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HEMOSTATIC DISTURBANCES IN ACUTE ISCHEMIC STROKE

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**Karolinska
Institutet**

Stockholm 2012

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ISBN 978-91-7457-557-6

Printed by



www.reproprint.se

Gårdsvägen 4, 169 70 Solna

*“Nog finns det mål och
mening i vår färd,
men det är vägen, som är
mödan värd”*

*”Yes, there is goal and
meaning in our path –
but it’s the way that is the
labour’s worth”*

(Karin Boye)

To Jens, Axel och Emelie

ABSTRACT

Background: Stroke is the 2nd most common cause of death after ischemic heart disease. About 85% of all strokes are caused by a thrombus or an embolus in the cerebral circulation. Stroke causes major handicap with impaired quality of life for the patients and their families and a large costs to society. Modern treatment of ischemic stroke includes thrombolytic and antithrombotic agents, but despite this treatment many patients do suffer a new ischemic stroke.

Overall aim: To study hemostasis with emphasis on global hemostatic methods and try to identify subgroups of ischemic stroke with a more activated hemostasis, thus at risk of cerebrovascular complications.

Paper I and II: 32 patients were recruited from the Stroke units at Danderyd Hospital and at Karolinska University Hospital. Blood samples was collected in the acute phase and in the convalescence of ischemic stroke. TAFI (an attenuator of fibrinolysis), Overall Hemostatic Potential (OHP; a global marker of hemostasis assessing both coagulation and fibrinolysis) and inflammatory markers were determined in plasma. We found an impaired fibrinolysis with increased levels of TAFI and a decreased fibrinolytic capacity assessed by the OHP-method (**paper I**). Furthermore, the fibrin network formed was found to be less permeable in ischemic stroke patients (n=20) as compared to controls, both in the acute phase and after two months. In addition, the network was more resistant to fibrinolysis (**paper II**) as measured by our global method of fibrinolysis.

Paper III and IV: 209 patients with ischemic stroke (67%) or transient ischemic attack (TIA) (33%) were recruited from the Stroke units at Danderyd Hospital and at the Southern Hospital in Stockholm. Thrombin generation was measured by the Calibrated automated Thrombogram (CAT) and platelet activity was assessed by flowcytometric measurements of platelet-derived microparticles (PMPs) in plasma. Peak thrombin concentrations were found to be elevated both in the acute phase of the event and at one month (**paper III**). In addition, an increase in PMPs was present in the acute phase and at one month. They exposed tissue factor and P-selectin on their surfaces and these molecules may contribute to the activation of hemostasis in acute ischemic stroke (**paper IV**).

Conclusion: Manifest ischemic stroke and TIA are conditions associated with an imbalance between coagulation and fibrinolysis, and elevated plasma levels of platelet-derived microparticles. Global methods of hemostasis may be useful in the evaluation of the hemostatic balance in ischemic stroke and a discrimination between high and low risk patients might be possible with standardized global assays in the future.

Keywords: ischemic stroke, acute phase, fibrinolysis, inflammation, thrombin activatable fibrinolysis inhibitor, fibrin network permeability, fibrinogen, PAI-1, fibrinolysis profile, thrombin generation, endogenous thrombin potential, cardioembolic, transient ischemic attack, paroxysmal atrial fibrillation, microparticles, tissue factor, P-selectin, flowcytometry

LIST OF PUBLICATIONS

- I. Thrombin activatable fibrinolysis inhibitor and its relationship to fibrinolysis and inflammation during the acute and convalescent phase of ischemic stroke.
Rooth E, Wallen NH, Antovic A, von Arbin M, Kaponides G, Wahlgren N, Margareta Blombäck, Antovic JP. Blood Coagulation and Fibrinolysis 2007 Jun 18 (4): 365-70.
- II. Decreased fibrin network permeability and impaired fibrinolysis in the acute and convalescent phase of ischemic stroke.
Rooth E, Wallen NH, Blombäck M, He S. Thrombosis Research 2011 Jan; 127(1): 51-6.
- III. Elevated thrombin generation in acute ischemic stroke and transient ischemic attack.
Rooth E, Sobocinski-Doliwa P, Antovic J, Frykman V, von Arbin M, Rosenqvist M, Wallén NH (Submitted).
- IV. Tissue factor and P-selectin expression on platelet-derived microparticles in patients with acute ischemic stroke or transient ischemic attack.
Rooth E, Mobarrez F, Sobocinski-Doliwa P, Antovic J, Frykman V, von Arbin M, Rosenqvist M, Wallén NH (In manuscript form).

