene variant is of the other gene variant, *i.e.* an r^2 of 1 indicates a perfect correlation.

SNPs are commonly used as markers to locate differences in the genome that might be associated with a trait or a disease. By genotyping SNPs within a gene or a chromosomal region, the frequencies of the genotypes can be compared between healthy individuals and individuals with a certain disease. Because SNPs are so common in the genome, it would be very costly to genotype all the SNPs in that region. Therefore, the HapMap project was launched (www.hapmap.org),³⁷ which takes advantage of the strong association between SNPs within a haplotype. Thus, only a few SNPs need to be selected to provide enough information about the other variants in that region. These *tagSNPs* can be selected through the HapMap database. To facilitate research on SNPs, the NCBI dbSNP (www.ncbi.nlm.nih.gov/snp) includes a broad collection of SNPs, and researchers are continuously reporting findings of new variants to this database.

Monogenic and complex inheritance

Monogenic, or Mendelian, inheritance describes the hereditary traits that are caused by a single locus. Many thousands of monogenic traits, or diseases, are known and information about them has been collected in the NCBI OMIM database (www.ncbi.nlm.nih.gov/omim). However, many diseases are more complex and are known as *multipfactorial* diseases. Such diseases have complex inheritance, which refers to the contribution of two or more loci, and are often also influenced by

environmental factors. Ischemic stroke can be caused by monogenic disorders, but it is usually multifactorial with both genetic and environmental contributions.

Strategies for the study of multifactorial diseases

There are several ways to try and determine the genetic variants that are responsible for causing a disease. For monogenic disorders, the first step in mapping the disease is usually a genome-wide *linkage study*. For multifactorial diseases, genetic *association studies*, have been more efficient.³⁸ With this approach, the aim is to find a correlation between the disease and a genetic variant. These studies usually involve a sample of unrelated individuals, such as a case-control study. A common approach for identification of genes associated with a disease is the *candidate gene* approach, which takes advantage of previous knowledge to select candidates that likely are involved in the pathophysiology of the disease under study. Thus, this method is hypothesis-driven. The ultimate goal is to find genetic variants that are more or less frequent in cases compared with controls. To reliably detect small relative risks, the sample size has to be fairly large. Candidate gene studies in case-control samples have typically been the method of choice for identification of ischemic stroke genes. In recent years, *genome-wide association studies* (GWAS), have gained popularity. This is a hypothesis-free approach, whereby a few hundred thousand SNPs, distributed throughout the whole genome, are investigated simultaneously. With this methodology, it becomes even more crucial to have a sufficient sample size, as the huge amount of tests performed generates lots of false positive results.

Hemostasis

Simply put, hemostasis is the process that prevents blood loss upon vessel injury. The hemostatic response involves narrowing of the blood vessel to prevent excessive hemorrhage (vasoconstriction), formation of a platelet plug (primary hemostasis), stabilization of the blood clot through formation of a protein network (secondary hemostasis), and moderating the size of the clot by dissolving it (fibrinolysis). Hemostasis is a heavily regulated process, with both positive feedback systems and inhibitors in most steps of the pathway. When these regulatory mechanisms are overwhelmed during pathologic conditions, excessive amounts of thrombin are formed, resulting in thrombosis. An overview of the prothrombotic mechanisms of hemostasis is shown in Figure 3, and below follows a description of these mechanisms, *i.e.* platelet activation and the coagulation cascade.

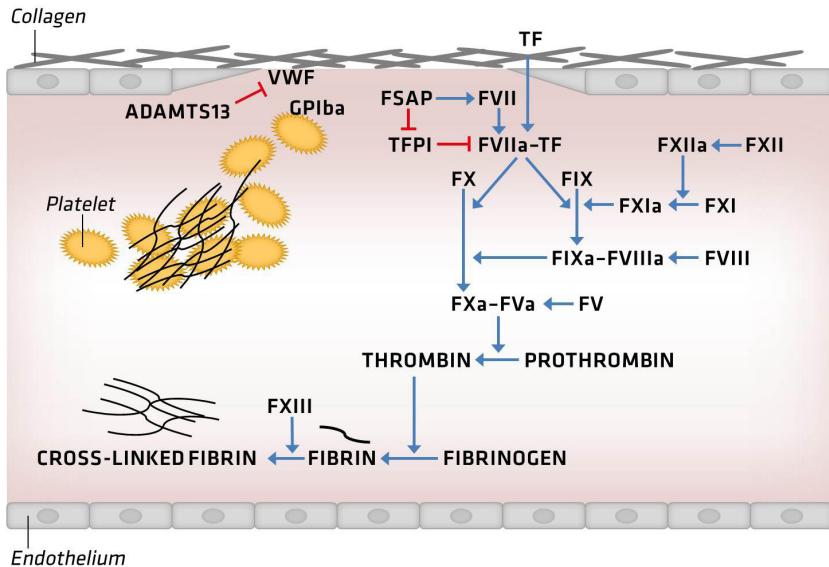


Figure 3. Overview of the prothrombotic mechanisms in hemostasis.

Platelet activation

Platelets, or thrombocytes, have a central role in primary hemostasis. During this step, the platelets become activated and form a primary loose platelet plug. Recent studies suggest that platelet activation is initiated through two separate pathways. Both can act in parallel or separately, and the process can be dominated by one of them, depending on the type of damage or disease.³⁹ The so called collagen pathway is initiated when a vessel wall injury occurs, damaging the endothelium and thereby exposing the subendothelial surface to the bloodstream. The collagen interacts with glycoprotein VI (GPVI) on the surface of circulating platelets, which adhere to the site of injury. Additionally, a complex (GPIb-V-IX) on the platelets interacts with the von Willebrand factor (VWF) which acts as a bridge between platelets and the exposed collagen fibrils.⁴⁰ Soon after adhesion, the activated platelets secrete the content of their granules which attracts more platelets to the lesion, and they in turn aggregate to the adhered platelets. The second platelet activation pathway does not directly require endothelium interruption, but is instead initiated by thrombin, generated in the coagulation cascade (please see next section). Thrombin cleaves protease-activated receptors on the platelet surface which activates the platelet and causes them to release agonists that, in turn, will activate other platelets.⁴¹

Coagulation cascade

Coagulation is initiated through two different pathways, the tissue factor (TF) pathway and the contact activation pathway. These two pathways ultimately lead to thrombin generation through a shared, common pathway. These pathways comprise a series of reactions, in which an inactive precursor of a serine protease is activated, which in turn catalyzes the next reaction in the cascade. The TF-pathway, also known as the extrinsic pathway, is initiated by TF forming a complex with coagulation factor VII (FVII). TF resides in the subendothelial tissue and vessel injury exposes TF to FVII in the flowing blood. Recent studies have shown that TF is also blood-borne, but its exact contribution to coagulation is unclear.⁴² In the coagulation cascade, the TF-FVII complex activates two different proteins, factor IX (FIX) and factor X (FX), through cleavage, both of which proceed into the common pathway.

The contact activation pathway, also called the intrinsic pathway, is initiated by the activation of factor XII (FXII). This then activates factor XI (FXI), which in turn activates FIX in the common pathway. The contact activation pathway is initiated when blood comes in contact with negatively charged surfaces *in vitro*, but it is still debated if this is a relevant mechanism *in vivo*.³⁹ A recent study has shown that nucleic acids, which are negatively charged, can activate proteins of this pathway both *in vitro* and *in vivo*.⁴³ As blood vessel injury likely would result in released extracellular RNA and DNA from damaged or necrotic cells, this is a plausible mechanism. Another hypothesis is that the main function of the contact activation pathway is to amplify the coagulation cascade, as initiated by the TF-pathway. FXI has an important role in this amplification as it is activated by thrombin via a feedback mechanism.⁴⁴

Both the TF-pathway and the contact activation pathway lead into the common pathway, with activation of FIX. Upon activation, FIX forms a complex with factor VIII (FVIII) that in turn activates FX. FX can also be activated directly by the TF-FVII complex. Activated FX forms a complex together with factor V (FV), which catalyzes the conversion of prothrombin into thrombin, via a series of cleavages. Thrombin has many functions. It can activate platelets by cleaving, as described above, but it can also activate FV, FVIII, FXI, and factor XIII (FXIII). The main function of thrombin, however, is to cleave soluble dimeric fibrinogen into insoluble fibrin. The fibrin aggregates with other fibrin molecules and is finally cross-linked by FXIII to form a network of fibrin strands.

The hemostatic proteins investigated in this thesis will be presented in more detail in the following sections.

von Willebrand factor (VWF)

In 1926, Dr. Erik von Willebrand described a bleeding disorder, distinct from hemophilia, in a family from Åland, Finland.⁴⁵ The disorder was later named von Willebrand disease (VWD), and has shown to be a common and complex hereditary bleeding disorder, which results from quantitative or qualitative defects of VWF.⁴⁶ The large VWF gene (*VWF*) maps to the short arm of chromosome 12, and encodes the VWF monomer. This monomer is predominantly synthesized in endothelial cells, and to some extent in megakaryocytes.⁴⁷ Once synthesized, the monomers dimerize and then undergo multimerization to create multimeric strings that can be extremely large. These multimers are either constitutively secreted or stored in Weibel-Palade (WP) bodies of endothelial cells or α -granules of megakaryocytes, from where they are released upon stimulation. The VWF multimers stored in granules are usually larger than the constitutively secreted forms.⁴⁸ A majority of circulating VWF is derived from the endothelium,⁴⁹ and consists of proteolytic fragments of larger multimers,⁵⁰ indicating that regulated release from WP bodies is the main determinant of plasma VWF levels. The larger, often ultra large VWF multimers, are biologically more potent than the smaller forms, and probably reside in the WP bodies for immediate secretion upon vascular injury.⁴⁸ In the circulation, the size of the VWF multimers are regulated by cleavage of ADAMTS13 (ADAM metallopeptidase with thrombospondin type 1 motif, 13), which is described in the next section.

VWF is an essential protein in hemostasis due to its role both in primary hemostasis with platelet adhesion and aggregation, and in coagulation as a carrier for FVIII. When the vascular wall is injured, ultra large VWF multimeric strings are released from granules and attach to endothelial cells and collagen in the subendothelial matrix. Adhesion of platelets to the lesion is then promoted by binding of VWF to platelet receptors GPIba in the GPIb-IX-V complex and the integrin α IIb β 3 in the GPIIb-IIIa complex.⁵¹ During high shear rate, the binding of VWF to GPIba is the essential interaction that can tether platelets to the lesion.⁵² The binding of VWF to the above platelet receptors is also necessary for platelet aggregation and thrombus growth. The second function of VWF in hemostasis is as a carrier for FVIII, thereby protecting the coagulation factor from proteolytic degradation as well as transporting it to the site of injury. A unique feature of VWF multimers is their responsiveness to high shear rate, which has been shown to expose key binding sites,⁵³ thereby facilitating the interactions with platelet receptors and ADAMTS13.

Plasma VWF has, to a quite large extent, been studied in relation to arterial thrombosis. High plasma levels of VWF have been shown to be a predictor of coronary heart disease (CHD),⁵⁴⁻⁵⁷ and more so in high-risk populations than in

the general population.⁵⁴ Fewer studies have been conducted on plasma VWF in ischemic stroke, but all in all the results from these studies indicate that VWF is a better predictor of stroke than of CHD.⁵⁸⁻⁶³ Among individuals, plasma VWF levels have a wide normal range, and they are influenced by both genetic and non-genetic factors. Twin studies have shown that genetic factors contribute to as much as 75% of the variation in VWF plasma levels.⁶⁴ The gene encoding ABO blood group is one such determinant of plasma VWF, as well as the *VWF* locus itself.⁶⁵ Non-genetic factors have also been shown to influence VWF levels, e.g. age, inflammation and diabetes.⁶⁵

Several experimental studies have indicated that VWF has a role in the development of atherosclerosis. For instance, several factors involved in atherogenesis induces VWF secretion.⁶⁵ *In vitro*, VWF has been shown to stimulate smooth muscle cell proliferation, the major constituent of atherosclerotic plaques.⁶⁶ Moreover, in an *in vivo* primate model, numerous WP bodies were found in endothelial cells at sites of atherosclerotic lesions.⁶⁷ Based on these studies, it seems plausible that individuals with VWD are protected from atherosclerosis. This hypothesis is supported by studies of VWF deficiency in animal models, but on the contrary, this has not been demonstrated in humans.⁶⁸

ADAMTS13

When ADAMTS13 was discovered by two separate labs in 1996, it was identified as the long sought after VWF-cleaving protease.^{69,70} A few years later, the gene sequence was revealed and the protease was found to be encoded by *ADAMTS13* on chromosome 9q34.⁷¹ In the same publication, it was shown that a number of mutations in *ADAMTS13* causes ADAMTS13 deficiency and that this deficiency is the molecular mechanism responsible for thrombotic thrombocytopenic purpura (TTP). TTP is a rare disorder characterized by thrombotic microangiopathy and hemolytic anemia, resulting from severely reduced ADAMTS13 activity followed by the accumulation of ultra large VWF multimers.⁷² In TTP, deficiency of ADAMTS13 is either caused by mutations in *ADAMTS13* or by antibodies that inhibit the activity of the protease.

ADAMTS13 is mainly synthesized and secreted by hepatic stellate cells,⁷³ but the protease has also been detected in endothelial cells,⁷⁴ and in tissues from the brain, heart, pancreas, kidney, spleen, adrenal glands, placenta, muscle, uterus, and testis.⁷⁵ In hemostasis, the main role of ADAMTS13 is to specifically cleave VWF and thereby inhibit excessive thrombus formation at sites of vascular injury. Upon endothelial stimulation, ultra large VWF multimer strings are released and become anchored to the endothelial surface.⁷⁶ The circulating ADAMTS13

molecules tether to the anchored VWF in competition with other molecules and cells, such as platelets, to rapidly cleave the VWF strings into less active forms.⁷⁶ The cleavage can occur in the absence of flow,⁷⁷ but the proteolysis seems to be facilitated by high shear stress, likely because the shear stress exposes the ADAMTS13 cleavage site.⁷⁸ Since ADAMTS13 and VWF can co-exist in plasma without proteolytic cleavage of VWF, it seems likely that high shear stress is needed for an efficient interaction.

The importance of this protease in the formation of a thrombus, as is evident in TTP, has led researchers to investigate whether ADAMTS13 deficiency is unique to TTP, or whether it also occurs in patients with a prothrombotic phenotype. Indeed, a few studies have found that decreased levels and/or reduced activity of ADAMTS13 are associated with myocardial infarction, CHD, and ischemic stroke.⁷⁹⁻⁸¹ ADAMTS13 has also been proposed as a link between thrombosis and inflammation. In an *in vivo* mouse model, ADAMTS13 deficiency resulted in increased leukocyte adhesion to inflamed vessel walls, a process that was dependent on the presence of VWF.⁸² In subsequent work in mice, ADAMTS13 has also been shown to down-regulate vascular inflammation and reduce plaque formation during early atherosclerosis.⁸³ Further support for a role of ADAMTS13 in inflammation comes from findings of reduced ADAMTS13 activity in patients with acute systemic inflammation or sepsis.^{84,85} Moreover, a more prominent activation of inflammatory cells, increased infarct volume, and more marked neuronal death in the ischemic penumbra, have been observed in experimental stroke models in *ADAMTS13* knock-out mice as compared with wild-type mice.⁸⁶⁻
⁸⁸

A rather large number of mutations and polymorphisms have been identified in *ADAMTS13*, and many of them seem to affect either synthesis, secretion, plasma levels, or activity of the protease.⁸⁹ For example, the minor allele of rs11575933 (also known as Pro475Ser) is associated with a markedly reduced ADAMTS13 activity in the Japanese population,⁹⁰ but this variant has not been detected in Caucasians.⁹¹ Interestingly, different combinations of *ADAMTS13* polymorphisms and mutations can modulate the protease secretion, expression and activity *in vitro*, even when the variants are individually non-functional.⁹² Despite the apparent functional effects of many *ADAMTS13* variants, very few studies have investigated this gene in relation to common thrombotic diseases.

Factor VII-activating protease (FSAP)

With the goal of identifying hyaluronic acid-binding proteins in human plasma, Choi-Miura *et al.*, in 1996, identified a novel protein which they called plasma

hyaluronan-binding protein,⁹³ later denoted hyaluronan-binding protein 2 (HABP2). The serine protease was cloned by the same group, and was found to be expressed in the human liver, pancreas, and kidney. A few years later, another group noted that a protease capable of potently activating FVII, termed FVII-activating protease (FSAP), was in fact identical to HABP2.⁹⁴ Hereafter, the protease will be referred to as FSAP.

FSAP circulates in plasma as an inactive zymogen that can be activated by factors released from apoptotic or dead cells, e.g. histones and nucleosomes.⁹⁵ In purified systems, FSAP can be auto-activated upon binding to negatively charged polyanions, such as hyaluronic acid, heparin, and nucleic acids.^{96,97} Apart from FVII, several other substrates for FSAP have been identified including fibrinogen, fibronectin, high molecular weight kininogen (HMWK), single-chain urokinase -type plasminogen activator (scu-PA), platelet-derived growth factor (PDGF) BB, FV, FVIII, and more recently the tissue factor pathway inhibitor (TFPI).⁹⁸⁻¹⁰³ A few plasmatic inhibitors of FSAP have also been identified, such as C1-inhibitor and α_2 -antiplasmin.¹⁰⁴

In recent years there has been debate as to whether FSAP really does what its name says – activate FVII. While early observations of FSAP-induced FVII activation⁹⁴ has been confirmed *in vivo*,¹⁰⁵ other studies have shown that FVII is a poor substrate for plasma derived FSAP.^{103,106} Instead, TFPI has been proposed as a physiological substrate for FSAP.¹⁰³ The procoagulant effect previously ascribed to FVII activation could thus rather be a result of TFPI inhibition. Furthermore, in *in vitro* experiments, FSAP has been shown to promote fibrinolysis through activation of scu-PA,¹⁰⁰ and scu-PA, in turn, can activate FSAP at a high enzyme to substrate ratio.⁹⁶ FSAP is also a potent inhibitor of PDGF-BB,¹⁰⁷ which has an important role in vascular smooth muscle cell proliferation and migration. Accordingly, FSAP has been localized in atherosclerotic plaques¹⁰¹ and found to reduce neointima formation *in vivo*.¹⁰⁷

The gene encoding FSAP, *HABP2*, is located on the long arm of chromosome 10. A SNP in *HABP2*, called the Marburg I SNP (MI-SNP), results in an amino acid exchange. The minor allele of the MI-SNP has reduced FSAP activity compared to the major allele,¹⁰⁸ and has also been reported to associate with an increased risk of venous thrombosis,¹⁰⁹ CHD,¹¹⁰ and ischemic stroke,¹¹¹ while other studies have not found an association.^{112,113} With regard to more local effects of the MI-SNP, a reduced scu-PA-activating capacity and an increased risk of progressive carotid stenosis has been shown in individuals carrying the rare variant of this SNP.¹¹⁴ Thus, based on the current understanding, a role for FSAP in both hemostasis and vascular remodeling processes emerges, although its physiological role remains unclear.

Coagulation factor XI (FXI)

FXI was first recognized in 1953 by Rosenthal *et al.*, who described a few cases with a history of mild hemorrhage.¹¹⁵ A closer examination of the patient's blood samples showed that this bleeding disorder was distinct from Hemophilia A and B and it was named Hemophilia C.¹¹⁵ This hemophilia factor was first referred to as thromboplastin antecedent, and later named FXI. Subsequent work has shown that Hemophilia C (more commonly known as FXI deficiency) is a rare bleeding disorder, but particularly prevalent in Jews.¹¹⁶ Patients with FXI deficiency generally present with mild to moderate bleeding tendencies, and bleeding is commonly associated with injuries, e.g. tooth extraction.¹¹⁷

FXI is produced in the liver and circulates in plasma as a complex with HMWK. FXI shares the same structure with its monomeric homolog prekallikrein, which also circulates in plasma in complex with HMWK.¹¹⁸ FXI is the zymogen of a serine protease (activated FXI, denoted FXIa), that is activated through cleavage by different substrates.¹¹⁸ In the contact activation pathway, FXI is activated by FXIIa. *In vitro* studies have shown that FXI can also be activated by thrombin, generated through the TF-pathway, via a feedback loop.^{119,120} At high concentrations of TF, the formation of fibrin was found to be independent of FXI, whereas at lower TF levels, the fibrin formation became FXI dependent. These results suggest that FXI is more important for the propagation and stabilization of the thrombus, than in the initial clot formation. Thrombin-induced activation of FXI also leads to the activation of thrombin-activated fibrinolysis inhibitor.¹²¹ Therefore, FXI can be regarded as both a prothrombotic and an antifibrinolytic factor.

The dual role of FXI in hemostasis makes it an interesting candidate to study in relation to thrombotic diseases. In animal models of FXI deficiency, thrombi that are formed upon vessel injury are unstable and dissolve before the blood vessel is occluded.^{122,123} In humans, increased FXI levels and/or activity have been observed in patients with venous thrombosis,^{124,125} and in younger subjects with ischemic stroke.¹²⁶⁻¹²⁸ A role for FXI in these diseases is further supported by the observation that individuals with FXI deficiency are protected against venous thrombosis¹²⁹ and ischemic stroke.¹³⁰ In the case of myocardial infarction, it does not seem that plasma levels of FXI are of equal importance,^{126,131,132} and FXI deficiency confers no protection against this disease.¹³³ Family-based studies have shown a rather strong heritability for FXI plasma levels.^{134,135} More recently, variants within the FXI gene (*F11*), located on chromosome 4q35, have been associated with increased FXI plasma levels.¹³⁶⁻¹³⁸ One of these studies was a very recent GWAS that aimed at identifying loci that influences FXI plasma levels. This study found an association with SNP rs4241824 in *F11*. An even stronger association was observed for a variant (rs710446) in *KNG1*, the gene encoding HMWK, which circulates in

plasma in complex with FXI.

As can be deduced from the above studies, FXI has several interesting properties that make it an attractive alternative target for anticoagulant therapy. Recently, several strategies to inhibit FXI/FXIIa have been investigated, which include neutralizing antibodies, antisense oligonucleotides, and small molecule inhibitors.¹³⁹ Many of these approaches have been successful in murine models, reducing thrombosis without causing bleeding.¹⁴⁰ Strategies for anticoagulant therapy by inhibiting FXIIa have also been developed.¹⁴¹ Similarly, these inhibitors have also been successfully effective in protection from thrombosis in several animal models. In addition to studies in animal models, a phase I clinical trial for an FXI inhibitor is currently underway.¹⁴¹

The ABO blood group system

Karl Landsteiner discovered the ABO blood group system in 1900.¹⁴² When serum from a few individuals was mixed individually with their red blood cells, Landsteiner observed agglutination in some of the mixtures but not in others. He concluded that an individual's serum contained antibodies to the antigen that is lacking from the individual's red blood cells, and was able to discriminate three different blood groups, which he named A, B and C (later renamed O from the German word *ohne*, meaning *without*).¹⁴³ Soon after, the blood group AB was described. The discovery was an important event in transfusion medicine, which had previously not been able to explain the often tragic circumstances of blood transfusion. At first, the ABO blood group system seemed uncomplicated, three antigens producing four phenotypes, but knowledge on the complexity has grown with time and a large number of subgroups have now been described.

It was early recognized that the ABO blood groups were inherited.¹⁴³ The antigens were first assumed to be encoded by separate genes, but later work by Felix Bernstein showed that it was in fact multiple alleles at one locus.¹⁴⁴ Though the work of Bernstein, the ABO blood group system became the first reliable human genetic marker to be used in forensic medicine and paternity testing.¹⁴³ In 1990, the gene encoding the ABO blood group system, *ABO* located on chromosome 9q34, was cloned.¹⁴⁵ The gene is highly polymorphic and there are numerous subgroups of the three main allelic forms *A*, *B*, and *O*. In Caucasians, the most common alleles are *A*¹, *A*², *B*, *O*¹, and *O*².¹⁴⁶ The *A* and *B* alleles encode *A* and *B* transferase, respectively, whereas the *O* allele encodes a non-active enzyme (Figure 4). The *A* and *B* transferases add specific monosaccharides to an acceptor substrate (called the H antigen), producing *A* and *B* antigens. Since the *O* enzyme is non-active,

the H antigen remains unconverted. Hence, the H antigen is an essential precursor of the ABO blood group antigens, so the ABO blood group system is not only governed by *ABO*.

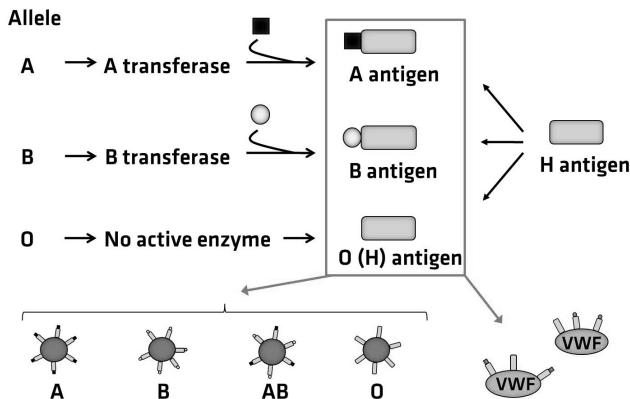


Figure 4. The ABO blood group system. The A and B transferases add monosaccharides to the H antigen, whereas the H antigen remains unconverted with the non-active O enzyme. The A, B, and H antigens are expressed on red blood cells and VWF multimers.

The A, B and H antigens are mainly expressed on the surface of red blood cells, but also in various other tissues, and on VWF multimers.¹⁴⁷ In several studies, the *ABO* locus has been shown to strongly influence the plasma levels of VWF (and consequently FVIII), with higher levels in individuals carrying one or two copies of the O allele.^{147,148} ABO blood group has been associated with myocardial infarction, venous thrombosis, and ischemic stroke, with an increased risk for the non-O phenotypes (*i.e.* A, B, and AB).¹⁴⁹ Therefore, increased VWF levels have been suggested as the mechanism behind the observed associations between the blood group non-O and several thrombotic diseases. The mechanism by which the ABO blood group exerts an effect on VWF plasma levels is thought to be attributed to a shorter VWF survival in the circulation of group O individuals.¹⁵⁰ Apart from influencing VWF levels, the *ABO* locus has also been associated with levels of soluble ICAM-1, P-selectin and E-selectin.¹⁵¹ Intriguingly, despite the strong associations between *ABO* and these proteins (particularly VWF), and the clinical importance of the ABO blood group system in transfusion medicine, the physiological function of this system is still not completely clear.

AIMS OF THE THESIS

The overall aim of this thesis was to increase our knowledge of the importance of the hemostatic pathway in ischemic stroke and the etiologic subtypes of ischemic stroke.

The specific aims were:

Paper I

- to investigate whether ADAMTS13 gene variants show association with ischemic stroke and/or any of the etiologic subtypes of ischemic stroke.

Paper II

- to investigate whether plasma levels of VWF are associated with ischemic stroke and/or any of the etiologic subtypes of ischemic stroke.
- if so, to explore if there are differences in VWF levels between the etiologic subtypes of ischemic stroke.

Paper III

- to investigate if there is an association between ABO blood group and overall ischemic stroke and/or any of the etiologic subtypes of ischemic stroke.

Paper IV

- to investigate whether plasma FSAP antigen levels and activity are associated with ischemic stroke and/or any of the etiologic subtypes of ischemic stroke.
- if so, to explore whether there are differences in FSAP antigen levels and/or activity between the etiologic subtypes of ischemic stroke.
- to investigate if FSAP gene variants are associated with (1) variation in FSAP antigen levels and/or activity, and (2) overall ischemic stroke and/or any of the etiologic subtypes.

Paper V

- to investigate if coagulation FXI gene variants show association with ischemic stroke and/or any of the etiologic subtypes of ischemic stroke.

SUBJECTS AND METHODS

The Sahlgrenska Academy Study on Ischemic Stroke (SAHLSIS)

For the purpose of studying genetic and hemostatic factors in ischemic stroke, our group initiated a large case-control study, the Sahlgrenska Academy Study on Ischemic Stroke (SAHLSIS),¹⁵² which constitutes a well-characterized and representative sample of patients with ischemic stroke and healthy controls from Western Sweden.

Patients

Between 1998 and 2008, consenting patients ($n=844$) younger than 70 years of age and presenting with first-ever ($n=732$) or recurrent ($n=112$) acute ischemic stroke were consecutively recruited at four Stroke Units in Western Sweden. Patients were excluded if they were >69 years, if the following evaluation showed another etiology than ischemic stroke, and if they had a diagnosis of cancer at advance stage, infectious hepatitis or HIV. The upper age limit is motivated by studies showing that the contribution of genetic and hemostatic factors varies with age, with a greater influence in the young.^{3,9} Baseline characteristics of this sample have been published previously^{152,153} and are illustrated in Table 1

Table 1. Baseline characteristics of the SAHLSIS participants.

	Controls $n=668$	Ischemic stroke $n=844$
Mean age, year (SD)	56 (10)	56 (11)
Male sex, n (%)	392 (59)	554 (66)
Hypertension, n (%)	230 (34)	487 (58)
Current smoking, n (%)	131 (20)	324 (38)
Diabetes mellitus, n (%)	33 (5)	153 (18)
Hyperlipidemia, n (%)	403 (67)†	413 (76)†

SD indicates standard deviation. †Numbers and percentages shown are for the initial 600 patients and 600 controls in SAHLSIS.

All patients were examined by a physician trained in stroke medicine during the acute phase of stroke, and all patients underwent electrocardiography and neuroimaging with computed tomography (CT) and/or magnetic resonance imaging (MRI). Extracranial carotid and vertebral duplex ultrasound, MR

angiography, catheter angiogram, transcranial Doppler ultrasound, transthoracic and/or transesophageal echo-cardiography were performed when clinically indicated. Stroke severity at inclusion was scored using the Scandinavian Stroke Scale.¹⁵⁴

To enable analysis of homogenous groups, a large effort has been put on the classification of etiologic subtypes of ischemic stroke. Based on clinical presentation and results from the diagnostic work-up, cases were classified into etiologic subtypes according to the TOAST criteria.¹⁰ In order to minimize interrater variability, the original TOAST criteria were refined according to a local protocol.¹⁵⁵ Risk factors, other than atrial fibrillation and carotid stenosis (*i.e.* hypertension and diabetes), were not included in the protocol. Adjudication of subtypes was performed by two neurologists. LVD was defined as either occlusive or significant stenosis (corresponding to >50% diameter reduction according to NASCET criteria) of a clinically relevant precerebral or cerebral artery, presumably due to atherosclerosis, or complex plaque (>4 mm thick, ulcerated or mobile) in the aortic arch. Potential causes of cardiac embolism should be excluded. SVD was defined as a clinical lacunar syndrome with a relevant infarct of <15 mm or normal CT/MRI in the absence of both a CE source and significant stenosis/occlusion due to atherosclerosis of an appropriate major brain artery. CE stroke was defined as the presence of atrial fibrillation, sick sinus syndrome, MI in the past four weeks, cardiac thrombus, infective endocarditis, atrial myxoma, prosthetic mitral or aortic valve, valvular vegetations, left ventricular akinetic segment, dilated cardiomyopathy, or patent foramen ovale in combination with either atrial septal aneurysm or deep venous thrombosis. Significant stenosis/occlusion due to atherosclerosis of an appropriate precerebral or cerebral artery should be excluded. Other determined cause of stroke included those with arterial dissection, vasculitis, hemathologic disorders, monogenic syndromes and complications of cardiovascular procedures. Cryptogenic stroke was defined when no cause was identified despite an extensive evaluation. Undetermined stroke included cases for whom more than one etiology was identified or when the evaluation was cursory.

Collection of the subject's vascular risk factors has been described in more detail previously.¹⁵² Hypertension was defined by pharmacological treatment for hypertension, systolic blood pressure ≥160 mm Hg, and/or diastolic blood pressure ≥90 mm Hg. Diabetes mellitus was defined by diet or pharmacological treatment, fasting plasma glucose ≥7.0 mmol/L, or fasting blood glucose ≥6.1 mmol/L. Smoking habit was coded as current versus never or former (smoking cessation at least one year before inclusion in the study). Information about hypertension was missing in 12 participants, diabetes mellitus in 12, and smoking habit in 4.

The first 600 patients enrolled in SAHLSIS were examined both in the acute phase and at a follow-up approximately three months after the index event. In connection with these examinations, plasma samples were obtained (please see section “Biochemical analysis of plasma proteins” for further details). Patients were also classified based on clinical presentation, as assessed using the OCSP system into the categories of TACI, PACI, POCI, and LACI.¹¹ The modified Rankin Scale (mRS) is commonly used to classify the outcome after a stroke.¹⁵⁶ At three months and at two years after the index stroke, the patients have been followed up regarding new vascular events or death, and functional outcome has been assessed with mRS. At the 3-month follow-up, functional outcome was assessed through examinations by a physician trained in stroke medicine. At the 2-year follow-up, all surviving patients were contacted by a research nurse trained in stroke medicine for a structured telephone interview that, among other things, involved the assessment of functional outcome.¹⁵⁷

Controls

Healthy population controls ($n=668$) without stroke, coronary artery disease (CAD), or peripheral artery disease (PAD), were randomly selected to match cases with regards to age and sex. The controls were from the same geographical region as the patients, and they were randomly recruited through a population-based health survey¹⁵⁸ or from the Swedish Population Registry (Skaraborg and Älvborg residents, and controls younger than 30 years). The controls were examined once by a research nurse trained in stroke medicine, using the same questionnaires and protocols as for patients. For the first 600 controls, plasma samples were obtained. To recruit these 600 controls, 1107 subjects had to be invited to participate, of which 208 did not respond, 191 were unwilling to participate, and 108 were excluded.

SAHLSIS is an ongoing study and patients are continuously being recruited at the Sahlgrenska University Hospital. To date, a total of 1085 patients are included. The study has been approved by the Ethics Committee of the University of Gothenburg and the management of collected data has been approved by the National Computer Data Inspection Board. All participants provided written informed consent prior to enrollment. Next-of-kin consented for those participants who were unable to communicate.

Methodological considerations: In SAHLSIS, all patients were recruited at Stroke Units in Western Sweden. The stroke admission rate in Sweden is high, with more than 87% of cases younger than 75 years being admitted to hospital.¹⁵⁹ In addition, the early case fatality rate in ischemic stroke is low, especially in the age group

studied here. Therefore, a patient selection bias is unlikely to influence our results. Regarding controls, selection bias cannot fully be excluded, as a significant proportion of those who were invited did not participate in the study. On the other hand, the controls were randomly sampled from the general population in Western Sweden, and the population in this geographical area is remarkably homogenous from an ethnic point of view.¹⁶⁰ Of note is that SAHLSIS includes participants younger than 70 years and is, of course, only representative for this age group. To better reveal the underlying pathophysiological mechanisms of ischemic stroke, TOAST is a widely used tool for classification of etiologic subtypes. However, as with most classification systems, there is room for interpretation and as long as there is no way to determine the mechanisms that occur *in vivo*, the pathophysiological mechanisms can only be inferred, not proven.

The Lund Stroke Register (LSR) and the Malmö Diet and Cancer study (MDC)

For validation of the genetic findings in SAHLSIS in Paper V, the Lund Stroke Register (LSR) and the Malmö Diet and Cancer study (MDC) were used as a replication sample (1213 patients and 788 controls ≤ 70 years of age, and in total 3145 patients and 1793 controls of all ages). Brief descriptions of the studies are given below, for detailed sample characteristics, data collection and clinical definitions please refer to Paper V and previous publications.^{159,161,162}

LSR is a consecutive, hospital-based case-control study initiated in 2001. All patients with first-ever stroke from the local catchment area of Skåne University Hospital, Lund, receiving hospital attention are included in this study. Patients 18 years and older with first-ever ischemic stroke between 2001 and 2009 were included. All patients underwent CT, MRI or post-mortem examination of the brain. Stroke-free control subjects were age- and sex matched to patients recruited during the first year of LSR (2001-2002), and were from the same geographic area.

MDC is a prospective, population based cohort study, which was established with the aim of studying the relationships between diet and health outcomes. A total of 28449 individuals, 45-73 years of age, participated in the baseline examinations between 1991 and 1996. Incidence of stroke after the baseline examination has been followed by data linkage with national and local registers. Validation of the stroke diagnosis, by review of hospital records, has been performed for 95% of all ischemic stroke in the cohort. The procedures for retrieval of incident cases of stroke, case ascertainment and stroke classification have been reported

elsewhere.¹⁶² For the purpose of studying genetic determinants of ischemic stroke in MDC, incident cases of ischemic stroke up to December 31th, 2006, were selected and matched (1:1) for age, sex and month of baseline examination in a nested case-control design. Control subjects were MDC participants who were alive and free from stroke at the time of the corresponding stroke event. Risk factors were assessed at the examination in 1991-1996.

Hypertension was defined by pharmacological treatment for hypertension, systolic blood pressure ≥ 160 mmHg, and/or diastolic blood pressure ≥ 90 mmHg. Diabetes mellitus was defined by diet or pharmacological treatment, fasting plasma glucose ≥ 7.0 mmol/L, and/or fasting blood glucose ≥ 6.1 mmol/L, or self-reported diabetes. Smoking history was coded as current versus never or former. In the combined sample of LSR and MDC ≤ 70 years of age, 17 participants had missing data for hypertension, 28 for diabetes mellitus, and 34 for smoking habit. The corresponding numbers for the whole sample including participants of all ages were 77 for hypertension, 111 for diabetes mellitus, and 87 for smoking habit.

The studies were approved by the Ethics Committee of Lund University. All participants provided informed consent prior to enrollment. For participants who were unable to communicate, consent was obtained from their next-of-kin.

Methodological considerations: SAHLSIS and LSR are both case-control studies, whereas MDC is a population based cohort study. Cohort studies are the ideal method for determining the incidence and natural history of a disease. These are usually prospective, meaning that a group of individuals are examined at baseline and then followed up for a long time. Hence, as the relevant variables are measured before the outcome (disease) has occurred, the “cause” rather than the effect is measured. However, the study has to be very large and proceed for a long time in order to have a sufficient number of patients with the outcome at follow-up. This study design is therefore not suitable for rare diseases. In case-control studies, on the other hand, it is easier to recruit larger numbers of patients. As case-control studies have comparatively few subjects, more resources can be put on examining them, and as a consequence a large number of variables can be considered. In the case of ischemic stroke, this could facilitate the classification of etiologic subtypes. A major drawback of the case-control design is that they are usually retrospective, *i.e.* variables are measured after the outcome (disease) occurred, thereby problematizing the question of cause and effect. For genetic factors, which remain the same throughout life, this is not an issue.

Studies of hemostatic protein levels and gene variants

In this thesis, the hemostatic pathway was investigated both at the protein and at the genotype level. These two approaches have different strengths. The major advantage of measuring plasma levels or activity of hemostatic proteins is that the proteins represent the actual functional molecules that circulate in the blood. However, these plasma levels are variable and are affected both in the short term, as in the acute phase by the ischemic injury itself, and in the long term by factors such as age and sex.¹⁶³ Furthermore, it is important that blood sampling and handling of plasma is done by a highly standardized protocol to avoid external influences on plasma quality.¹⁶⁴ In SAHLSIS, several measures have been taken to minimize these factors, such as age and sex matching between cases and controls and standardized protocols for blood sampling (please see next section). In addition, blood sampling in patients was performed both in the acute phase of the stroke event and in the convalescent phase at follow-up approximately three months after the index event. This enables us to compare the levels of hemostatic proteins with and without major influence of the acute-phase event itself.

On the other hand, the advantage of conducting studies at the genotype level is that these do not change over time, thus, they reflect life-long exposure. Genetic variation can also help in identifying other (local) mechanisms in contrast to the systemic effects that are measured by plasma levels. In the lab, DNA is favorable for practical reasons because it is relatively stable, easy to handle, and is less susceptible to external influences as compared to proteins. Ideally, the genotype and protein level approaches can be used together to complement each other, as additional information may be provided if there is an association between the two.

Biochemical analysis of plasma proteins

Laboratory protein measurements are delicate and complex procedures, and careful consideration must be made to ensure that errors are minimized. Much emphasis has been placed on the analytical phase with advances in instrument technology and automation, which has simplified the analytical procedure and improved the quality of the analyses.¹⁶⁵ However, a large proportion of mistakes occur in the pre- and post-analytical phases.^{165,166} Consequently, each step from patient to final data file must be handled with care using highly standardized procedures.

When SAHLSIS was initiated, detailed protocols were made for the collection of plasma. For the first 600 patients, blood sampling was performed in the acute

phase within 10 days (median four days) of the stroke event, and at follow-up approximately three months after the event (median 101 days, range 85–125 days). For the first 600 controls, blood sampling was performed once. Blood sampling was performed between 8:30 AM and 10:30 AM after overnight fasting, and after at least 10 minutes of rest in the supine position. Venous blood was collected in tubes that contained 10% by volume of 0.13 mol/L sodium citrate. Plasma was isolated within two hours by centrifugation $2000 \times g$ at 4°C for 20 minutes, aliquoted, and stored at -80°C. Other pre-analytic measures performed before the analyses included calibration of pipettes and careful selection and testing of the assays before commencing the analytical phase.

The enzyme-linked immunosorbent assay (ELISA) principle is an antibody-based method commonly used to detect the amount of a specific antigen in plasma. For the present thesis, sandwich ELISAs were used (Papers II and IV). Please see Figure 5 for a detailed description of this method. During the analytical procedures, careful measures involved accurate pipetting (*e.g.* avoiding air bubbles), vortexing samples just before pipetting, and including quality controls on each microtiter plate.