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LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
AF	Atrial fibrillation
APC	Activated protein C
APTT	Activated partial thromboplastin time
APS	Antiphospholipid syndrome
ASA	Acetylsalicylic acid, aspirin
BMI	Body mass index
CAT	Calibrated automated thrombogram
CD62P	P-selectin specific, Cluster of Differentiation
CD142	Tissue factor specific, Cluster of Differentiation
CD41	Glycoprotein IIb specific, Cluster of Differentiation
Cp	Coagulation profile
CRF	Case report form
CT	Computer tomography
DIC	Disseminated intravascular coagulation
ELISA	Enzyme-linked immunosorbent assay
ETP	Endogenous thrombin potential
Fp	Fibrinolysis profile
IS	Ischemic stroke
MP/PMP	Microparticle/platelet-derived microparticle
MRI	Magnetic resonance imaging
NINDS	National Institute of Neurologic Disorders and Stroke
OCP	Overall coagulation potential
OD	Optical density
OHP	Overall hemostatic potential
OFP	Overall fibrinolytic potential
PAI-1	Plasminogen activator-1
PC-INR	Prothrombin complex-international normalized ratio
PFO	Patent foramen ovale
SLE	Systemic lupus erythematosus
TAFI	Thrombin-activatable fibrinolysis inhibitor
TF	Tissue factor
TGA	Thrombin generation assay
TIA	Transient ischemic attack
tPA	Tissue plasminogen activator
VTE	Venous thromboembolism
VWF	Von Willebrand factor

1 INTRODUCTION

Stroke is the 2nd most common cause of death after ischemic heart disease ¹. About 85% of all strokes are caused by thrombosis or thromboembolism of an artery in the brain. This condition causes major handicap with impaired quality of life for the patients and their families and to a large cost for society. With a growing aging population we will be challenged to prevent age-related diseases such as stroke. Modern treatment of acute brain infarction includes thrombolytic as well as antithrombotic treatment in order to dissolve the thrombus and prevent thrombus formation, respectively. However, despite secondary prophylaxis, as many as 30% of the patients will suffer a new ischemic stroke or a transient ischemic attack (TIA) ². The annual risk of recurrence is about 4 %, with the highest risk during the 1st year (around 12%) and further research is needed in the area of biological markers and the possibility of their being targets of interventions in a clinical setting ³.

There are currently few opportunities to identify individuals who will suffer from a new ischemic stroke. It would be convenient to have biomarkers as guidance and inflammatory markers such as C-reactive protein (CRP high sensitive) have been associated with recurrent cardiovascular events ⁴, but measurements of such a marker has not yet been applied in routine stroke practice ³. In general, the stroke patients are treated in the same manner with the same antithrombotic agents with the same dosage regardless of ischemic subtype. One exception is cardioembolic stroke where oral anticoagulation has been shown to be the treatment of choice ^{5 6 7}. However, this type of stroke is commonly due to “silent” atrial fibrillation (AF), and thus identification and diagnosis may be difficult, and correct treatment may not be initiated ⁸.

In about 40% of cases of ischemic strokes the pathogenesis is atherothrombotic ⁹. It is noteworthy that the old view of arterial thrombotic disease and venous thromboembolism (VTE) being two separate entities has been challenged as studies suggest they may share a common pathophysiology ¹⁰. VTE has been associated with an increased risk of cardiovascular disease ¹¹ and vice versa ¹². The possible mechanisms and clinical implications of this phenomenon is yet to be explored but these intriguing findings definitely raises interest in the coagulation system as the target of research in an ischemic stroke population, as these patients share the same risk factors as patients with cardiovascular disease.