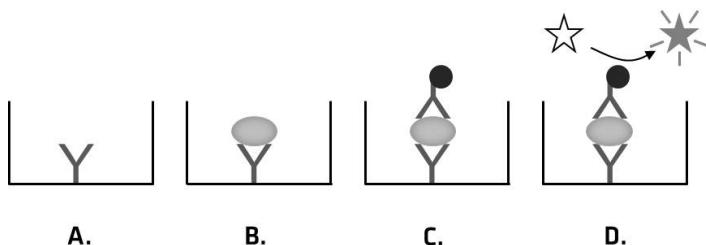


Figure 5. The principle of sandwich ELISA. **A.** The surface of each well in a 96-well microtiter plate is coated with a capture antibody, and nonspecific binding sites on the surface are then blocked. **B.** The diluted plasma samples are added and the antigen present in the plasma binds specifically to the capture antibody. This is followed by incubation and washing to remove unbound antigen. **C.** An enzyme-linked detecting antibody is applied that binds to the antigen, followed by incubation and washing to remove unbound antibody-enzyme conjugates. **D.** A substrate is added which is converted by the enzyme to a detectable form. The absorbance is measured to quantify the amount of substrate, which is directly proportional to the antigen concentration initially present in the plasma sample.

Plasma levels of VWF antigen were determined in our lab by sandwich ELISA according to the principle described above and using the manufacturer's instructions (Asserachrom VWF:Ag, Diagnostica Stago, Asnières, France). In brief, the plasma samples were defrosted in a 37°C water bath, diluted 1:102 (samples with

very high levels were further diluted), and added to a microtiter plate coated with rabbit polyclonal antibodies. VWF can be present in plasma in a wide variety of sizes. As such, the VWF molecules can stratify into layers with the largest multimers at the bottom of the test tube. Therefore, the plasma samples were always vortexed just prior to pipetting. Bound VWF antigen was detected with a peroxidase-coupled polyclonal rabbit antibody. The quality controls consisted of pooled human plasma, one with low and one with high values, which were always run in duplicate. A calibration curve was established with a dilution series of reference standard plasma, which corresponds to a VWF antigen value assigned by the WHO International Standard, established in 2003 (NIBSC code 02/150). Accordingly, the VWF levels were expressed in IU/dL. The optical density was measured at 450 nm, and the detection limit for the assay was 0.02 IU/dL. The intra- and inter-assay coefficients of variation (CV) were calculated by the mean values for the quality controls for all plates, and they were 5.3% and 13.3%, respectively.

For the measurement of FSAP, citrated plasma was aliquoted in our lab and the plasma levels and activity of FSAP were measured in Prof. Sandip Kanse's lab in Giessen, Germany. Total FSAP concentration was detected by an FSAP-specific sandwich ELISA, as previously described,¹⁶⁷ with minor modifications. Shortly, the plasma samples were defrosted on ice, diluted (1:2000), and applied to a microtiter plate coated with rabbit polyclonal antibodies. For detection of bound antigen, an FSAP-specific monoclonal antibody was added, followed by addition of a peroxidase-labeled mouse polyclonal antibody that binds to the FSAP-specific detecting antibody. An internal control consisting of one sample with normal FSAP plasma levels was run in duplicate on each plate. A calibration curve was established through a dilution series of Standard Human Plasma (SHP, Siemens Diagnostics, Marburg, Germany), and this preparation was assumed to contain approximately 12 µg/ml FSAP as described.¹⁶⁷ The absorbance was measured at 405 nm. The intra- and inter-assay CV were calculated by the mean values for the quality control for all plates, and were 4.1% and 13.2%, respectively.

In addition to measuring total FSAP concentration in plasma, the FSAP activity was measured by an immunocapture activity assay as described,¹⁶⁷ with minor modifications (Figure 6). For the FSAP activity assay, the plasma samples were diluted 1:100. As for the FSAP ELISA, a dilution series of SHP was used to establish a calibration curve. This preparation served as reference for the measured FSAP activity, which was defined as one plasma equivalent unit (1000 mU/ml). The quality controls were the same as detailed for the FSAP ELISA above. The optical density was measured every minute over one hour at 405 nm. The maximal velocity (substrate turnover with time) was determined over 8 min, and this was invariably always the initial reaction velocity. The intra- and inter-assay CV were

calculated by the mean values for the quality control for all plates, and they were 4.7% and 14.7%, respectively.

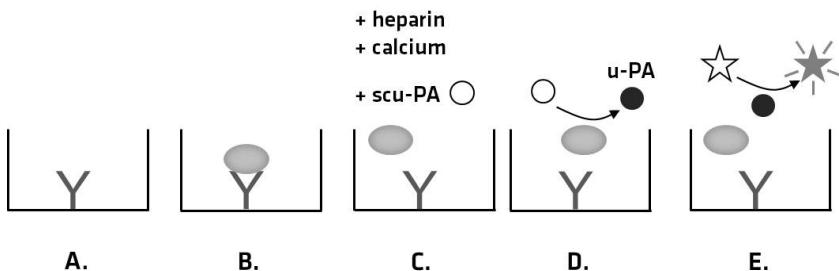


Figure 6. The FSAP activity assay principle. **A.** The surface of each well in a 96-well microtiter plate is coated with a monoclonal capture antibody, and nonspecific binding sites on the surface are then blocked. **B.** The diluted plasma samples are added and the FSAP antigen present in the plasma binds specifically to the capture antibody. This is followed by incubation and washing to remove unbound antigen. **C.** A buffer containing heparin and calcium is applied, which leads to the release of bound FSAP into the fluid phase. Recombinant scu-PA is also added, followed by incubation. **D.** The released FSAP is activated through an autocatalytic mechanism and then activates scu-PA, generating active u-PA. At high concentrations of u-PA, FSAP is activated by u-PA in a backloop activation potentiating further activation of scu-PA. **E.** A chromogenic substrate is added, which is cleaved by u-PA and followed by measuring the absorbance over a period of time. The intensity of color produced is proportional to the activity of u-PA, and thus to FSAP activity.

For all three assays described above, the acute phase and 3-month samples from each patient were analyzed together with the sample from the matching control on the same microtiter plate. In this way, the analysis is less susceptible to inter-assay variations. To avoid post-analytical errors arising from the preparation of data files, a random control was made of about 10% of the data which was compared with the raw data.

Methodological considerations: Measurement of VWF plasma levels with ELISA is the commonly used method in epidemiological and clinical studies. This approach mainly reflects VWF synthesis and secretion from vascular endothelial cells, but does not measure VWF from platelets. On the other hand, the endothelium is the predominant source of VWF. Moreover, this type of assay does not differ between the sizes of the VWF multimers, meaning that even though two individuals have similar plasma VWF levels, the biologic activity of their multimers can differ. Thus, the prothrombotic risk may be better identified in the VWF multimer pattern or by

measuring VWF activity with, for instance, the ristocetin cofactor assay. However, this assay does not actually measure the physiologic function of VWF, and it is also labor-intensive, and imprecise with high intra- and interassay CVs.¹⁶⁸ As VWF interacts with several different substrates, there is no single ultimate test to determine an individual's quantitative and qualitative properties of VWF. In the clinic, VWF is therefore measured with a panel of assays. For FSAP, we measured both the total plasma concentration as well as the activity. From a biological point of view, it may be more interesting to measure the activity. In this particular assay, the activity of FSAP is measured through its ability to activate scu-PA. Thus, this assumes that the activation of scu-PA mirrors FSAP's activity in the circulation. An advantage of this assay is that FSAP is not only auto-activated; the active u-PA provides a feedback activation of FSAP. This efficiency of FSAP activation probably contributes to a more constant and reproducible maximum activity.

Genetic variation

DNA extraction and quantification

In SAHLSIS, DNA was extracted from venous whole blood using either QIAamp® 96DNA Blood Kit (QIAGEN GmbH, Hilden, Germany) in our lab with the manufacturer's reagents as directed (Papers I and III), or with Maxi DNA Isolation PLUS at AGOWA GmbH in Berlin, Germany (Papers III-V). When the DNA yield with these methods was low, an additional extraction was performed for a few samples with a phenol-chloroform method using a local protocol in our lab. DNA concentration was quantified with PicoGreen® (Molecular Probes, Invitrogen, Paisley, UK) on a fluorometer (ABI PRISM® 7900HT Sequence Detection System, Applied Biosystems, Foster City, CA, USA) or with a ND-1000 spectrophotometer (NanoDrop Products, Thermo Fisher Scientific, Wilmington, DE, USA). DNA samples were diluted in TE-buffer to a concentration of 10 ng/µL or 50 ng/µL, depending on the genotyping method.

Selection of gene variants

The selection of SNPs was either based on a tagging approach (Papers I, III-V) or from the literature (Paper IV). For the tagSNPs, genotype information from the CEU population in HapMap was used to capture the genetic variation within the respective genes using pair-wise tagging with an $r^2 > 0.8$ and minor allele frequency

(MAF)>0.1. In Paper III, the five most common ABO alleles in Caucasians were chosen based on the literature.¹⁴⁶

Methodological considerations: The tagSNP strategy takes advantage of our recent understanding of the fine LD structure in the human genome. In this way, the SNP that might be associated with the disease may not be selected as a tagSNP, but this information can be indirectly deduced from its associated tagSNP. The major challenge is to construct algorithms to efficiently capture the genetic variation. When selecting a SNP from the literature, no new information is gained. If the literature is more or less consistent regarding the effect of the SNP on a phenotype, it can function as a quality check of your own data. If there is no clarity, your study contributes to the collective knowledge of the SNP, which can be used to conduct a meta-analysis.

Genotyping

Genotyping was performed using TaqMan® chemistry (Applied Biosystems, Foster City, CA, USA) in Papers I and III, restriction fragment length polymorphism polymerase chain reaction (RFLP-PCR) in Paper III, the Illumina GoldenGate™ assay (Illumina, San Diego, CA, USA) in Papers IV and V, and with KASP On Demand assays (KBioscience, Hertfordshire, UK) in Papers IV and V. Below is a general description of these four genotyping methods.

The TaqMan® principle uses the 5'→3' exonuclease activity of the *Taq* DNA polymerase to cleave a fluorescently labeled probe during PCR amplification.¹⁶⁹⁻¹⁷¹ The set-up of the reaction is very similar to a conventional PCR, but it also includes two oligonucleotide probes with a specific sequence for each allelic variant of the SNP (Figure 7). Each probe is labeled with a fluorescent reporter dye at one end and a quencher dye at the other end. When the probe is intact, the quencher absorbs the fluorescent light emitted from the reporter. The probes are designed to anneal to the genomic region surrounding the SNP, in between the two primers. During DNA amplification, if the probe is perfectly hybridized to the DNA, the activity of the *Taq* polymerase will cleave the probe. This releases the reporter dye and results in an increase in fluorescence, as the quencher is no longer in close proximity of the reporter dye. A mismatch between the probe and the target sequence leads to displacement and a sustained quenching effect on the intact probe. The fluorescence detected at the end of the PCR is directly proportional to the amount of fluorophore released from each probe. This allows for determination of the different homozygote and heterozygote individuals, as visualized in a scatter plot (Figure 7).

Methodological considerations: The advantages of the TaqMan® assays are that the genotyping success rate is usually high, a fluorescent signal is generated only if the probes hybridize specifically to the target sequence (thereby reducing background), the whole assay is performed in a closed tube system and does not require post-PCR handling, the design of probes is fairly flexible, and the assays can be multiplexed using distinguishable reporter dyes. On the other hand, the possibilities for multiplexing are limited, and the assay is only suitable for a smaller number of SNPs as a different probe has to be synthesized for each SNP, which is time consuming and increases the cost per genotype, compared to more high-throughput genotyping methods.

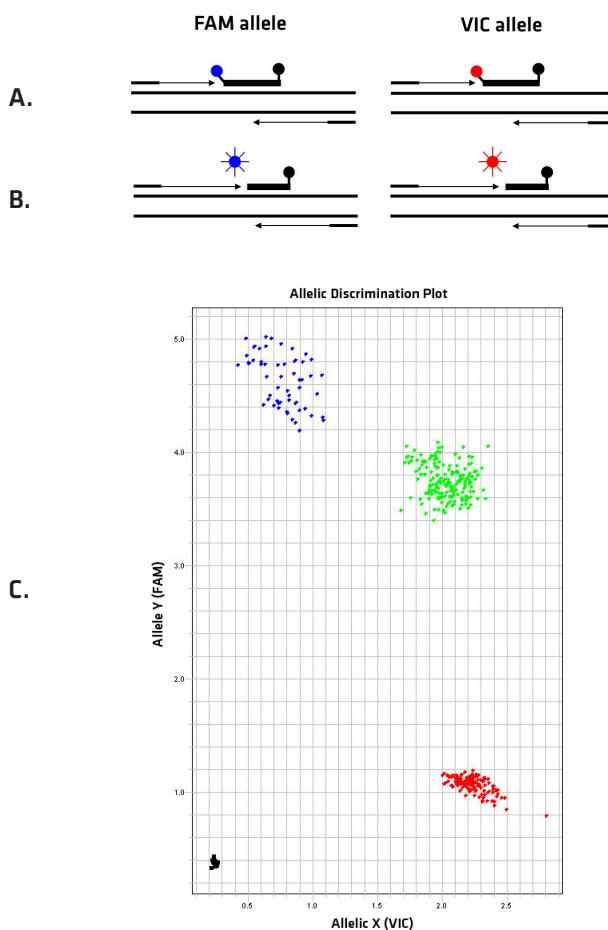


Figure 7. The principle of the TaqMan® assay with a typical scatter plot. **A.** The allele-specific probes and the primers anneal. **B.** During PCR, the probe is cleaved, releasing the fluorescent dye. **C.** The fluorescent signals are visualized in a scatter plot.

RFLP-PCR was used for genotyping of the 15 different *ABO* genotypes that are possible when combining the five most common *ABO* alleles (A^I , A^2 , B , O^I , and O^2). These alleles are a result of a number of polymorphisms residing in exons 6 and 7 of *ABO*. The principle of RFLP-PCR, in which DNA is amplified, digested with restriction endonucleases, and size-fractionated in a gel electrophoresis, is depicted in Figure 8. Essentially, we used the same primers and protocol as described by Olsson *et al.*¹⁷² Two allele-specific primer pairs were used to amplify exons 6 and 7 followed by digestion of the amplified DNA with two restriction enzymes (*Hpa*II and *Kpn*I). The cleaved PCR product was then separated by agarose gel electrophoresis, revealing a unique digestion pattern characteristic for each of the 15 genotypes.

Methodological considerations: RFLP-PCR is a robust method with good reproducibility, and the banding patterns produced are unambiguous due to the large amount of DNA being cut and visualized following electrophoresis and staining. However, this method requires large amounts of DNA, and because it cannot be automated, it is labor-intensive and therefore both time consuming and costly.

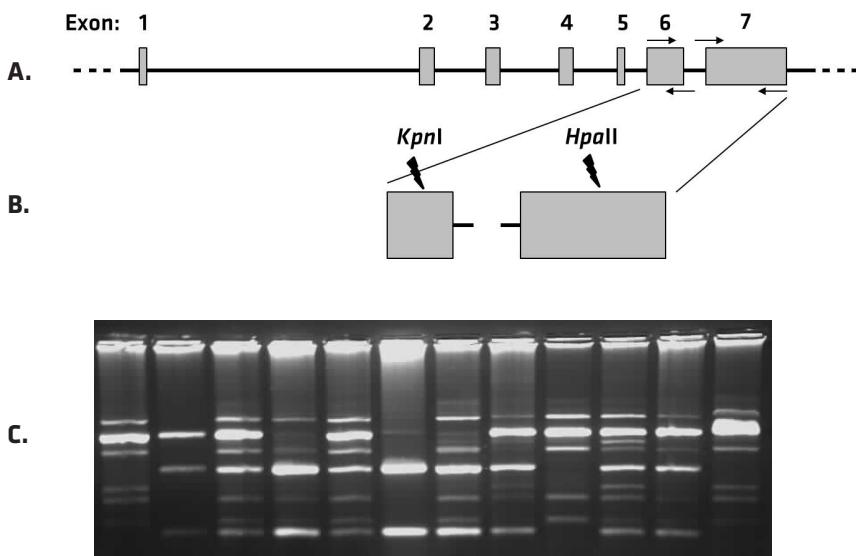


Figure 8. An overview of the RFLP-PCR method used to determine the five most common alleles in *ABO*, with a typical example of a gel electrophoresis photo. **A.** Amplification of *ABO* exons 6 and 7 (including the intermediate intron). **B.** Cleavage of the amplified DNA by restriction enzymes. **C.** The cleaved fragments are size-fractionated in a gel electrophoresis for visualization of the different banding patterns.

For the GoldenGateTM assay,¹⁷³ we selected a set of SNPs in the inflammatory and hemostatic pathways. With this method, allele-specific primers are hybridized directly to genomic DNA that has been attached to a solid support. This attachment allows unbound and non-specifically annealed primers to be removed during washing. In case of a perfect match, the primer is extended and the extension product is ligated to a probe hybridized downstream of the SNP position. The joining of the two fragments to create a PCR template provides an additional genomic specificity. The ligated template is then amplified by PCR using universal primers that are complementary to a universal sequence in the 3'-end of the ligation probes and 5'-ends of the allele-specific primers, respectively. Each SNP is assigned a different Tag-sequence that is contained within the ligation probe, and the universal allele-specific primers carry an allele-specific fluorescent dye in their 5' end. After PCR, the amplified products are captured on beads carrying complementary sequences for the SNP-specific Tag-sequences. The beads are kept in fiber-optic array bundles, the fluorescent signals from the amplified extension-ligation products are read through the fiber bundles, and the results are displayed in a scatter plot. An overview of the GoldenGateTM assay is depicted in Figure 9.

Methodological considerations: The GoldenGateTM assay is a fast method for acquisition of large-scale genotype data with a low cost per data point, the call rate of genotypes is usually high, the selection of SNPs is highly flexible, and the use of universal primers for PCR eliminates sequence-related differences in amplification between SNPs. The disadvantages are that the analysis, on the whole, is expensive, and requires DNA of relatively high concentrations.

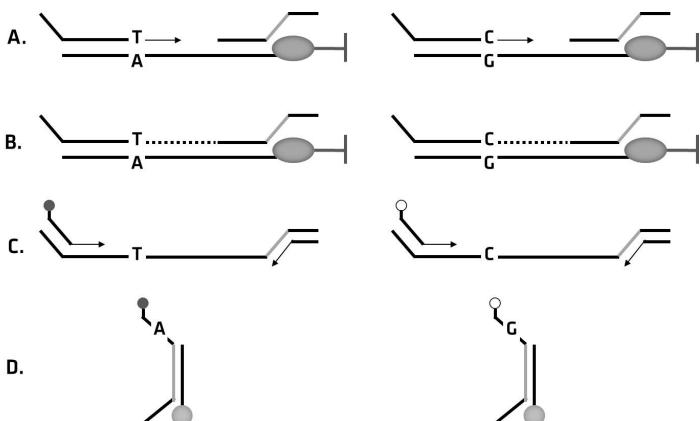


Figure 9. The GoldenGateTM assay principle. **A.** Hybridization of the allele-specific primers and the probe. **B.** Ligation of the extended product to the probe. **C.** The ligated template is amplified by an allele-specific primer with a fluorescent dye. **D.** The amplified products are captured on beads for reading of fluorescent signals.

The KASP genotyping system (www.kbioscience.co.uk) is similar to TaqMan® chemistry, but uses a different strategy for the design and labeling of oligonucleotides (Figure 10). In the initial stage of PCR, two allele-specific primers bind to their complementary sequence upstream of the SNP position so that the 3'-end of the primer is positioned at the SNP nucleotide. A common reverse primer anneals and the PCR proceeds. The reaction mix also includes two oligonucleotides, each labeled with an allele-specific fluorescent dye in the 5'-end, which are hybridized to oligonucleotide sequences with quenchers bound at the 3'-end. Thus, no fluorescent signal is generated during this stage. As the PCR proceeds further, the quenched oligonucleotide cassettes are denatured, and the 5' fluorescent-labeled oligonucleotides are incorporated into the PCR template. The detected fluorescent signals determine the homozygous and heterozygous genotypes, visualized in a scatter plot.

Methodological considerations: The advantages of the KASP assays are that the genotyping success rate is usually high, they are easy to design for most SNPs (high SNP to assay conversion rate), the whole assay is performed in a closed tube system and does not require post-PCR handling, and the assays are cheaper than TaqMan® as they do not need allele-specific fluorescent dual labeled probes. However, this assay does not have any multiplexing capabilities, and the assay is only suitable for a smaller number of SNPs as it is time consuming and has a rather high cost per genotype compared to more high-throughput genotyping methods.

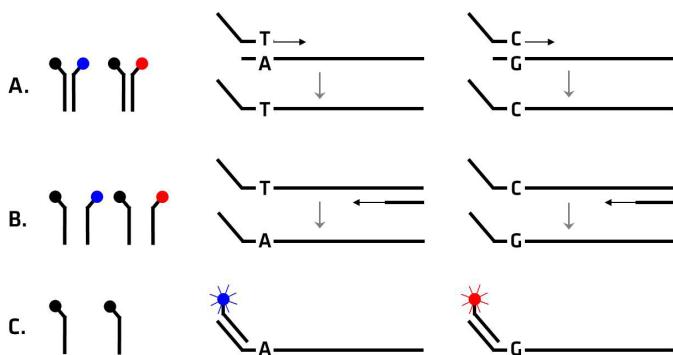


Figure 10. Principle of the KASP genotyping system. **A.** Binding of allele-specific primers. The reaction mix also contains allele-specific oligonucleotides with a fluorescent label. **B.** The common reverse primer anneals. The labeled oligonucleotides are denatured, and then **C.** incorporated into the PCR template.

Haplotype phasing

The genotype data of SNPs at a certain locus provides us with information on whether the individuals are homozygous or heterozygous for the various polymorphisms. These data do not, however, tell us the *haplotype phase*, *i.e.* how the alleles of the different SNPs co-segregate on the two chromosomes. Thus, in the case of multiple heterozygous genotypes, it is not possible to determine the specific haplotypes that each individual carries. There are some molecular techniques to achieve this, but they are both expensive and laborious, especially for large-scale genotyping data.¹⁷⁴ To resolve the haplotype phase, several computational methods have been developed that estimate haplotypes from unphased genotype data. One commonly used algorithm is the expectation-maximization algorithm,¹⁷⁵ which was used for haplotype phasing in the present thesis.

Statistical concepts in genetic association studies

Power and effect measure

In the context of genetics, the effect of a variation on a disease is often quite small. Therefore, it is important to have a large study population to get sufficient *power*, *i.e.* the chance to get a significant finding given a true small effect. Thus, a high power, usually set to above 80%, means that a study has good ability to identify a true association. Tests of significance should always be accompanied by an effect measure, to be able to practically determine the importance of the difference. In all the papers included in this thesis, we have consistently used *odds ratios* (ORs) as measures of effect.

Correction for multiple testing

In genetic association studies, it is usual to include many genetic markers which are also tested in several different statistical analysis models. With the number of statistical analyzes being performed, the risk of false positive results increases. Under these circumstances, it is necessary to correct for multiple testing, which is also becoming increasingly recognized in medical research. There are different approaches to correction and many methods have been developed. Below follows a description of two common methods for correction of multiple testing, which both have been used in this thesis.

Bonferroni correction is a traditional and common method where the p-value is

simply multiplied by the number of tests performed.¹⁷⁶ In terms of genetic data, this method is overly conservative when gene variants are in tight LD, since the tests performed for each genetic variant are not independent of each other.

Permutation is a more suitable method when the tests are not independent, as is the case with many SNPs in tight LD. It works by comparing the observed p-values with the p-values calculated through a large number of repeated random rearrangement of the original data points (for example phenotype and genotype data).¹⁷⁷

Replication

Another way of handling the problem of false positives when performing multiple tests is *replication* of the most significant findings. Replication has become the gold standard in genetic association studies, and is now commonly requested by medical journals for publication of new findings. Replication requires that an association that has been identified in one study population should also be demonstrated in at least one independent population. In this way, one has stronger support for the discrimination between a true and a false association.

Hardy-Weinberg equilibrium (HWE)

In a randomly mating population that is not under any evolutionary pressure, the genotype frequencies are determined by the allele frequencies.¹⁷⁸ A population that is under these conditions is said to be in *Hardy-Weinberg equilibrium* (HWE). The HWE balance can be easily assessed with χ^2 statistics. A deviation from the balance can be caused by non-random mating, but it could also be the result of a genotyping error.¹⁷⁹ In this thesis, HWE was calculated for all genetic markers included, for patients and controls separately. If the genotype frequencies did not conform to HWE in the control group, they were excluded from further analysis.

RESULTS AND DISCUSSION

Characteristics of SAHLSIS

All analyzes of the etiologic subtypes of ischemic stroke in this thesis was confined to LVD, SVD, CE stroke and cryptogenic stroke. The distribution of all etiologic subtypes in SAHLSIS is shown in Table 2.

Table 2. Distribution of the etiologic subtypes of ischemic stroke in SAHLSIS in the first 600 patients (Papers I-IV) and in all patients (Papers IV and V).

	Ischemic stroke n=600	Ischemic stroke n=844
LVD, n (%)	73 (12)	111 (13)
SVD, n (%)	124 (21)	165 (20)
CE stroke, n (%)	98 (16)	151 (18)
Cryptogenic stroke, n (%)	162 (27)	206 (24)
Arterial dissection, n (%)	32 (6)	59 (7)
Other determined cause of stroke, n (%)	19 (3)	33 (4)
Undetermined cause of stroke, n (%)	92 (15)	119 (14)

Of the first 600 patients in SAHLSIS, 114 (19%) had a history of stroke (ischemic or hemorrhagic stroke), and 31 (5%) suffered a recurrent stroke within three months of inclusion. Information regarding medications was obtained at the 3-month follow-up. The number of individuals using statins, anti-hypertensive drugs, and anticoagulant drugs in controls, overall ischemic stroke and the four major etiologic subtypes is displayed in Table 3. In this group of patients, functional outcome was assessed with mRS at three months and at two years after index stroke. At three months, 130 patients were dependent or deceased (mRS score 3-6) and 438 patients had a favorable outcome (mRS score 0-2). The corresponding numbers were 134 and 458 patients at two years after index stroke (missing scores for 32 and 8 patients, respectively, at the two time-points).

Table 3. Distribution of statins, antihypertensive drugs and anticoagulant drugs in SAHLSIS.

	Controls n=600	Ischemic stroke n=600	LVD n=73	SVD n=142	CE stroke n=98	Cryptogenic stroke n=162
Statinst, n (%)	31 (5)	198 (33)	36 (49)	40 (32)	32 (33)	55 (34)
Anti-hypertensive drugst, n (%)	89 (15)	292 (49)	35 (48)	71 (57)	65 (66)	61 (38)
Anticoagulant drugst, n (%)	-	113 (29)	8 (11)	2 (2)	61 (62)	18 (11)

†Information obtained three months after index stroke.

ADAMTS13 gene variation in ischemic stroke (Paper I)

In Paper I, we found three *ADAMTS13* SNPs that showed association with overall ischemic stroke in the univariable conditional logistic regression. One of these was rs4962153, where the minor allele (A) was associated with an increased risk of overall ischemic stroke (Table 4). For rs2285489 and rs2301612, the minor alleles (T and G, respectively) were associated with a decreased risk. After inclusion of hypertension, diabetes mellitus, hyperlipidemia, and smoking as covariates, none of the associations remained although there was a trend for rs4962153 ($p=0.07$).

Table 4. Uni- and multivariable ORs (95% CI) for *ADAMTS13* single SNPs in overall ischemic stroke and the four major etiologic subtypes, compared to controls.

	Ischemic stroke <i>n</i> =600	LVD <i>n</i> =73	SVD <i>n</i> =142	CE stroke <i>n</i> =98	Cryptogenic stroke <i>n</i> =162
rs652600					
OR (95 % CI)	1.0 (0.8-1.2)	1.2 (0.8-1.8)	1.1 (0.8-1.5)	0.9 (0.7-1.4)	1.0 (0.7-1.3)
OR (95 % CI)†	1.0 (0.8-1.2)	1.3 (0.8-2.0)	1.1 (0.8-1.6)	1.0 (0.7-1.5)	0.9 (0.7-1.3)
rs739469					
OR (95 % CI)	1.1 (1.0-1.3)	1.1 (0.8-1.6)	1.1 (0.9-1.5)	1.0 (0.8-1.4)	1.2 (1.0-1.6)
OR (95 % CI)†	1.1 (1.0-1.3)	1.2 (0.8-1.8)	1.2 (0.9-1.6)	1.1 (0.8-1.5)	1.2 (1.0-1.6)
rs2285489					
OR (95 % CI)	0.8 (0.7-1.0)*	0.9 (0.7-1.4)	0.8 (0.6-1.0)	0.9 (0.6-1.2)	0.8 (0.6-1.0)
OR (95 % CI)†	0.9 (0.7-1.1)	1.0 (0.7-1.5)	0.8 (0.6-1.1)	0.9 (0.6-1.2)	0.8 (0.6-1.1)
rs2301612					
OR (95 % CI)	0.9 (0.7-1.0)*	0.8 (0.6-1.2)	0.8 (0.6-1.1)	1.0 (0.7-1.3)	0.8 (0.6-1.1)
OR (95 % CI)†	0.9 (0.7-1.1)	0.8 (0.5-1.2)	0.8 (0.6-1.1)	1.0 (0.7-1.3)	0.8 (0.6-1.1)
rs4962153					
OR (95 % CI)	1.3 (1.0-1.5)*	0.8 (0.5-1.3)	1.3 (0.9-1.8)	1.2 (0.8-1.8)	1.5 (1.1-2.1)*
OR (95 % CI)†	1.3 (1.0-1.6)	0.9 (0.5-1.6)	1.3 (0.9-2.0)	1.2 (0.8-1.9)	1.6 (1.1-2.2)*

An additive model in conditional logistic regression was applied for overall ischemic stroke, and an unconditional binary logistic regression for the etiologic subtypes. †Adjusted for hypertension, diabetes mellitus, hyperlipidemia, and smoking for overall ischemic stroke, as well as age, sex, and geographic area for the etiologic subtypes. * $p<0.05$

In *ADAMTS13*, seven haplotypes with a frequency >1% were found (Table 5). The SNPs were distributed in one haplotype block. One haplotype (H3) was more common in patients, as compared with controls. This association remained after adjustment for vascular risk factors. H3 is the only haplotype containing the minor allele (A) of rs4962153, implying that the association is driven by this SNP or an unknown marker in tight LD with this variant.

Table 5. Estimated haplotype frequencies, and uni- and multivariable ORs (95% CI) for overall ischemic stroke, as compared to controls.

Number	Haplotype	Estimated frequency (%)	Univariable OR (95 % CI)	Multivariable OR (95 % CI)†
H1	TCGAG	35.7	ref	ref
H2	CGCGG	19.8	1.1 (0.9-1.4)	1.1 (0.9-1.4)
H3	CGCAA	15.2	1.3 (1.1-1.7)*	1.4 (1.1-1.8)*
H4	CCCAG	12.9	1.2 (0.9-1.5)	1.1 (0.8-1.5)
H5	CCGAG	9.3	1.1 (0.8-1.4)	1.0 (0.7-1.4)
H6	TGCGG	2.4	0.7 (0.4-1.3)	0.7 (0.4-1.3)
H7	CGCAG	1.9	1.0 (0.6-1.8)	1.0 (0.5-2.0)

The order of the alleles in the haplotypes is in accordance with the relative chromosomal positions of the SNPs, from left to right: rs2285489, rs739469, rs2301612, rs65200, rs4962153.

†Adjusted for age, sex, hypertension, diabetes mellitus, hyperlipidemia, and smoking. *p<0.05.

Regarding the etiologic subtypes of ischemic stroke, the minor allele of rs4962153 showed association with cryptogenic stroke both in the univariable and multivariable regression analysis (Table 4). No association was detected for the other subtypes. As the cryptogenic stroke group includes the largest number of patients of the four etiologic subtypes, these results may simply be due to a limited power in the other subtypes. On the other hand, the fact that the OR was even larger for cryptogenic stroke than for overall ischemic stroke makes this less likely. Our finding for cryptogenic stroke is interesting as prothrombotic genetic variants (FV Leiden and prothrombin G20210A polymorphisms) have been suggested in the pathophysiology of this subtype.¹⁸⁰⁻¹⁸² However, these studies included a small number of patients with cryptogenic stroke ($n=49-94$), so no firm conclusions can be drawn. In addition, there is no data as to whether the minor allele of rs4962153 (or a variant in LD with this SNP) is in fact associated with a prothrombotic phenotype. Therefore, one must be cautious when interpreting these findings, and independent large studies must be carried out both regarding ADAMTS13 variants in relation to ischemic stroke and on possible mechanisms behind the etiology of cryptogenic stroke.

Although some years have passed since this paper was published, there are still very few clinical studies investigating ADAMTS13 in ischemic stroke. In a study by Bongers *et al.*, no association was detected between cerebrovascular disease and ADAMTS13 gene variants.⁸¹ Only 52 patients with ischemic stroke and 57 patients with transient ischemic attack were included in the aforementioned study, and the lead SNP in our study (rs4962153) was not included, nor was any SNP in strong LD with this variant. Apart from our study, rs4962153 has only been investigated in one other study where the minor allele of this SNP was

associated with a decreased risk of cerebral malaria, a serious complication of malaria that is characterized by sequestration of parasitized red blood cells to brain microvascular endothelium.¹⁸³ Not much is known about rs4962153. It is an intronic SNP with no known functionality. Possibly, this SNP could be in LD with a variant that regulates gene expression, or with a non-synonymous coding SNP. There could also be an alternative splice site spanning the location of rs4962153, or the SNP could possibly affect an enhancer/silencer encoded within the intron.

In conclusion, there is very limited knowledge on the role of ADAMTS13 in ischemic stroke. Our results imply that variation at the *ADAMTS13* locus could play a greater role in cryptogenic stroke, but these findings must be verified. As discussed in the introduction of this thesis, this protease appears to have several diverse roles in thrombosis, inflammation, atherosclerosis, and neuroprotection. Altogether, this makes ADAMTS13 an interesting candidate for further studies in ischemic stroke.

VWF plasma levels in the etiologic subtypes of ischemic stroke (Paper II)

In conjunction with our study of *ADAMTS13*, we also investigated plasma levels of VWF. We found that VWF levels were significantly increased in the overall ischemic stroke group, both in the acute phase and at the 3-month follow-up, as compared with controls (Figure 11). In patients, the acute phase VWF levels were significantly higher compared with the follow-up levels. In the regression analysis, significant associations between overall ischemic stroke and VWF were observed for both time-points (Figure 12).

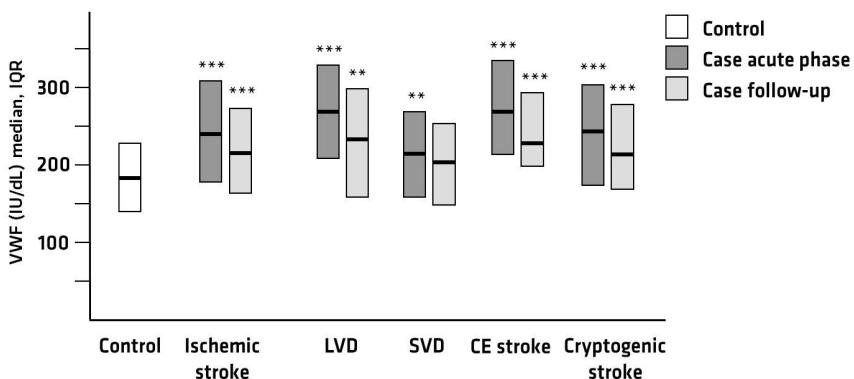


Figure 11. Median and interquartile ranges (IQR) of acute phase and 3-month follow-up VWF plasma levels in controls, overall ischemic stroke and in the four major etiologic subtypes. Cases vs. controls (Student's t-test, or ANCOVA adjusting for age with Bonferroni correction). **p<0.01, ***p<0.001

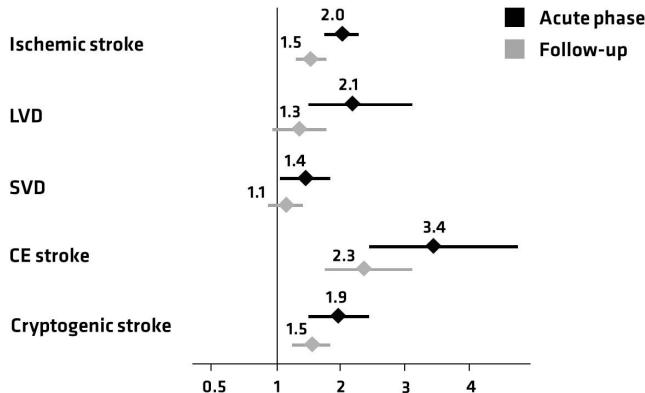


Figure 12. Multivariable ORs (95% CI) for overall ischemic stroke and the four major etiologic subtypes per 1 SD increase in the acute phase and follow-up log VWF levels. Conditional and unconditional logistic regression for overall ischemic stroke and for subtypes, respectively. Adjusted for hypertension, diabetes mellitus, hyperlipidemia, and smoking for overall ischemic stroke, as well as age, sex, and geographic area for subtypes.

It is reasonable to presume that the acute phase VWF levels, at least to some extent, are influenced by the ischemic event in the brain itself. To investigate whether the above associations were independent of markers of the acute phase response, we made further adjustment for levels of high sensitive C-reactive protein (hsCRP) and fibrinogen. In this analysis, the ORs were slightly diminished, and the associations remained significant. However, it is worth noting that both hsCRP and fibrinogen are synthesized by the liver, whereas VWF is synthesized by endothelial cells. Thus, while the acute phase response may be over, one might speculate that there could be a more prolonged influence from the ischemic event in the brain on the endothelium resulting in enhanced VWF synthesis and release. Consequently, adjustment for an acute phase response using hsCRP and fibrinogen might not be ideal. In light of this, we also adjusted for tissue-type plasminogen activator (t-PA) antigen levels, which is a possible marker for endothelial damage. This did not diminish the association between ischemic stroke and VWF to a larger extent than the inflammatory markers.

Increased levels of VWF have been suggested as a predictor of CHD,⁶⁵ and therefore, we investigated whether preexisting clinical vascular disease contributed to the association between overall ischemic stroke and VWF. When patients with a history of stroke, CAD, or PAD ($n=196$) were excluded, the associations remained for both the acute phase and 3-month VWF levels. No significant differences in VWF levels were observed between patients with or without a history of vascular

disease at any of the two time-points. One could also speculate that various drugs may influence the result, but we found no significant differences in VWF levels between patients with or without statins or anti-hypertensive drugs at follow-up. With regard to anticoagulant therapy, patients with this treatment at follow-up had significantly higher VWF levels compared to patients without. The only subtype with a significant proportion of patients on anticoagulant therapy was CE stroke (62%), and this group also displays high VWF levels. Within this subtype, there were no significant differences in VWF levels between patients with or without anticoagulant therapy at follow-up. These results suggest that these medications are not major confounders for the increased VWF levels.

Table 6. Differences in geometric mean VWF levels between the four major etiologic subtypes in the acute phase and at the 3-month follow-up.

		Acute phase ΔVWF (IU/dL)	Follow-up ΔVWF (IU/dL)
CE stroke >	LVD	3.3	11.3
	Cryptogenic stroke	20.5	16.1
	SVD	27.8***	20.4**
LVD >	Cryptogenic stroke	17.1	4.8
	SVD	24.5*	9.1
Cryptogenic stroke >	SVD	7.4	4.2

Differences between subtypes was calculated with ANCOVA adjusting for age and using Bonferroni's correction. *p<0.05, **p<0.01, ***p<0.001

Regarding the etiologic subtypes, the acute phase VWF levels were significantly higher in all subtypes, as compared with controls (Figure 11), and in the logistic regression we found independent associations with all four subtypes (Figure 12). For the convalescent VWF levels, all subtypes except SVD displayed significantly higher levels than the controls, and independent associations were observed for CE stroke and cryptogenic stroke. Further adjustment for hsCRP and fibrinogen levels, and t-PA antigen levels, attenuated the associations between acute phase VWF and LVD and CE stroke, but had almost no effect on the associations for the 3-month levels. The highest VWF levels were observed for LVD and CE stroke, and the lowest levels for SVD. As a consequence, the CE stroke group (and the LVD group in the acute phase) had significantly higher levels as compared to the SVD group (Table 6).

Our finding of increased VWF levels in ischemic stroke is in line with the results obtained in several prospective studies,⁵⁸⁻⁶³ although there are also contradictory results.¹⁸⁴ Regarding the etiologic subtypes, we found subtype-specific differences

in VWF levels, with the highest levels in the LVD and CE stroke groups, and lowest levels in the SVD group. A possible explanation for this could be that the increased VWF plasma levels to a higher degree reflect endothelial damage in many vascular beds, which can be expected in LVD and CE stroke patients. In SVD, there could be more regional endothelial damage that is not reflected in the same way in the total circulation. However, the association between CE stroke and convalescent phase VWF was independent of vascular risk factors, hsCRP and fibrinogen levels, and t-PA antigen levels. The same was true for the association with cryptogenic stroke. This indicates that the prothrombotic effects of VWF may play a role independent of atherosclerosis/inflammation or endothelial damage in these subtypes. In favor of this interpretation, there are data suggesting that VWF is of major importance for arterial thrombosis, rather than the atherosclerotic process itself.¹⁸⁵ In one study, the intima-media thickness of the atherosclerotic plaques in the carotid and femoral arteries of patients with severe VWD, did not differ from those of healthy controls.¹⁸⁶ This could also explain the lack of an independent association between follow-up VWF levels and LVD. For LVD, we have previously shown that hsCRP levels are exclusively associated with this subtype.¹⁸⁷ Collectively, our findings suggest that inflammation/atherosclerosis is of importance for LVD, while prothrombotic mechanisms may be involved in CE stroke and cryptogenic stroke.

We found no influence of statins, anti-hypertensive drugs or anticoagulant drugs on the convalescent VWF plasma levels. Similarly, in studies of patients with atrial fibrillation, no difference in VWF levels was found between those with compared to those without warfarin or aspirin,¹⁸⁸ and initiating treatment with warfarin and/or aspirin did not influence VWF levels after a few weeks of treatment.¹⁸⁹ Intriguingly, a recent study of patients receiving anticoagulant treatment found that high VWF levels were associated with an increased risk of cardiovascular mortality, all-cause mortality, and also with bleeding complications.¹⁹⁰ Given the low VWF levels associated with bleeding in VWD, the mechanism by which high VWF levels increased the risk of bleeding is not clear.

In summary, the plasma levels of VWF in overall ischemic stroke were not majorly influenced by either a history of clinical vascular disease, medications, or levels of markers of inflammation/endothelial damage. We found subtype-specific differences in VWF levels, pointing to the importance of considering the underlying etiology in studies of VWF plasma levels and ischemic stroke.

ABO blood group in ischemic stroke (Paper III)

As discussed in the introduction of this thesis, there is a strong correlation between VWF levels and ABO blood group. Therefore, following Paper II, it was a natural next step to investigate ABO blood group in ischemic stroke.

The observed distribution of the ABO phenotypes (O, A, B and AB) in our study is similar to that reported for the Swedish population (www.geblod.nu/fakta). Since there were few individuals with blood group AB, no comparison between blood groups O and AB was made. When comparing phenotypes non-O vs. O, A vs. O, and B vs. O, there were no significant associations with overall ischemic stroke in the univariable regression analysis, for any of the three comparisons (Table 7). Similar results were obtained after inclusion of vascular risk factors in the model. Moreover, no association with ABO phenotype was detected for any of the four major etiologic subtypes. Consistent with our results, a lack of association between ABO phenotype and overall ischemic stroke has been observed in a few early studies.¹⁹¹⁻¹⁹³ Other studies, including a meta-analysis, have found a small but significant increased risk of stroke for the non-O phenotypes.^{149,194} Although our study is underpowered to detect small effect sizes, differences in the definition of stroke and clinical characteristics of the study populations might account for some of these discrepancies. A more recent study showed an association between phenotype B and ischemic stroke,¹⁹⁵ a finding that could not be replicated in our study. Very recently, the combined results from two large prospective studies

Table 7. ABO phenotype frequencies in controls, overall ischemic stroke and the etiologic subtypes, and multivariable ORs (95% CI) for overall ischemic stroke and the subtypes.

	Control n=600	Ischemic stroke n=600	LVD n=73	SVD n=124	CE stroke n=98	Cryptogenic stroke n=162
Phenotype						
O, n (%)	214 (36)	206 (34)	30 (41)	45 (36)	32 (33)	52 (32)
A, n (%)	269 (45)	284 (47)	30 (41)	60 (48)	51 (52)	69 (43)
B, n (%)	77 (13)	78 (13)	9 (12)	15 (12)	11 (11)	26 (16)
AB, n (%)	40 (7)	32 (5)	4 (6)	4 (3)	4 (4)	15 (9)
OR (95% CI)						
O vs. non-O †	ref	0.9 (0.7-1.2)	1.7 (0.6-4.4)	1.1 (0.5-2.1)	0.7 (0.3-1.4)	0.9 (0.5-1.5)
O vs. A	ref	1.1 (0.8-1.5)	0.6 (0.2-1.6)	1.0 (0.5-2.1)	1.5 (0.7-3.0)	1.0 (0.6-1.9)
O vs. B	ref	1.3 (0.7-1.6)	0.6 (0.1-3.0)	1.2 (0.4-3.9)	1.8 (0.6-6.0)	1.0 (0.5-2.1)

Conditional logistic regression adjusted for hypertension, diabetes mellitus, and smoking was used for overall ischemic stroke, and subtypes. †Non-O includes phenotype A, B and AB.

showed that individuals with phenotype AB had an increased risk of suffering an ischemic stroke, compared to individuals with phenotype O.¹⁹⁶ In women, but not in men, phenotype B was associated with an increased risk of stroke. Altogether, these findings may suggest that phenotype B and/or AB confers an increased risk for ischemic stroke. These blood groups are rather unusual in Caucasians so the lack of association for phenotype B in our study could be due to low statistical power.

In recent GWASes, significant associations between SNPs at the *ABO* locus and CAD, or myocardial infarction in the presence of CAD, were identified.^{197,198} Therefore, we also investigated *ABO* with a tagSNP approach. In line with our results described above, we did not detect a significant association between any of the SNPs and overall ischemic stroke or any of the etiologic subtypes. The SNP rs687621 included in our study was one of the top hits in one GWAS.¹⁹⁷ In that study, the minor allele of this SNP was associated with an increased risk of myocardial infarction in patients with CAD. Mechanistically, the ischemic stroke subtype that is most similar to CAD is LVD, since both have an atherosclerotic plaque as the main pathophysiologic mechanism. Therefore, one can speculate that *ABO* variants, especially rs687621, would be most likely to associate with the ischemic stroke subtype of LVD. In our study, we could not find support for this hypothesis, although the LVD group is small and includes only 73 patients. Thus, small or modest associations with stroke in this subtype cannot be excluded, and larger studies on *ABO* blood group in LVD are thus clearly warranted.

In conclusion, we could not find any association between *ABO* blood group and ischemic stroke. Although there is recent evidence to suggest that the rare blood groups B and AB (in Caucasians) are associated with stroke, based on the current results it seems unlikely that the *ABO* blood group system is a major contributing factor to the pathophysiologic mechanisms of ischemic stroke.

Determinants of VWF plasma levels (Papers I-III)

To connect all data from the above publications, we examined whether a combination of VWF plasma levels and *ADAMTS13* genotype shows association with ischemic stroke. We also investigated whether *ADAMTS13* gene variation and/or *ABO* blood group shows association with VWF plasma levels.

Data on convalescent VWF levels were dichotomized to above or below the median. An increased risk of overall ischemic stroke (multivariable OR of 1.91, 95% CI 1.47-2.84, p<0.001) was observed for individuals with convalescent VWF levels above the median, compared to those with VWF levels below the median.

The VWF data were then combined with genotypes of the *ADAMTS13* lead SNP rs4962153. Having both VWF levels above the median and being a carrier of the minor allele of rs4962153 was associated with an increased risk of ischemic stroke (Figure 13). However, an increased risk of ischemic stroke was also observed for individuals with high VWF levels and the wild-type GG genotype, but with lower OR.

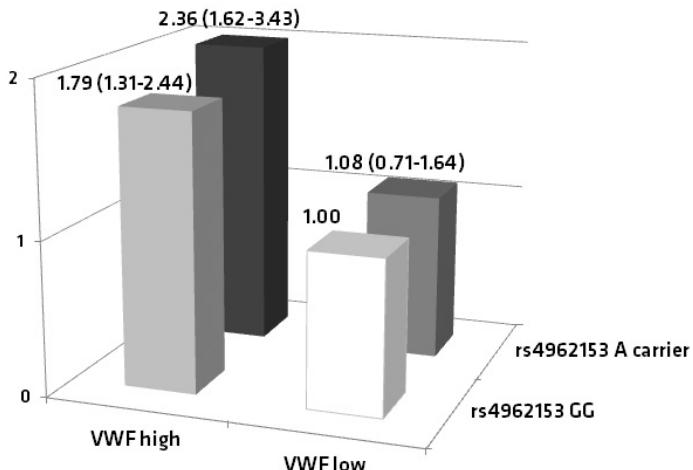


Figure 13. Multivariable ORs (95% CI) for overall ischemic stroke for the combination of the *ADAMTS13* genotype and convalescent VWF levels dichotomized to above or below the median. Number of cases/controls for the different combinations was: 168/260 for VWF low/rs4962153 GG; 55/88 for VWF low/rs4962153 A carrier; 202/165 for VWF high/rs4962153 GG; and 125/79 for VWF high/rs4962153 A carrier. Unconditional logistic regression adjusted for age, sex, hypertension, diabetes mellitus, hyperlipidemia, and smoking.

For the VWF plasma levels, independent associations were observed for both time-points with the etiologic subtype cryptogenic stroke. This result is of course interesting in view of our finding of *ADAMTS13* gene variation with respect to this subtype. It is also of interest because relatively little, in terms of risk factors, is known for this subtype. In the cryptogenic stroke group, carriers of the minor allele A of rs4962153 ($n=94$) have significantly higher VWF levels than those with two copies of the major allele ($n=59$), 240 IU/dL vs. 192 IU/dL, $p<0.001$. No difference in VWF levels between carriers of the two alleles were detected for the other major etiologic subtypes ($p>0.41$). Collectively, our findings suggest that a prothrombotic phenotype, by high VWF levels and the rare allele of rs4962153, is of importance for cryptogenic stroke.

Since we found increased VWF levels in patients with ischemic stroke, and no

association between ABO blood group and ischemic stroke was found, we wanted to investigate whether ABO phenotype showed the expected association with VWF,^{147,148} in SAHLSIS. Indeed, controls with phenotype O had significantly lower VWF levels as compared to individuals with phenotype non-O (Figure 14). This was also observed for phenotype O vs. non-O for patients, both in the acute phase and at the 3-month follow-up. To explore whether ABO blood group would diminish the observed association between VWF levels and ischemic stroke, a logistic regression model with adjustment for age, sex, hypertension, diabetes mellitus, hyperlipidemia, smoking and ABO phenotype was applied. In this analysis, similar ORs were observed as when ABO was not included in the model (ORs of 2.13, 95% CI 1.76-2.59, and 1.53, 95% CI 1.29-1.82, for acute phase and convalescent VWF levels, respectively, $p<0.001$ for both). This implies that our observed association between VWF levels and ischemic stroke is not explained by ABO blood group.

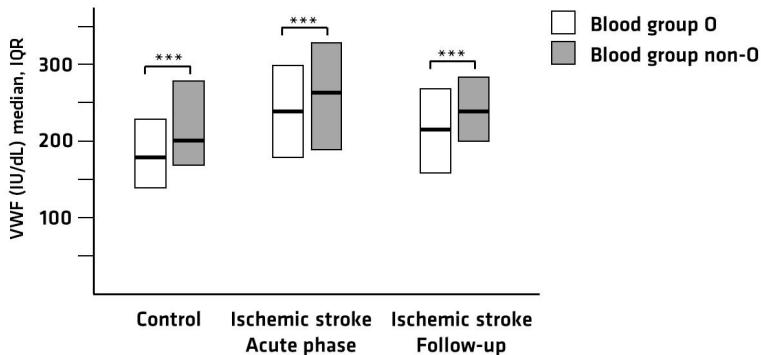


Figure 14. Median and interquartile ranges (IQR) of VWF plasma levels in controls and in overall ischemic stroke in the acute phase and at the 3-month follow-up. Phenotype O vs. non-O (A, B, and AB), (Student's t-test). *** $p<0.001$

FSAP plasma levels and gene variants in ischemic stroke (Paper IV)

In Paper IV, we set out to investigate a novel discovered protein in the hemostatic pathway, FSAP, both at the plasma and genotype levels. We found increased plasma FSAP antigen levels and activity in patients with ischemic stroke as compared to controls, at both time-points (Figure 15). For FSAP activity, the acute phase levels were significantly higher than the follow-up levels, whereas no such difference could be observed for the FSAP antigen levels. Independent associations were

detected for both the acute and convalescent phase FSAP measures with overall ischemic stroke (Figure 16).

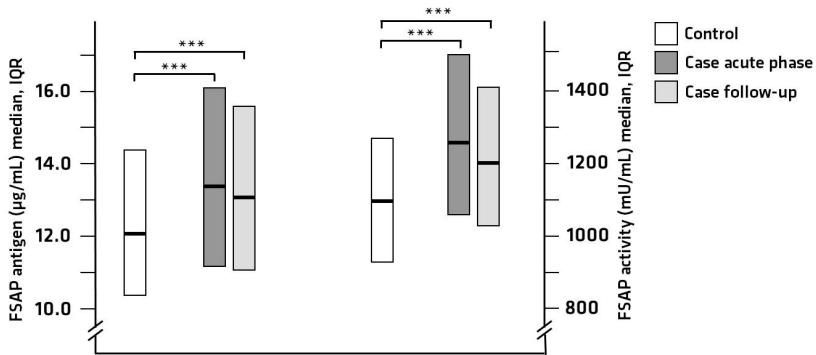


Figure 15. Median and interquartile ranges (IQR) of plasma FSAP antigen levels (left) and activity (right) in the control and overall ischemic stroke groups. Cases vs. controls (Student's t-test). ***p<0.001

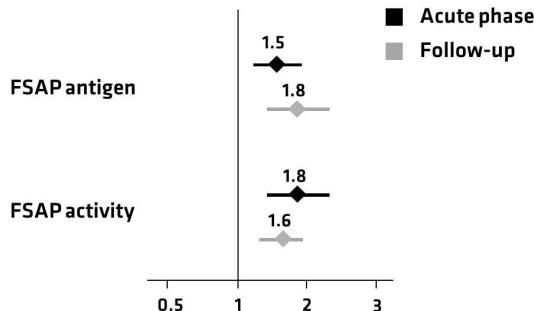


Figure 16. Multivariable ORs (95% CI) for overall ischemic stroke per 1 SD increase in the acute phase and follow-up log FSAP antigen levels and FSAP activity. Conditional logistic regression adjusted for hypertension, diabetes mellitus, hyperlipidemia, smoking, and systolic blood pressure.

When compared to controls, all four major ischemic stroke subtypes showed increased FSAP activity at both time-points. However, the acute phase FSAP antigen levels were only significantly increased in the SVD group, and the convalescent FSAP antigen levels were increased in the LVD, SVD, and CE stroke groups, as compared to controls. No significant differences in FSAP antigen levels or activity were observed between the subtypes at any of the two time-points. Both FSAP antigen and activity showed independent associations with all subtypes at both time-points, except for LVD and CE stroke for acute phase FSAP antigen

levels (Figure 17).

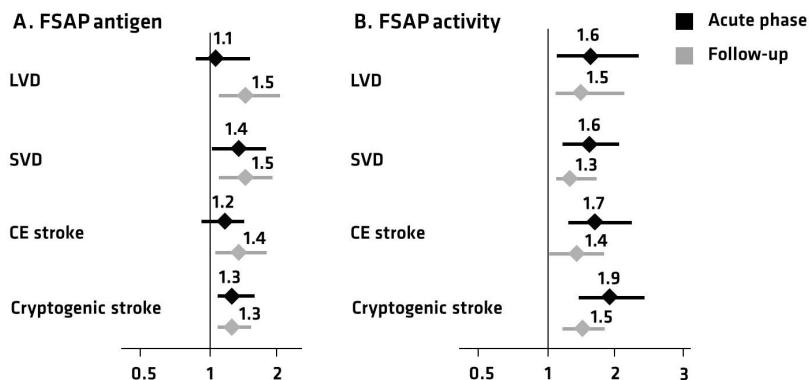


Figure 17. Multivariable ORs (95% CI) for the four major etiologic subtypes per SD increase in the acute phase and follow-up log FSAP antigen levels **A** and FSAP activity **B**. Unconditional logistic regression adjusted for age, sex, geographic area, hypertension, diabetes mellitus, hyperlipidemia, smoking, and systolic blood pressure.

There is very limited knowledge on the genetic and non-genetic determinants of FSAP levels in plasma. Therefore, we explored the possible relations with classic vascular risk factors, other biomarkers, and FSAP gene variants in the controls. Congruent with what has been previously reported,¹⁶⁷ women had higher plasma FSAP antigen levels than men. We found several significant but weak correlations, of which the strongest was with cholesterol for FSAP antigen, and systolic blood pressure for FSAP activity (Pearson correlation coefficients of 0.20, and 0.18 respectively, $p<0.001$ for both). The risk factor explaining most of the variation in FSAP antigen and activity was sex, but in general, traditional vascular risk factors explained a very small part of the variation in plasma FSAP (8.9% and 4.7%, respectively). When excluding controls undergoing treatment for hypertension, diabetes, or hyperlipidemia, classic risk factors (as measured with continuous variables) accounted for slightly more of the variance in FSAP antigen and activity (12.0% and 6.4%, respectively). As expected, the minor allele of the MI-SNP was associated with reduced FSAP activity, but also with reduced FSAP antigen levels, as compared to the wild-type allele. In addition, we also found a few novel associations between SNPs and both FSAP measures. All associated genotypes collectively explained 7.8% and 20.5% of the total variance in FSAP antigen and activity, respectively. Thus, in comparison, the FSAP gene variants actually explained more of the variation in FSAP antigen levels and activity, than the vascular risk factors.

There are some interesting publications on FSAP and estrogen use, which show increased circulating FSAP antigen and activity in individuals on hormonal therapy.^{105,199,200} These studies were either designed to follow a group of women from baseline over a time period of treatment, or samples were drawn at a specific time in their menstrual cycle. In SAHLSIS, there are only 27 women (9 controls and 18 cases) who received oral contraceptives and 112 women (52 controls and 60 cases) who received postmenopausal hormone replacement therapy at the time of inclusion. Unfortunately, information is lacking regarding when the treatment was initiated in relation to their stroke, or on the phase of their menstrual cycle at the time of blood sampling. Hence, we cannot perform a reliable analysis on the influence of sex hormones on FSAP levels in our study.

In patients, the increase in FSAP activity was most pronounced in the acute phase. FSAP in plasma can be activated by factors released from apoptotic or dead cells.⁹⁵ Thus, it is plausible that the increased acute phase FSAP activity is, to some extent, a result of tissue injury after stroke. On the other hand, FSAP in plasma binds rapidly to protease inhibitors, and the resulting complexes are removed by internalization via LRP.¹⁰⁴ It is therefore a fair assumption that the half-life of active FSAP in plasma is very short. However, in the case of chronic FSAP activation the scenario might be different. In this case, active FSAP will be continuously generated, some of which would escape the inhibitory mechanisms. In addition, both the FSAP activity and antigen levels were increased, both in the acute phase and three months after stroke. Therefore, an interpretation of our results could be that the increased plasma FSAP levels, at least in part, preceded the stroke. The mechanism may be through increased synthesis and/or decreased clearance. Thus, one can speculate that FSAP through prothrombotic mechanisms, perhaps by activation of FVII^{94,105} and/or inhibition of TFPI,¹⁰³ could contribute to the pathophysiology of ischemic stroke.

In our study, neither the MI-SNP nor any tagSNP was associated with overall ischemic stroke or any of the etiologic subtypes. Unlike these results, two previous publications have found associations between FSAP SNPs and stroke.^{111,201} In one of these studies, the minor allele of the MI-SNP was associated with an increased risk of stroke (ischemic and hemorrhagic) and mortality.¹¹¹ This is not consistent with our findings of increased FSAP activity in patients with ischemic stroke. In the original publications, summarized in Römisch *et al.*,¹⁰⁴ it was shown that MI-FSAP has lower enzymatic activity towards scuPA, but FVII activation is unchanged in comparison to wild-type FSAP. However, more novel data demonstrate that FVII activation by MI-FSAP is in fact lower than wild-type FSAP, hence, the SNP results in a protein with overall lower enzymatic activity.¹⁰³ Moreover, this study showed that FSAP inactivates TFPI, and that MI-FSAP was also weaker in this respect. If we hypothetically project these new findings into the clinical setting, then we

would expect that MI-FSAP protects from thrombosis. On the other hand, high FSAP activity in patients with ischemic stroke would lead to reduced TFPI activity and a shift towards thrombosis.

For the first time, we could show that plasma FSAP levels are associated with ischemic stroke. We investigated several potential determinants of FSAP in plasma, but a relatively small part of the variation could be explained by these factors. Given the possible involvement of FSAP in prothrombotic mechanisms, this protease could have a role in the pathophysiology of ischemic stroke.

FXI gene variants in ischemic stroke up to 70 years of age (Paper V)

Like FSAP, coagulation FXI is a hemostatic protein that has been less well studied. For variants in the gene encoding this protein, we detected an interesting association in SAHLSIS. We therefore sought to replicate this association in an independent sample.

Table 8. Uni- and multivariable ORs (95% CI) for three *F11* SNPs for overall ischemic stroke in the discovery and replication samples, as compared to controls.

	Discovery sample (SAHLSIS)	Replication sample (LSR, MDC ≤ 70 years)	Replication sample (LSR, MDC)
	Ischemic stroke n=844	Ischemic stroke n=1213	Ischemic stroke n=3145
rs3733403			
OR (95 % CI)	0.76 (0.61-0.95)*	0.88 (0.73-1.06)	0.97 (0.96-1.10)
OR (95 % CI)†	0.74 (0.59-0.94)*	0.86 (0.70-1.06)	0.99 (0.86-1.12)
rs925451			
OR (95 % CI)	1.23 (1.06-1.43)**	1.11 (0.97-1.26)	1.02 (0.94-1.11)
OR (95 % CI)†	1.24 (1.06-1.46)**	1.16 (1.00-1.34)*	1.02 (0.93-1.11)
rs1593			
OR (95 % CI)	0.74 (0.59-0.93)**	1.02 (0.84-1.23)	1.05 (0.93-1.18)
OR (95 % CI)†	0.70 (0.55-0.90)**	1.01 (0.82-1.25)	1.03 (0.91-1.18)

†Adjusted for age, sex, hypertension, diabetes mellitus, and smoking. *p<0.05, **p<0.01

In SAHLSIS, we found independent associations between the minor alleles (G) of rs3733403, (A) of rs925451, and (T) of rs1593 with overall ischemic stroke (Table 8). The former two were associated with a decreased risk, whereas the latter was associated with an increased risk of overall ischemic stroke. For SNPs rs2036914,

rs4253423, rs3822058, and rs4253431, we found no association with overall ischemic stroke. The haplotype analysis was confined to rs3733403, rs925451 and rs1593, and four haplotypes with a frequency >1% were identified. Haplotypes CAA, GGA, and CGT were independently associated with overall ischemic stroke. The strongest association was observed for haplotype CAA (multivariable OR of 1.26, 95% CI 1.08-1.46, p=0.003). The haplotype analysis was congruent with the results for the single SNPs, e.g. haplotype CAA comprises the major alleles (C) of rs3733403 and (A) of rs1593, and minor allele (A) of rs925451, and was associated with an increased risk of IS.

In order to investigate whether we could replicate the above findings in an independent sample of patients with ischemic stroke up to 70 years of age, we genotyped rs3733403, rs1593, and rs925451 in LSR and MDC. We found an independent association for the minor allele of rs925451, whereas no association was detected for rs3733403 or rs1593 (Table 8). When including participants of all ages in the analysis, we observed no significant association for any of the three SNPs, either in the uni- or multivariable analyzes. Similarly, haplotype CAA was independently associated with overall ischemic stroke in the sample up to 70 years of age (multivariable OR of 1.17, 95% CI 1.02-1.36, p=0.03), whereas no association was observed in the replication sample of all ages (multivariable OR of 1.03, 95% CI 0.94-1.13, p=0.56). Haplotype CAA is the only haplotype containing the minor (A) allele of rs925451, so the association is probably driven by this allele. Of note is that the cases were 48-70 years in the MDC sample, which could potentially contribute to the weaker association observed in the replication sample compared to the discovery sample (aged 18-70 years).

In SAHLSIS, we also investigated whether *F11* SNPs show association with the four major etiologic subtypes of ischemic stroke, but no subtype-specific differences were found for any SNP. Furthermore, none of the *F11* SNPs were significantly associated with functional outcome, either at three months or at two years after index stroke.

Our observed associations between rs925451 and haplotype CAA and overall ischemic stroke was restricted to ischemic stroke up to 70 years of age, suggesting that variation within the *F11* locus might contribute to the pathophysiology of ischemic stroke in younger rather than older subjects. This hypothesis is supported by findings of increased FXI plasma levels in young patients with ischemic stroke, compared to controls.¹²⁶⁻¹²⁸ What could then be the reason that FXI seems more important in younger than in older individuals with ischemic stroke? One possibility is that the role of FXI in thrombosis varies depending on the event. In older subjects with ischemic stroke, atherosclerosis is more common than in younger individuals. In atherosclerotic plaques, there are high levels of

TF.²⁰² Thus, when thrombosis occurs after plaque rupture, the contact activation pathway could be of less importance, as the large amount of TF may independently trigger thrombin generation. In younger individuals with ischemic stroke, there might be a general prothrombotic tendency, and this age group usually has less atherosclerotic plaques. So in this case, the contact activation pathway may have a greater impact on thrombosis than the TF-pathway.

Variation at the *F11* locus has not been investigated in ischemic stroke before. A few studies have found associations between *F11* SNPs and venous thrombosis,^{136,137,203-205} one of these variants was rs925451, the lead SNP in our study. This variant is located within the intronic region of *F11*, and is not known to be functional. Interestingly, rs925451 is in tight LD with rs2289252 ($r^2=0.87$ in HapMap), of which the minor allele has been strongly associated with both an increased risk of venous thrombosis and with increased FXI plasma levels.¹³⁷ Hence, it is plausible that the minor allele of rs925451 is also associated with high FXI levels, which could in part explain the association we found with ischemic stroke. On the other hand, the association of rs2289252 with venous thrombosis was independent of FXI levels, implying that other disease-associated mechanisms are also at play. As there is a rather strong heritability for FXI plasma levels,^{134,135} another explanation is that rs2289252 (or a variant in LD) better reflects life-long FXI level exposure than a single plasma measurement. Moreover, rs2036914 has also been strongly associated with venous thrombosis and FXI plasma levels,^{136,137} but for this SNP we could not detect an association with ischemic stroke. In a recent GWAS, the *F11* rs4241824 was one of the top SNPs associated with FXI plasma levels.¹³⁸ This SNP is almost perfectly correlated with rs2036914 ($r^2=0.94$ in HapMap). In the same study, a weak but significant association was found between rs2289252 and FXI plasma levels.

Taken together, the genetic regulation of plasma FXI expression seems to be rather strong, but if this is a relevant mechanism for our observed association between FXI gene variation and ischemic stroke remains to be shown.

Future perspectives

In the present thesis, we investigated the different hemostatic proteins individually, which could give us some information about the prothrombotic state in patients with ischemic stroke. From a clinical point of view, it would be interesting to determine a more global function of prothrombotic mechanisms in these patients. There are a few available coagulation and fibrinolysis screening methods, for example activated partial thromboplastin time, but these methods have shown to

be more efficient for bleeding disorders than for hypercoagulable disorders.²⁰⁶ A more global assay of hemostatic activation is thromboelastography, but a major drawback of this method is the limited time window between sampling and analysis and that freeze-thawed samples cannot be used.²⁰⁷ Other methods examine the fibrin network, which can be visualized microscopically and examined through its morphology, or evaluated through its functional properties using for example the fibrin network permeability technique.²⁰⁸ However, for a large number of patients these methods would be very laborious, so in this situation, there are no really good tests. In the future, it would be desirable to have a feasible global measure of prothrombotic status that could be used for analysis of a large number of freeze-thawed samples.

For the genetic studies in this work we used a candidate gene approach, which is the preferred method for a thorough investigation of the genetic variation in a specific gene or locus in relation to a disease. However, this method requires prior knowledge pointing towards a role for this gene in the disease of interest, in this case ischemic stroke. In contrast, in a GWAS, genetic variations spread over the entire genome are analyzed in a hypothesis-free approach, enabling detection of completely novel genetic loci. Therefore, we will now, in collaboration with several other groups, participate in GWAS on ischemic stroke and ischemic stroke subtypes. In addition to find novel genetic variants associated with ischemic stroke these studies could reveal novel loci that regulate plasma levels of hemostatic proteins. Subsequently, these regions then need to be analyzed in more depth, in studies with denser SNP coverage, to learn more about the potential associations and their role in ischemic stroke.

Another interesting approach for detection of genetic variants associated with a disease is whole genome sequencing, which is a method used to determine the complete DNA sequence of an individual. While a GWAS is designed to detect common disease-associated variants, whole genome sequencing allows detection of rare variants that occur infrequently in the population. One can hypothesize that multiple rare variants contribute to the pathophysiology of a complex disease, and identification of these rare variants may then lead to new insights. Today, whole genome sequencing is very expensive and produces vast amounts of data. Some researchers are instead focusing on sequencing only the protein-coding parts of the genome, the so called exome. Earlier this year, the first exome sequencing pilot study of ischemic stroke was published.²⁰⁹ However, a large proportion of human genetic variation reside in the non-coding parts, so hopefully, whole genome sequencing will be more feasible in the near future.

For a variety of diseases, such as breast cancer and heart diseases, studies have been conducted that combine genetic variants (*i.e.* SNPs) to form a genetic risk

score. As a next step I would like to construct such a score for hemostatic genes of which there is a variant that is clearly associated with a prothrombotic phenotype. This prothrombotic genetic risk score could be an alternative approach to a global assay of prothrombotic status, that seems more feasible in the near future than a protein based score. In the current situation, we realize that there is not sufficient knowledge to build a solid prothrombotic genetic risk score. Hence, we need more biological studies to functionally characterize hemostatic gene variants.

The field of genetics is moving fast forward, with constant development of new techniques and algorithms. In search of variants having only a small effect on a disease, the study samples grow bigger and bigger in order to achieve statistical power. This may lead to large samples with less well-characterized patients and controls. For ischemic stroke, this could mean that classification of etiologic subtypes is not performed. As is exemplified in our group's studies, a good phenotyping is important since we show that some mechanisms are shared between the etiologic subtypes of ischemic stroke, while others are subtype-specific. Hopefully, the demand for larger study populations will not hamper genetic studies in clinically well-defined samples, but rather lead to collaborations to help improve genetic research on ischemic stroke.

CONCLUSIONS

The focus of this thesis is on the role of hemostatic genes and proteins in ischemic stroke and in the etiologic subtypes of ischemic stroke. Our group has previously investigated well-known hemostatic proteins (t-PA, PAI-1, fibrinogen), while I have chosen to examine proteins that have been less well-studied. VWF and ABO blood group have been recognized for a long time, but despite this, data on their role in ischemic stroke is scarce, especially regarding etiologic subtypes. ADAMTS13, FSAP, and FXI are more recently discovered, and knowledge of their role in ischemic stroke is very limited, or virtually non-existent.

We could detect a weak association between variation in the ADAMTS13 gene and overall ischemic stroke, and an independent association with the etiologic subtype cryptogenic stroke. These results suggest that ADAMTS13 may be of importance in the pathophysiology of ischemic stroke, perhaps most notably for cryptogenic stroke (Paper I).

Approximately three months after ischemic stroke, the plasma VWF levels remain increased in patients, as compared to controls. At this time-point, we also observed strong and independent associations between the VWF levels and CE stroke and cryptogenic stroke. There are significant differences in VWF levels between the four major etiologic subtypes of ischemic stroke, which underlines the importance of considering these subtypes in studies of VWF plasma levels in patients with ischemic stroke (Paper II).

We found no association between ABO blood group and overall ischemic stroke or any of the four major etiologic subtypes, either by ABO phenotype or genotype. However, due to the limited sample size in our study, smaller effects, especially of the rare blood groups B and AB in ischemic stroke, cannot be excluded (Paper III).

Plasma FSAP antigen levels and activity are influenced by genetic variation in the FSAP gene, but we could not detect any association between these variants and ischemic stroke. Increased FSAP antigen levels and activity are independently associated with all major etiologic subtypes, indicating a role for FSAP in ischemic stroke irrespective of the underlying etiology. Despite previous findings of FSAP in unstable atherosclerotic plaques, we did not find the highest FSAP levels in LVD. This suggests that FSAP is involved in both arterial wall processes and systemic prothrombotic mechanisms in ischemic stroke pathophysiology (Paper IV).

In two independent samples of patients with ischemic stroke and healthy controls, aged 18-70 years, we found an independent association between FXI gene varia-

tion and overall ischemic stroke. No association was observed when including participants of all ages, indicating that FXI may have a role in ischemic stroke at younger, rather than an older ages (Paper V).

The present work lends support for the hypothesis that prothrombotic mechanisms are involved in the pathophysiology of ischemic stroke. These mechanisms appear to be of importance for all four major etiologic subtypes of ischemic stroke, but are perhaps most interesting for cryptogenic stroke since we know so little about this subtype.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Stroke, eller slaganfall, är en av våra stora folksjukdomar. I Sverige drabbas cirka 30 000 personer varje år av en stroke varav hälften avlidet eller får svåra funktionshinder. Trots detta finns det relativt få studier på stroke jämfört med många andra vanliga sjukdomar. En orsak till detta kan vara att stroke är ett syndrom snarare än en enskild sjukdom och denna heterogenitet försvårar designen av kliniska studier. Stroke är ett samlingsnamn för det tillstånd med plötsliga symptom som uppstår när blodtillförseln till hjärnan hindras och den efterföljande bristen på syre och näringssämnen orsakar skador på hjärnvävnaden. Det finns två huvudtyper av stroke, ischemisk stroke som orsakas av en blodpropp och hemorragisk stroke som orsakas av en blödning. Men även inom dessa grupper är sjukdomsetiologin heterogen. I denna avhandling studeras ischemisk stroke som är den vanligaste formen av stroke.

Ischemisk stroke orsakas både av miljöfaktorer och av ärfliga faktorer. Väl-establerade riskfaktorer utgörs bland annat av högt blodtryck, diabetes och rökning. Kunskapen om vilka ärfliga faktorer som är involverade är dock begränsad. Dessutom varierar betydelsen av ärfliga faktorer med åldern, med ett större inflytande hos yngre som drabbas av stroke. I majoriteten av fallen orsakas ischemisk stroke av en blodpropp som förhindrar blodtillförseln i ett av hjärnans kärl. Således finns det skäl att anta att faktorer som påverkar blodproppsbildning, så kallade hemostasfaktorer, kan påverka risken att insjukna i ischemisk stroke.

Syftet med den aktuella avhandlingen var att undersöka om variation i hemostasgener och/eller plasmanivåer av hemostasproteiner är associerade med ischemisk stroke. Ytterligare ett mål var att undersöka om dessa samband skiljer sig mellan de etiologiska subtyperna av ischemisk stroke. Studierna baseras på en fall-kontroll studie av ischemisk stroke, the Sahlgrenska Academy Study on Ischemic Stroke (SAHLSIS). SAHLSIS omfattar 844 patienter med ischemisk stroke och 668 friska kontroller. Studien fokuserar patienter som insjuknat i relativt unga år, före 70 års ålder, och kontrollerna har samma åldersfördelning. För att möjliggöra analyser av homogena grupper har stor vikt lagts vid att klassificera etiologiska subtyper av ischemisk stroke. De fyra vanligaste subtyperna, som undersökts i denna avhandling, är storkärlssjuka (ischemisk stroke orsakad av ateroskleros i ett större blodkärl i hjärnan), småkärlssjuka (ischemisk stroke orsakad av förträngning av ett mindre blodkärl i hjärnan), kardioembolisk stroke (ischemisk stroke orsakad av en blodpropp som bildats i hjärtat och sedan täppt till ett blodkärl i hjärnan) och kryptogen stroke (någon orsak kan inte identifieras trots extensiv utredning).

I delarbete I och II studerades hemostasfaktorerna von Willebrand faktor (VWF)

och det VWF-klyvande enzymet ADAMTS13, vilka båda är involverade i de initiala stegen i uppkomsten av en blodpropp efter en kärlskada. Vi fann att genetisk variation i ADAMTS13 var associerad med ischemisk stroke och även med den etiologiska subtypen kryptogen stroke. Inget samband mellan ADAMTS13 och någon av de andra etiologiska subtyperna kunde observeras. Dessa resultat tyder på att ADAMTS13 kan vara av betydelse för patofysiologin vid ischemisk stroke och är kanske av särskild vikt för kryptogen stroke. Vad gäller VWF-proteinet, fann vi förhöjda plasmanivåer hos patienter med ischemisk stroke, jämfört med kontroller. VWF-nivåerna var förhöjda i alla de fyra etiologiska subtyperna, med högst nivåer hos patienter med storkärlssjuka och kardioembolisk stroke och lägst nivåer hos patienter med småkärlssjuka. Vi fann också signifikanta skillnader i VWF-nivåer mellan subtyperna, vilket betonar vikten av att ta hänsyn till de etiologiska subtyperna i studier av patienter med ischemisk stroke. ABO-blodgrupp har en stark påverkan på VWF-nivåer och därför studerade vi ABO i relation till ischemisk stroke i delarbete III. Vi kunde inte hitta något samband mellan ABO-blodgrupp och ischemisk stroke eller någon av subtyperna. Dessa resultat talar således för att ABO-blodgrupp inte är av någon större betydelse för risken att insjukna i ischemisk stroke.

Vi fortsatte sedan genom att analysera både plasmanivåer och genvarianter av det nyligen upptäckta hemostasproteinet factor VII-activating protease (FSAP) i delarbete IV. Vi fann att variation i genen kodande för FSAP påverkar plasmanivåerna, men inte är associerad med ischemisk stroke. Däremot var plasmanivåerna av FSAP associerade med ischemisk stroke och med alla de fyra etiologiska subtyperna, vilket indikerar att FSAP är involverad i ischemisk stroke oberoende av den underliggande etiologin. I delarbete V studerades genetisk variation i koagulationsfaktor XI (FXI) som bidrar till att stabilisera den nybildade blodproppen vid en kärlskada. Vi fann en intressant association mellan FXI-genvarianter och ischemisk stroke i SAHLSIS. Vi gick sedan vidare genom att analysera dessa varianter i ett oberoende material av patienter med ischemisk stroke och friska kontroller från Skåne. Vi kunde replikera vårt tidigare fynd av ett samband mellan genetisk variation i FXI och ischemisk stroke upp till 70 års ålder, medan detta samband inte kunde detekteras om även äldre individer inkluderades i analysen av replikationsmaterialet. Detta tyder på att FXI kan vara involverad i ischemisk stroke framförallt hos yngre individer.

Sammanfattningsvis talar våra resultat för att hemostasfaktorer som har betydelse för uppkomsten av en blodpropp är involverade i patofysiologin vid ischemisk stroke. Dessa faktorer verkar ha betydelse för alla fyra vanliga etiologiska subtyper av ischemisk stroke. Våra fynd avseende kryptogen stroke är särskilt intressanta eftersom kunskapen om orsakerna till denna subtyp är så begränsad.

ACKNOWLEDGMENTS

First I would like to thank my supervisor *Christina Jern*, for great mentoring and for always taking time to give me feedback despite your many other engagements. Your devotion and expertise is truly inspirational and creates a pleasant research environment. I always leave our meetings with a lot of new energy and a long list of “to do” things.

Katarina Jood, my co-supervisor, for sharing your comprehensive knowledge on stroke and all the details of SAHLSIS. You always have well thought-out comments, and you have been a great company on travels to conferences.

Staffan Nilsson, my co-supervisor, for our great discussions on statistics, genetics, and other matters, and for all the laughs. You have always had valuable inputs on my work, which has led me to really think things through.

I would also like to thank *Agneta Holmäng*, head of the Institute of Neuroscience and Physiology, and *Lars Rönnbäck* head of the Department of Clinical Neuroscience and Rehabilitation, for creating excellent working conditions and a creative atmosphere at the Institute.

Through these years, I have met and gotten to know many warm friends and colleagues in the SAHLSIS study group. I would especially like to thank; *Christian Blomstrand*, for your enthusiastic work of connecting stroke researchers in Göteborg, and the friendly meetings at Amundövik. *Ingrid Eriksson*, for your dedication and incredible work with the study patients. My colleague and friend, *Sandra Olsson*, for many interesting discussions, company at conferences, and not to mention all the good laughs, both within and outside of work. *Karin Hultman*, for great fun at work and for all your support on my way to my PhD. *Sara Karlsson*, for excellent laboratory work, your never ending curiosity, and for being a true and wonderful friend. I would also like to acknowledge the work of; *Tara Stanne*, for excellent proofreading of my thesis, *Annie Pedersen*, for being a pleasant travel companion, *Petra Redfors*, *Lukas Holmegaard*, *Anna Wolf*, *Martina Olsson*, *Jenny Ödqvist Pettersson*, *Sven-Öjvind Swahn* and all past and present colleagues who have worked in the extended SAHLSIS group for longer or shorter periods of time. Thanks also to the master thesis students who have done a very fine work in our group.

In addition to those who have been directly involved in my work, I am most grateful to my colleagues at the Department of Clinical Genetics, you all contribute to a nice work environment. I would especially like to thank *Åsa Naluai* and *Svetlana*

Adamovic for tutoring me during my master thesis and introducing me to genetic research, which led me to begin my PhD project. *Anna Linder*, for interesting discussions about genetics and because you are a lovely friend, and thank you *Hanna Kryh*, for nice discussions and for sharing the stressful process of thesis writing. A collective thanks to all current and former members at floor 3, for all the fun times at coffee and lunch breaks, and for always being helpful and friendly. Many thanks to the past and present staff at the Genomics Core Facility, in particular *Frida Abel*, *Camilla Stiller*, *Annica Wilzén*, *Catrine Forssén*, and *Elham Rekabdar*, you all took good care of me during my master project and have always met me with a smile when I have had questions.

I would like to thank my co-authors and collaborators *Sandip Kanse*, *Amit Joshi*, *Arne Lindgren*, *Olle Melander*, *Bo Norrvling*, and *Gunnar Engström*, for fruitful discussions and invaluable comments that helped improve our work.

To my fellow members of the PhD committee and the Doctoral Student Council, you all do a fantastic work for PhD students. I am glad to see that through hard work, several goals have been achieved, and I want to thank you for an enjoyable time.

My loving family and friends, I am fortunate to have you in my life. Thank you for reminding me that there are other things in life than work, for lots of laughs and for the happiness you give me. I have really been a bit absent during this intense period, thanks for all your love, patience, and encouragement! To my love *Christoffer*, for giving me the greatest joy in life.

Finally, I would also like to express my deepest gratitude to all the participants of our studies. Without your contribution this thesis would never have been possible.

The work presented in this thesis was supported by the Swedish Research Council, the Swedish Heart-Lung Foundation, grants from the Swedish State (LUA-ALF), the Swedish Stroke Association, the Rune and Ulla Amlöv Foundation, the John and Brit Wennerström Foundation, the Per-Olov Ahl Foundation, the Märtha Lundqvist Foundation, the Emelle Foundation, and the Wilhelm and Martina Lundgren Foundation.

REFERENCES

1. WHO. The global burden of disease: 2004 update. Geneva, World Health Organization, 2008.
2. Pendlebury ST. Worldwide under-funding of stroke research. *Int J Stroke*. 2007;2:80-84.
3. Flossmann E, Schulz UG, Rothwell PM. Systematic review of methods and results of studies of the genetic epidemiology of ischemic stroke. *Stroke*. 2004;35:212-227.
4. Kasiman K, Lundholm C, Sandin S, Malki N, Sparén P, Ingelsson E. Familial Effects on Ischemic Stroke / Clinical Perspective. *Circ Cardiovasc Genet*. 2012;5:226-233.
5. Jeffs B, Clark JS, Anderson NH, Gratton J, Brosnan MJ, Gauguier D, et al. Sensitivity to cerebral ischaemic insult in a rat model of stroke is determined by a single genetic locus. *Nat Genet*. 1997;16:364-367.
6. Lane DA, Grant PJ. Role of hemostatic gene polymorphisms in venous and arterial thrombotic disease. *Blood*. 2000;95:1517-1532.
7. Lee KW, Lip GYH. Effects of Lifestyle on Hemostasis, Fibrinolysis, and Platelet Reactivity: A Systematic Review. *Arch Intern Med*. 2003;163:2368-2392.
8. Franchini M, Mannucci PM. Interactions between genotype and phenotype in bleeding and thrombosis. *Haematologica*. 2008;93:649-652.
9. de Moerloose P, Boehlen F. Inherited thrombophilia in arterial disease: a selective review. *Semin Hematol*. 2007;44:106-113.
10. Adams HP, Jr., Bendixen BH, Kappelle LJ, Biller J, Love BB, Gordon DL, et al. 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.
11. Bamford J, Sandercock P, Dennis M, Burn J, Warlow C. Classification and natural history of clinically identifiable subtypes of cerebral infarction. *Lancet*. 1991;337:1521-1526.
12. Amarenco P, Bogousslavsky J, Caplan LR, Donnan GA, Hennerici MG. Classification of stroke subtypes. *Cerebrovasc Dis*. 2009;27:493-501.
13. Kirshner HS. Differentiating ischemic stroke subtypes: Risk factors and secondary prevention. *J Neurol Sci*. 2009;279:1-8.
14. Rovira A, Grive E, Alvarez-Sabin J. Distribution territories and causative mechanisms of ischemic stroke. *Eur Radiol*. 2005;15:416-426.
15. Kolominsky-Rabas PL, Weber M, Gefeller O, Neundoerfer B, Heuschmann PU. Epidemiology of Ischemic Stroke Subtypes According to TOAST Criteria. *Stroke*. 2001;32:2735-2740.

16. de Jong G, Kessels F, Lodder J. Two Types of Lacunar Infarcts. *Stroke*. 2002;33:2072-2076.
17. Micheli S, Corea F. Lacunar versus non-lacunar syndromes. *Front Neurol Neurosci*. 2012;30:94-98.
18. Bamford J, Sandercock P, Jones L, Warlow C. The natural history of lacunar infarction: The Oxfordshire Community Stroke Project. *Stroke*. 1987;18:545-551.
19. Arboix A, Martí-Vilalta JL. New concepts in lacunar stroke etiology: the constellation of small-vessel arterial disease. *Cerebrovasc Dis*. 2004;17 Suppl 1:58-62.
20. Weir NU. An update on cardioembolic stroke. *Postgrad Med J*. 2008;84:133-142; quiz 139-140.
21. Freeman WD, Aguilar MI. Prevention of cardioembolic stroke. *Neurother*. 2011;8:488-502.
22. Dahabreh IJ, Kent DM. Towards an epidemiology of the known unknowns in cryptogenic stroke. *Heart*. 2012
23. Guercini F, Acciaresi M, Agnelli G, Paciaroni M. Cryptogenic stroke: time to determine aetiology. *J Thromb Haemost*. 2008;6:549-554.
24. Kurth T, Chabriat H, Bousser MG. Migraine and stroke: a complex association with clinical implications. *Lancet Neurol*. 2012;11:92-100.
25. Ballabio E, Bersano A, Bresolin N, Candelise L. Monogenic vessel diseases related to ischemic stroke: a clinical approach. *J Cereb Blood Flow Metab*. 2007;27:1649-1662.
26. Levine SR. Hypercoagulable states and stroke: a selective review. *CNS Spectr*. 2005;10:567-578.
27. Ferro JM, Massaro AR, Mas J-L. Aetiological diagnosis of ischaemic stroke in young adults. *Lancet Neurol*. 2010;9:1085-1096.
28. Goldstein LB, Adams R, Alberts MJ, Appel LJ, Brass LM, Bushnell CD, et al. 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.
29. Rashid P, Leonardi-Bee J, Bath P. Blood pressure reduction and secondary prevention of stroke and other vascular events: a systematic review. *Stroke*. 2003;34:2741-2748.
30. Galimianis A, Mono ML, Arnold M, Nedeltchev K, Mattle HP. Lifestyle and stroke risk: a review. *Curr Opin Neurol*. 2009;22:60-68.
31. Kokubo Y. Traditional risk factor management for stroke: a never-ending challenge for health behaviors of diet and physical activity. *Curr Opin*

- Neurol.* 2012;25:11-17.
32. Hankey GJ. Potential new risk factors for ischemic stroke: what is their potential? *Stroke*. 2006;37:2181-2188.
 33. Wanscher JH. The history of Wilhelm Johannsen's genetical terms and concepts from the period 1903 to 1926. *Centaurus*. 1975;19:125-147.
 34. Watson JD, Crick FH. Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature*. 1953;171:737-738.
 35. Finishing the euchromatic sequence of the human genome. *Nature*. 2004;431:931-945.
 36. Pearson H. Genetics: what is a gene? *Nature*. 2006;441:398-401.
 37. HapMap. The International HapMap Project. *Nature*. 2003;426:789-796.
 38. Cardon LR, Bell JI. Association study designs for complex diseases. *Nat Rev Genet*. 2001;2:91-99.
 39. Furie B, Furie BC. Mechanisms of thrombus formation. *N Engl J Med*. 2008;359:938-949.
 40. Ruggeri ZM. Old concepts and new developments in the study of platelet aggregation. *J Clin Invest*. 2000;105:699-701.
 41. De Candia E. Mechanisms of platelet activation by thrombin: a short history. *Thromb Res*. 2012;129:250-256.
 42. Grabowski EF, Yam K, Gerace M. Evaluation of hemostasis in flowing blood. *Am J Hematol*. 2012;87 Suppl 1:S51-55.
 43. Kannemeier C, Shibamiya A, Nakazawa F, Trusheim H, Ruppert C, Markart P, et al. Extracellular RNA constitutes a natural procoagulant cofactor in blood coagulation. *Proc Natl Acad Sci*. 2007;104:6388-6393.
 44. Kravtsov DV, Matafonov A, Tucker EI, Sun MF, Walsh PN, Gruber A, et al. Factor XI contributes to thrombin generation in the absence of factor XII. *Blood*. 2009;114:452-458.
 45. von Willebrand EA. Hereditär pseudohemofili. *Finska Läkaresällskapets Handlingar*. 1926;68:87-112.
 46. Schneppenheim R. The pathophysiology of von Willebrand disease: therapeutic implications. *Thromb Res*. 2011;128 Suppl 1:S3-7.
 47. Wagner DD. Cell biology of von Willebrand factor. *Annu Rev Cell Biol*. 1990;6:217-246.
 48. Sporn LA, Marder VJ, Wagner DD. Inducible secretion of large, biologically potent von Willebrand factor multimers. *Cell*. 1986;46:185-190.
 49. Bowie EJ, Solberg LA, Jr., Fass DN, Johnson CM, Knutson GJ, Stewart ML, et al. Transplantation of normal bone marrow into a pig with severe von Willebrand's disease. *J Clin Invest*. 1986;78:26-30.
 50. Furlan M, Robles R, Affolter D, Meyer D, Baillod P, Lammle B. Triplet structure of von Willebrand factor reflects proteolytic degradation of high molecular weight multimers. *Proc Natl Acad Sci*. 1993;90:7503-

- 7507.
51. Ruggeri ZM. Von Willebrand factor, platelets and endothelial cell interactions. *J Thromb Haemost*. 2003;1:1335-1342.
 52. Ruggeri ZM. The role of von Willebrand factor in thrombus formation. *Thromb Res*. 2007;120 Suppl 1:S5-9.
 53. Schneider SW, Nuschele S, Wixforth A, Gorzelanny C, Alexander-Katz A, Netz RR, et al. Shear-induced unfolding triggers adhesion of von Willebrand factor fibers. *Proc Natl Acad Sci*. 2007;104:7899-7903.
 54. Whincup PH, Danesh J, Walker M, Lennon L, Thomson A, Appleby P, et al. von Willebrand factor and coronary heart disease: prospective study and meta-analysis. *Eur Heart J*. 2002;23:1764-1770.
 55. Danesh J, Wheeler JG, Hirschfield GM, Eda S, Eiriksdottir G, Rumley A, et al. C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease. *N Engl J Med*. 2004;350:1387-1397.
 56. Morange PE, Simon C, Alessi MC, Luc G, Arveiler D, Ferrieres J, et al. Endothelial cell markers and the risk of coronary heart disease: the Prospective Epidemiological Study of Myocardial Infarction (PRIME) study. *Circulation*. 2004;109:1343-1348.
 57. Wennberg P, Wensley F, Di Angelantonio E, Johansson L, Boman K, Rumley A, et al. Haemostatic and inflammatory markers are independently associated with myocardial infarction in men and women. *Thromb Res*. 2012;129:68-73.
 58. Folsom AR, Rosamond WD, Shahar E, Cooper LS, Aleksic N, Nieto FJ, et al. Prospective study of markers of hemostatic function with risk of ischemic stroke. The Atherosclerosis Risk in Communities (ARIC) Study Investigators. *Circulation*. 1999;100:736-742.
 59. Conway DS, Pearce LA, Chin BS, Hart RG, Lip GY. Prognostic value of plasma von Willebrand factor and soluble P-selectin as indices of endothelial damage and platelet activation in 994 patients with nonvalvular atrial fibrillation. *Circulation*. 2003;107:3141-3145.
 60. Ohira T, Shahar E, Chambliss LE, Rosamond WD, Mosley TH, Jr., Folsom AR. Risk factors for ischemic stroke subtypes: the Atherosclerosis Risk in Communities study. *Stroke*. 2006;37:2493-2498.
 61. Pinto A, Tuttolomondo A, Casuccio A, Di Raimondo D, Di Sciacca R, Arnao V, et al. Immuno-inflammatory predictors of stroke at follow-up in patients with chronic non-valvular atrial fibrillation (NVAF). *Clin Sci*. 2009;116:781-789.
 62. Tzoulaki I, Murray GD, Lee AJ, Rumley A, Lowe GD, Fowkes FG. Relative value of inflammatory, hemostatic, and rheological factors for incident myocardial infarction and stroke: the Edinburgh Artery Study. *Circulation*. 2007;115:2119-2127.

63. Wieberdink RG, van Schie MC, Koudstaal PJ, Hofman A, Witteman JC, de Maat MP, et al. High von Willebrand factor levels increase the risk of stroke: the Rotterdam study. *Stroke*. 2010;41:2151-2156.
64. de Lange M, Snieder H, Ariens RA, Spector TD, Grant PJ. The genetics of haemostasis: a twin study. *Lancet*. 2001;357:101-105.
65. Vischer UM. von Willebrand factor, endothelial dysfunction, and cardiovascular disease. *J Thromb Haemost*. 2006;4:1186-1193.
66. Qin F, Impeduglia T, Schaffer P, Dardik H. Overexpression of von Willebrand factor is an independent risk factor for pathogenesis of intimal hyperplasia: preliminary studies. *J Vasc Surg*. 2003;37:433-439.
67. Trillo AA, Prichard RW. Early endothelial changes in experimental primate atherosclerosis. *Lab Invest*. 1979;41:294-302.
68. van Galen KP, Tuinenburg A, Smeets EM, Schutgens RE. Von Willebrand factor deficiency and atherosclerosis. *Blood Rev*. 2012
69. Furlan M, Robles R, Lamie B. Partial purification and characterization of a protease from human plasma cleaving von Willebrand factor to fragments produced by in vivo proteolysis. *Blood*. 1996;87:4223-4234.
70. Tsai HM. Physiologic cleavage of von Willebrand factor by a plasma protease is dependent on its conformation and requires calcium ion. *Blood*. 1996;87:4235-4244.
71. Levy GG, Nichols WC, Lian EC, Foroud T, McClintick JN, McGee BM, et al. Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. *Nature*. 2001;413:488-494.
72. Koifman A, Brem E, Chiang VW. Thrombotic thrombocytopenic purpura. *Ped Emer Care*. 2011;27:1085-1088; quiz 1089-1090.
73. Zhou W, Inada M, Lee TP, Benten D, Lyubsky S, Bouhassira EE, et al. ADAMTS13 is expressed in hepatic stellate cells. *Lab Invest*. 2005;85:780-788.
74. Turner N, Nolasco L, Tao Z, Dong JF, Moake J. Human endothelial cells synthesize and release ADAMTS-13. *J Thromb Haemost*. 2006;4:1396-1404.
75. Plaimauer B, Zimmermann K, Volkel D, Antoine G, Kerschbaumer R, Jenab P, et al. Cloning, expression, and functional characterization of the von Willebrand factor-cleaving protease (ADAMTS13). *Blood*. 2002;100:3626-3632.
76. Dong JF, Moake JL, Nolasco L, Bernardo A, Arceneaux W, Shrimpton CN, et al. ADAMTS-13 rapidly cleaves newly secreted ultralarge von Willebrand factor multimers on the endothelial surface under flowing conditions. *Blood*. 2002;100:4033-4039.
77. Turner N, Nolasco L, Dong JF, Moake J. ADAMTS-13 cleaves long von Willebrand factor multimeric strings anchored to endothelial cells in the absence of flow, platelets or conformation-altering chemicals. *J Thromb*

- Haemost.* 2009;7:229-232.
78. Sutherland JJ, O'Brien LA, Lillicrap D, Weaver DF. Molecular modeling of the von Willebrand factor A2 Domain and the effects of associated type 2A von Willebrand disease mutations. *J Mol Model.* 2004;10:259-270.
79. Crawley JT, Lane DA, Woodward M, Rumley A, Lowe GD. Evidence that high von Willebrand factor and low ADAMTS-13 levels independently increase the risk of a non-fatal heart attack. *J Thromb Haemost.* 2008;6:583-588.
80. Andersson HM, Siegerink B, Luken BM, Crawley JT, Algra A, Lane DA, et al. High VWF, low ADAMTS13, and oral contraceptives increase the risk of ischemic stroke and myocardial infarction in young women. *Blood.* 2012;119:1555-1560.
81. Bongers TN, de Bruijne EL, Dippel DW, de Jong AJ, Deckers JW, Poldermans D, et al. Lower levels of ADAMTS13 are associated with cardiovascular disease in young patients. *Atherosclerosis.* 2009;207:250-254.
82. Chauhan AK, Kisucka J, Brill A, Walsh MT, Scheiflinger F, Wagner DD. ADAMTS13: a new link between thrombosis and inflammation. *J Exp Med.* 2008;205:2065-2074.
83. Gandhi C, Khan MM, Lentz SR, Chauhan AK. ADAMTS13 reduces vascular inflammation and the development of early atherosclerosis in mice. *Blood.* 2011
84. Reiter RA, Varadi K, Turecek PL, Jilma B, Knobl P. Changes in ADAMTS13 (von-Willebrand-factor-cleaving protease) activity after induced release of von Willebrand factor during acute systemic inflammation. *Thromb Haemost.* 2005;93:554-558.
85. Ono T, Mimuro J, Madoiwa S, Soejima K, Kashiwakura Y, Ishiwata A, et al. Severe secondary deficiency of von Willebrand factor-cleaving protease (ADAMTS13) in patients with sepsis-induced disseminated intravascular coagulation: its correlation with development of renal failure. *Blood.* 2006;107:528-534.
86. Fujioka M, Nakano T, Hayakawa K, Irie K, Akitake Y, Sakamoto Y, et al. ADAMTS13 gene deletion enhances plasma high-mobility group box1 elevation and neuroinflammation in brain ischemia-reperfusion injury. *Neurol Sci.* 2012; [Epub ahead of print].
87. Fujioka M, Hayakawa K, Mishima K, Kunizawa A, Irie K, Higuchi S, et al. ADAMTS13 gene deletion aggravates ischemic brain damage: a possible neuroprotective role of ADAMTS13 by ameliorating postischemic hypoperfusion. *Blood.* 2010;115:1650-1653.
88. Zhao BQ, Chauhan AK, Canault M, Patten IS, Yang JJ, Dockal M, et al. von Willebrand factor-cleaving protease ADAMTS13 reduces ischemic

- brain injury in experimental stroke. *Blood*. 2009;114:3329-3334.
89. Dong JF. Structural and functional correlation of ADAMTS13. *Curr Opin Hematol*. 2007;14:270-276.
90. Kokame K, Matsumoto M, Soejima K, Yagi H, Ishizashi H, Funato M, et al. Mutations and common polymorphisms in ADAMTS13 gene responsible for von Willebrand factor-cleaving protease activity. *Proc Natl Acad Sci*. 2002;99:11902-11907.
91. Bongers TN, De Maat MP, Dippel DW, Uitterlinden AG, Leebeek FW. Absence of Pro475Ser polymorphism in ADAMTS-13 in Caucasians. *J Thromb Haemost*. 2005;3:805.
92. Plaimauer B, Fuhrmann J, Mohr G, Wernhart W, Bruno K, Ferrari S, et al. Modulation of ADAMTS13 secretion and specific activity by a combination of common amino acid polymorphisms and a missense mutation. *Blood*. 2006;107:118-125.
93. Choi-Miura NH, Tobe T, Sumiya J, Nakano Y, Sano Y, Mazda T, et al. Purification and characterization of a novel hyaluronan-binding protein (PHBP) from human plasma: it has three EGF, a kringle and a serine protease domain, similar to hepatocyte growth factor activator. *J Biochem*. 1996;119:1157-1165.
94. Römisch J, Feussner A, Vermöhlen S, Stöhr HA. A protease isolated from human plasma activating Factor VII independent of tissue factor. *Blood Coagul Fibrinolysis*. 1999;10:471-479.
95. Stephan F, Hazelzet JA, Bulder I, Boermeester MA, van Till JO, van der Poll T, et al. Activation of factor VII-activating protease in human inflammation: a sensor for cell death. *Crit Care*. 2011;15:R110.
96. Kannemeier C, Feussner A, Stohr HA, Weisse J, Preissner KT, Romisch J. Factor VII and single-chain plasminogen activator-activating protease: activation and autoactivation of the proenzyme. *Eur J Biochem*. 2001;268:3789-3796.
97. Shibamiya A, Muhl L, Tannert-Otto S, Preissner KT, Kanse SM. Nucleic acids potentiate factor VII-activating protease (FSAP)-mediated cleavage of platelet-derived growth factor-BB and inhibition of vascular smooth muscle cell proliferation. *Biochem J*. 2007;404:45-50.
98. Choi-Miura NH, Yoda M, Saito K, Takahashi K, Tomita M. Identification of the substrates for plasma hyaluronan binding protein. *Biol Pharmaceut Bull*. 2001;24:140-143.
99. Etscheid M, Beer N, Fink E, Seitz R, Johannes D. The hyaluronan-binding serine protease from human plasma cleaves HMW and LMW kininogen and releases bradykinin. *Biol Chem*. 2002;383:1633-1643.
100. Römisch J, Vermöhlen S, Feussner A, Stöhr H. The FVII activating protease cleaves single-chain plasminogen activators. *Haemostasis*. 1999;29:292-299.

101. Kannemeier C, Al-Fakhri N, Preissner KT, Kanse SM. Factor VII-activating protease (FSAP) inhibits growth factor-mediated cell proliferation and migration of vascular smooth muscle cells. *FASEB J.* 2004;18:728-730.
102. Vostrov AA, Quitschke WW. Plasma hyaluronan-binding protein is a serine protease. *J Biol Chem.* 2000;275:22978-22985.
103. Kanse SM, Declerck PJ, Ruf W, Broze G, Etscheid M. Factor VII-Activating Protease Promotes the Proteolysis and Inhibition of Tissue Factor Pathway Inhibitor. *Arterioscler Thromb Vasc Biol.* 2012;32:427-433.
104. Romisch J. Factor VII activating protease (FSAP): a novel protease in hemostasis. *Biol Chem.* 2002;383:1119-1124.
105. Sidelmann JJ, Skouby SO, Kluft C, Winkler U, Vitzthum F, Schwarz H, et al. Plasma factor VII-activating protease is increased by oral contraceptives and induces factor VII activation in-vivo. *Thromb Res.* 2011;128:e67-72.
106. Stavenuiter F, Dienava-Verdoold I, Boon-Spijker MG, Brinkman HJ, Meijer AB, Mertens K. Factor seven activating protease (FSAP): does it activate factor VII? *J Thromb Haemost.* 2012;10:859-866.
107. Sedding D, Daniel JM, Muhl L, Hersemeyer K, Brunsch H, Kemkes-Matthes B, et al. The G534E polymorphism of the gene encoding the factor VII-activating protease is associated with cardiovascular risk due to increased neointima formation. *J Exp Med.* 2006;203:2801-2807.
108. Roemisch J, Feussner A, Nerlich C, Stoehr HA, Weimer T. The frequent Marburg I polymorphism impairs the pro-urokinase activating potency of the factor VII activating protease (FSAP). *Blood Coagul Fibrinolysis.* 2002;13:433-441.
109. Hoppe B, Tolou F, Radtke H, Kiesewetter H, Dörner T, Salama A. Marburg I polymorphism of factor VII-activating protease is associated with idiopathic venous thromboembolism. *Blood.* 2005;105:1549-1551.
110. Ireland H, Miller GJ, Webb KE, Cooper JA, Humphries SE. The factor VII activating protease G511E (Marburg) variant and cardiovascular risk. *Thromb Haemost.* 2004;92:986-992.
111. Trompet S, Pons D, Kanse SM, de Craen AJ, Ikram MA, Verschuren JJ, et al. Factor VII activating protease polymorphism (G534E) is associated with increased risk for stroke and mortality. *Stroke Res Treat.* 2011;2011:424759.
112. van Minkelen R, de Visser MC, Vos HL, Bertina RM, Rosendaal FR. The Marburg I polymorphism of factor VII-activating protease is not associated with venous thrombosis. *Blood.* 2005;105:4898; author reply 4899.
113. Sidelmann JJ, Vitzthum F, Funding E, Munster AM, Gram J, Jespersen

- J. Factor VII-activating protease in patients with acute deep venous thrombosis. *Thromb Res.* 2008;122:848-853.
114. Willeit J, Kiechl S, Weimer T, Mair A, Santer P, Wiedermann CJ, et al. Marburg I polymorphism of factor VII-activating protease: a prominent risk predictor of carotid stenosis. *Circulation.* 2003;107:667-670.
115. Rosenthal RL, Dreskin OH, Rosenthal N. New hemophilia-like disease caused by deficiency of a third plasma thromboplastin factor. *Proc Soc Exper Biol Med.* 1953;82:171-174.
116. Seligsohn U. High gene frequency of factor XI (PTA) deficiency in Ashkenazi Jews. *Blood.* 1978;51:1223-1228.
117. Seligsohn U. Factor XI in haemostasis and thrombosis: past, present and future. *Thromb Haemost.* 2007;98:84-89.
118. Emsley J, McEwan PA, Gailani D. Structure and function of factor XI. *Blood.* 2010;115:2569-2577.
119. von dem Borne PA, Meijers JC, Bouma BN. Feedback activation of factor XI by thrombin in plasma results in additional formation of thrombin that protects fibrin clots from fibrinolysis. *Blood.* 1995;86:3035-3042.
120. von dem Borne PA, Cox LM, Bouma BN. Factor XI enhances fibrin generation and inhibits fibrinolysis in a coagulation model initiated by surface-coated tissue factor. *Blood Coagul Fibrinolysis.* 2006;17:251-257.
121. Von dem Borne PA, Bajzar L, Meijers JC, Nesheim ME, Bouma BN. Thrombin-mediated activation of factor XI results in a thrombin-activatable fibrinolysis inhibitor-dependent inhibition of fibrinolysis. *J Clin Invest.* 1997;99:2323-2327.
122. Wang X, Smith PL, Hsu MY, Gailani D, Schumacher WA, Ogletree ML, et al. Effects of factor XI deficiency on ferric chloride-induced vena cava thrombosis in mice. *J Thromb Haemost.* 2006;4:1982-1988.
123. Rosen ED, Gailani D, Castellino FJ. FXI is essential for thrombus formation following FeCl₃-induced injury of the carotid artery in the mouse. *Thromb Haemost.* 2002;87:774-776.
124. Meijers JC, Tekelenburg WL, Bouma BN, Bertina RM, Rosendaal FR. High levels of coagulation factor XI as a risk factor for venous thrombosis. *N Engl J Med.* 2000;342:696-701.
125. Cushman M, O'Meara ES, Folsom AR, Heckbert SR. Coagulation factors IX through XIII and the risk of future venous thrombosis: the Longitudinal Investigation of Thromboembolism Etiology. *Blood.* 2009;114:2878-2883.
126. Siegerink B, Govers-Riemslag JW, Rosendaal FR, Ten Cate H, Algra A. Intrinsic coagulation activation and the risk of arterial thrombosis in young women: results from the Risk of Arterial Thrombosis in relation to Oral contraceptives (RATIO) case-control study. *Circulation.* 2010;122:1854-1861.

127. Suri MF, Yamagishi K, Aleksic N, Hannan PJ, Folsom AR. Novel hemostatic factor levels and risk of ischemic stroke: the Atherosclerosis Risk in Communities (ARIC) Study. *Cerebrovasc Dis.* 2010;29:497-502.
128. Yang DT, Flanders MM, Kim H, Rodgers GM. Elevated factor XI activity levels are associated with an increased odds ratio for cerebrovascular events. *Am J Clin Pathol.* 2006;126:411-415.
129. Salomon O, Steinberg DM, Zucker M, Varon D, Zivelin A, Seligsohn U. Patients with severe factor XI deficiency have a reduced incidence of deep-vein thrombosis. *Thromb Haemost.* 2011;105:269-273.
130. Salomon O, Steinberg DM, Koren-Morag N, Tanne D, Seligsohn U. Reduced incidence of ischemic stroke in patients with severe factor XI deficiency. *Blood.* 2008;111:4113-4117.
131. Tanis B, Algra A, van der Graaf Y, Helmerhorst F, Rosendaal F. Procoagulant factors and the risk of myocardial infarction in young women. *Eur J Haematol.* 2006;77:67-73.
132. Doggen CJ, Rosendaal FR, Meijers JC. Levels of intrinsic coagulation factors and the risk of myocardial infarction among men: Opposite and synergistic effects of factors XI and XII. *Blood.* 2006;108:4045-4051.
133. Salomon O, Steinberg DM, Dardik R, Rosenberg N, Zivelin A, Tamarin I, et al. Inherited factor XI deficiency confers no protection against acute myocardial infarction. *J Thromb Haemost.* 2003;1:658-661.
134. Souto JC, Almasy L, Borrell M, Blanco-Vaca F, Mateo J, Soria JM, et al. Genetic susceptibility to thrombosis and its relationship to physiological risk factors: the GAIT study. Genetic Analysis of Idiopathic Thrombophilia. *Am J Hum Genet.* 2000;67:1452-1459.
135. Souto JC, Almasy L, Borrell M, Gari M, Martinez E, Mateo J, et al. Genetic determinants of hemostasis phenotypes in Spanish families. *Circulation.* 2000;101:1546-1551.
136. Bezemer ID, Bare LA, Doggen CJ, Arellano AR, Tong C, Rowland CM, et al. Gene variants associated with deep vein thrombosis. *JAMA.* 2008;299:1306-1314.
137. Li Y, Bezemer ID, Rowland CM, Tong CH, Arellano AR, Catanese JJ, et al. Genetic variants associated with deep vein thrombosis: the F11 locus. *J Thromb Haemost.* 2009;7:1802-1808.
138. Sabater-Lleal M, Martinez-Perez A, Buil A, Folkersen L, Souto JC, Bruzelius M, et al. A genome-wide association study identifies KNG1 as a genetic determinant of plasma factor XI level and activated partial thromboplastin time. *Arterioscler Thromb Vasc Biol.* 2012
139. Woodruff RS, Sullenger B, Becker RC. The many faces of the contact pathway and their role in thrombosis. *J Thromb Thrombolysis.* 2011;32:9-20.
140. Schumacher WA, Luettgen JM, Quan ML, Seiffert DA. Inhibition of

- factor XIa as a new approach to anticoagulation. *Arterioscler Thromb Vasc Biol.* 2010;30:388-392.
141. Muller F, Gailani D, Renne T. Factor XI and XII as antithrombotic targets. *Curr Opin Hematol.* 2011;18:349-355.
142. Landsteiner K. Zur Kenntnis der antifermentativen, lytischen und agglutinierenden Wirkungen des Blutserums und der Lymphe. *Zbl Bakt.* 1900;27:357-362.
143. Storry JR, Olsson ML. The ABO blood group system revisited: A review and update. *Immunohematology.* 2009;25:48-59.
144. Crow JF. Felix Bernstein and the first human marker locus. *Genetics.* 1993;133:4-7.
145. Yamamoto F, Clausen H, White T, Marken J, Hakomori S. Molecular genetic basis of the histo-blood group ABO system. *Nature.* 1990;345:229-233.
146. Chester MA, Olsson ML. The ABO blood group gene: A locus of considerable genetic diversity. *Transfus Med Rev.* 2001;15:177-200.
147. Jenkins PV, O'Donnell JS. ABO blood group determines plasma von Willebrand factor levels: a biologic function after all? *Transfusion.* 2006;46:1836-1844.
148. Schleef M, Strobel E, Dick A, Frank J, Schramm W, Spannagl M. Relationship between ABO and Secretor genotype with plasma levels of factor VIII and von Willebrand factor in thrombosis patients and control individuals. *Br J Haematol.* 2005;128:100-107.
149. Wu O, Bayoumi N, Vickers MA, Clark P. ABO(H) blood groups and vascular disease: a systematic review and meta-analysis. *J Thromb Haemost.* 2008;6:62-69.
150. Gallinaro L, Cattini MG, Sztukowska M, Padrini R, Sartorello F, Pontara E, et al. A shorter von Willebrand factor survival in O blood group subjects explains how ABO determinants influence plasma von Willebrand factor. *Blood.* 2008;111:3540-3545.
151. Kiechl S, Pare G, Barbalic M, Qi L, Dupuis J, Dehghan A, et al. Association of Variation at the ABO Locus with Circulating Levels of sICAM-1, sP-selectin and sE-selectin: A Meta-Analysis. *Circ Cardiovasc Genet.* 2011;1:505-513.
152. 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. *Stroke.* 2005;36:1383-1387.
153. Olsson S, Stokowska A, Holmegaard L, Jood K, Blomstrand C, Pekna M, et al. Genetic variation in complement component C3 shows association with ischaemic stroke. *Eur J Neurol.* 2011;18:1272-1274.
154. Scandinavian Stroke Study Group. Multicenter trial of hemodilution in ischemic stroke--background and study protocol. Scandinavian Stroke

- Study Group. *Stroke*. 1985;16:885-890.
155. Jood K. Risk factors in ischemic stroke. *Dissertation, University of Gothenburg*. 2005
156. van Swieten JC, Koudstaal PJ, Visser MC, Schouten HJ, van Gijn J. Interobserver agreement for the assessment of handicap in stroke patients. *Stroke*. 1988;19:604-607.
157. Redfors P, Jood K, Holmegaard L, Rosengren A, Blomstrand C, Jern C. Stroke subtype predicts outcome in young and middle-aged stroke sufferers. *Acta Neurol Scand*. 2012
158. 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.
159. Hallstrom B, Jonsson AC, Nerbrand C, Petersen B, Norrving B, Lindgren A. Lund Stroke Register: hospitalization pattern and yield of different screening methods for first-ever stroke. *Acta Neurol Scand*. 2007;115:49-54.
160. Bergman A, Einbeigi Z, Olofsson U, Taib Z, Wallgren A, Karlsson P, et al. The western Swedish BRCA1 founder mutation 3171ins5; a 3.7 cM conserved haplotype of today is a reminiscence of a 1500-year-old mutation. *Eur J Hum Genet*. 2001;9:787-793.
161. Li C, Engstrom G, Hedblad B, Berglund G, Janzon L. Risk factors for stroke in subjects with normal blood pressure: a prospective cohort study. *Stroke*. 2005;36:234-238.
162. Zia E, Hedblad B, Pessah-Rasmussen H, Berglund G, Janzon L, Engstrom G. Blood pressure in relation to the incidence of cerebral infarction and intracerebral hemorrhage. Hypertensive hemorrhage: debated nomenclature is still relevant. *Stroke*. 2007;38:2681-2685.
163. Eliasson M, Evrin PE, Lundblad D, Asplund K, Rånby M. Influence of gender, age and sampling time on plasma fibrinolytic variables and fibrinogen: A population study. *Fibrinolysis*. 1993;7:316-323.
164. Lippi G, Franchini M, Montagnana M, Salvagno GL, Poli G, Guidi GC. Quality and reliability of routine coagulation testing: can we trust that sample? *Blood Coagul Fibrinolysis*. 2006;17:513-519.
165. Plebani M. Errors in clinical laboratories or errors in laboratory medicine? *Clin Chem Lab Med*. 2006;44:750-759.
166. Livesey JH, Ellis MJ, Evans MJ. Pre-analytical requirements. *Clin Biochem Rev*. 2008;29 Suppl 1:S11-15.
167. Römisch J, Feussner A, Stöhr HA. Quantitation of the factor VII- and single-chain plasminogen activator-activating protease in plasmas of healthy subjects. *Blood Coagul Fibrinolysis*. 2001;12:375-383.
168. Kitchen S, Jennings I, Woods TA, Kitchen DP, Walker ID, Preston FE.

- Laboratory tests for measurement of von Willebrand factor show poor agreement among different centers: results from the United Kingdom National External Quality Assessment Scheme for Blood Coagulation. *Semin Thromb Hemost*. 2006;32:492-498.
169. 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*. 1991;88:7276-7280.
170. Livak KJ, Marmaro J, Todd JA. Towards fully automated genome-wide polymorphism screening. *Nat Genet*. 1995;9:341-342.
171. Lee LG, Connell CR, Bloch W. Allelic discrimination by nick-translation PCR with fluorogenic probes. *Nucl Acids Res*. 1993;21:3761-3766.
172. Olsson ML, Chester MA. A rapid and simple ABO genotype screening method using a novel B/O₂ versus A/O₂ discriminating nucleotide substitution at the ABO locus. *Vox Sang*. 1995;69:242-247.
173. Fan JB, Olliphant A, Shen R, Kermani BG, Garcia F, Gunderson KL, et al. Highly parallel SNP genotyping. *Cold Spring Harb Symp Quant Biol*. 2003;68:69-78.
174. Browning SR, Browning BL. Haplotype phasing: existing methods and new developments. *Nat Rev Genet*. 2011;12:703-714.
175. Excoffier L, Slatkin M. Maximum-likelihood estimation of molecular haplotype frequencies in a diploid population. *Mol Biol Evol*. 1995;12:921-927.
176. Bonferroni CE. Teoria statistica delle classi e calcolo delle probabilità. *Pubblicazioni del R Istituto Superiore di Scienze Economiche e Commerciali di Firenze*. 1936;8:3-62.
177. Rice TK, Schork NJ, Rao DC. Methods for handling multiple testing. *Adv Genet*. 2008;60:293-308.
178. Andrews C. The Hardy-Weinberg Principle. *Nat Education Knowledge*. 2010;1:65.
179. Leal SM. Detection of genotyping errors and pseudo-SNPs via deviations from Hardy-Weinberg equilibrium. *Genet Epidemiol*. 2005;29:204-214.
180. Nabavi DG, Junker R, Wolff E, Ludemann P, Doherty C, Evers S, et al. Prevalence of factor V Leiden mutation in young adults with cerebral ischaemia: a case-control study on 225 patients. *J Neurol*. 1998;245:653-658.
181. Aznar J, Mira Y, Vaya A, Corella D, Ferrando F, Villa P, et al. Factor V Leiden and prothrombin G20210A mutations in young adults with cryptogenic ischemic stroke. *Thromb Haemost*. 2004;91:1031-1034.
182. Belvis R, Santamaria A, Martí-Fabregas J, Cocho D, Borrell M, Fontcuberta J, et al. Diagnostic yield of prothrombotic state studies in cryptogenic stroke. *Acta Neurol Scand*. 2006;114:250-253.

183. Kraisin S, Naka I, Patarapotikul J, Nantakomol D, Nuchnoi P, Hananantachai H, et al. Association of ADAMTS13 polymorphism with cerebral malaria. *Malaria J.* 2011;10:366.
184. Johansson L, Jansson JH, Boman K, Nilsson TK, Stegmayr B, Hallmans G. Prospective study on soluble thrombomodulin and von Willebrand factor and the risk of ischemic and hemorrhagic stroke. *Thromb Haemost.* 2002;87:211-217.
185. Franchini M, Coppola A. Atherothrombosis in von willebrand disease: An analysis of the literature and implications for clinical management. *Semin Thromb Hemost.* 2012;38:185-199.
186. Sramek A, Bucciarelli P, Federici AB, Mannucci PM, De Rosa V, Castaman G, et al. Patients with type 3 severe von Willebrand disease are not protected against atherosclerosis: results from a multicenter study in 47 patients. *Circulation.* 2004;109:740-744.
187. 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.
188. Lip GY, Lowe GD, Rumley A, Dunn FG. Increased markers of thrombogenesis in chronic atrial fibrillation: effects of warfarin treatment. *Br Heart J.* 1995;73:527-533.
189. Li-Saw-Hee FL, Blann AD, Lip GY. Effects of fixed low-dose warfarin, aspirin-warfarin combination therapy, and dose-adjusted warfarin on thrombogenesis in chronic atrial fibrillation. *Stroke.* 2000;31:828-833.
190. Lind M, Boman K, Johansson L, Nilsson TK, Jarvholm LS, Jansson JH. Von Willebrand factor predicts major bleeding and mortality during oral anticoagulant treatment. *J Intern Med.* 2012;271:239-246.
191. Herman B, Schmitz PI, Leyten AC, Van Luijk JH, Frenken CW, Op De Coul AA, et al. Multivariate logistic analysis of risk factors for stroke in Tilburg, The Netherlands. *Am J Epidemiol.* 1983;118:514-525.
192. Ionescu DA, Marcu I, Bicescu E. Cerebral thrombosis, cerebral haemorrhage, and ABO blood-groups. *Lancet.* 1976;1:278-280.
193. Larsen S, Anthonisen B, Marquardsen J. Cerebral infarction, cerebral haemorrhage, and ABO blood-groups. *Lancet.* 1977;1:1064.
194. Clark P, Meiklejohn DJ, O'Sullivan A, Vickers MA, Greaves M. The relationships of ABO, Lewis and Secretor blood groups with cerebral ischaemia of arterial origin. *J Thromb Haemost.* 2005;3:2105-2108.
195. Wiggins KL, Smith NL, Glazer NL, Rosendaal FR, Heckbert SR, Psaty BM, et al. ABO genotype and risk of thrombotic events and hemorrhagic stroke. *J Thromb Haemost.* 2009;7:263-269.
196. Qi L, Rimm E, Manson JE, Hu FB, Rexrode KM. ABO blood group and risk of stroke in US men and women *Abstract.* 2011;AHA meeting Nov 2011.

197. Reilly MP, Li M, He J, Ferguson JF, Stylianou IM, Mehta NN, et al. Identification of ADAMTS7 as a novel locus for coronary atherosclerosis and association of ABO with myocardial infarction in the presence of coronary atherosclerosis: two genome-wide association studies. *Lancet*. 2011;377:383-392.
198. Schunkert H, Konig IR, Kathiresan S, Reilly MP, Assimes TL, Holm H, et al. Large-scale association analysis identifies 13 new susceptibility loci for coronary artery disease. *Nat Genet*. 2011;43:333-338.
199. Parahuleva MS, Hölschermann H, Erdogan A, Langanke E, Prickartz I, Parviz B, et al. Factor seven activating protease (FSAP) levels during normal pregnancy and in women using oral contraceptives. *Thromb Res*. 2010;126:e36-40.
200. Sidelmann JJ, Skouby SO, Vitzthum F, Schwarz H, Jespersen J. Hormone therapy affects plasma measures of factor VII-activating protease in younger postmenopausal women. *Climacteric*. 2010;13:340-346.
201. Zakai NA, Lange L, Longstreth WT, Jr, O'Meara ES, Kelley JL, Fornage M, et al. Association of coagulation-related and inflammation-related genes and factor VIIc levels with stroke: the Cardiovascular Health Study. *J Thromb Haemost*. 2011;9:267-274.
202. Owens AP, 3rd, Mackman N. Sources of tissue factor that contribute to thrombosis after rupture of an atherosclerotic plaque. *Thromb Res*. 2012;129 Suppl 2:S30-33.
203. Austin H, De Staercke C, Lally C, Bezemer ID, Rosendaal FR, Hooper WC. New gene variants associated with venous thrombosis: a replication study in White and Black Americans. *J Thromb Haemost*. 2011;9:489-495.
204. Delluc A, Gourhant L, Lacut K, Mercier B, Audrezet MP, Nowak E, et al. Association of common genetic variations and idiopathic venous thromboembolism. Results from EDITH, a hospital-based case-control study. *Thromb Haemost*. 2010;103:1161-1169.
205. Smith NL, Hindorff LA, Heckbert SR, Lemaitre RN, Marcante KD, Rice K, et al. Association of genetic variations with nonfatal venous thrombosis in postmenopausal women. *JAMA*. 2007;297:489-498.
206. Antovic A. Screening haemostasis--looking for global assays: the Overall Haemostasis Potential (OHP) method--a possible tool for laboratory investigation of global haemostasis in both hypo- and hypercoagulable conditions. *Curr Vasc Pharmacol*. 2008;6:173-185.
207. Hartert H. Thrombelastography, a method for physical analysis of blood coagulation. *Zeitschrift fur die gesamte experimentelle Medizin*. 1951;117:189-203.
208. Blomback B, Okada M. Fibrin gel structure and clotting time. *Thromb Res*. 1982;25:51-70.

209. Cole JW, Stine OC, Liu X, Pratap A, Cheng Y, Tallon LJ, et al. Rare variants in ischemic stroke: an exome pilot study. *PLoS ONE*. 2012;7:e35591.