As for AF and stroke prevention, the use of risk scores, CHADS₂ ¹³ or the more recent CHA₂DS₂-VASC ¹⁴ to estimate the individual risk of ischemic stroke has reached a wide acceptance in the clinic. Unfortunately, these score-systems are restricted to patients with AF and not directed at the remaining part of patients who may also have a high risk for a recurrent ischemic stroke. As for TIA, a simple score system is available to assess early risk of stroke (ABCD²) ¹⁵ but for long-term risk assessments and for manifest ischemic strokes, better risk-evaluation tools are demanded.

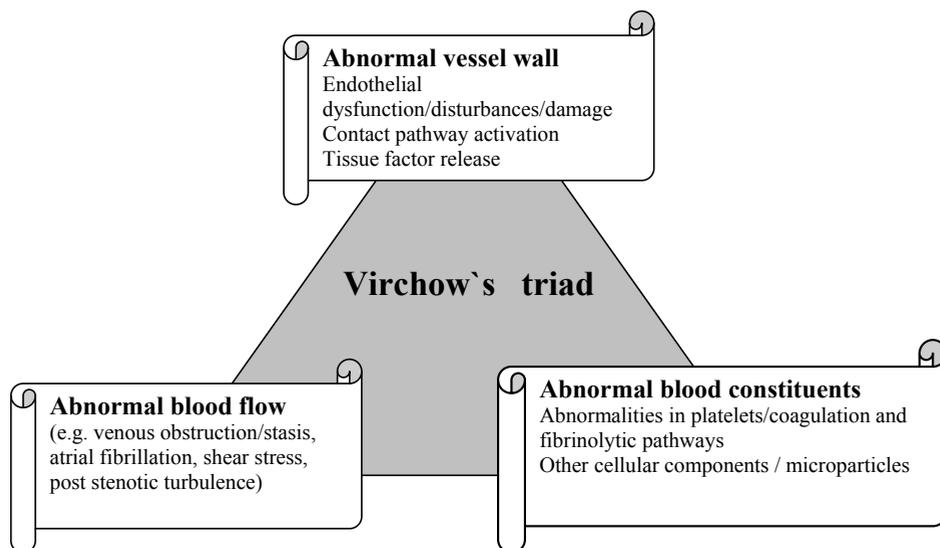
The risk of bleeding complications is the obvious drawback of anticoagulants and antiplatelet treatment. Even though a new user-friendly algorithm has been developed as an aid in the bleeding risk assessment of individual patients as regards to anticoagulation ¹⁶, the bleeding risk of those given antiplatelet agents are assessed mainly on the basis of clinical experience. Hence, limited means of evaluating a patient from different aspects opens up an avenue for new research within the area of hemostasis. One approach to evaluate “high-” and “low-risk” patients may be through laboratory tests with special focus on the hemostatic system ¹⁷. In the younger stroke population several coagulation factors have been associated with the ischemic stroke disease. Thus, when a hereditary or an acquired coagulation disorder is suspected an

investigation is usually performed with a directed assessment of single coagulation factors that could possibly alter the treatment strategy if they are significantly changed¹⁸. However, this particular group of patients is in the minority as the mean age of ischemic stroke patients in the western world is well above seventy years of age. This excludes a large proportion of patients for whom one is “blind” to their individual “hemostatic risk pattern”.

A tendency to treat elderly patients more cautiously with antithrombotic medication¹⁹ is probably justified by the potential risk of bleeding complications. However, this might result in suboptimal medication for a patient with a very high recurrence rate of an ischemic stroke or TIA. Thus, we have a need for tools to allow us to judge whether the patient in front of us is at a high risk of a new event. One approach might be to evaluate patients through more global laboratory tests with special focus on the hemostatic balance²⁰.

The main focus of the present work is on abnormalities of the blood constituents including the secondary hemostasis with fibrin network formation and lysis. Primary hemostasis is investigated by a study on microparticles from activated platelets. Below, a short introduction is given concerning the disturbances responsible for the formation of a thrombus.

Fig 1. Virchow`s triad is applicable both in arterial and venous thrombosis.



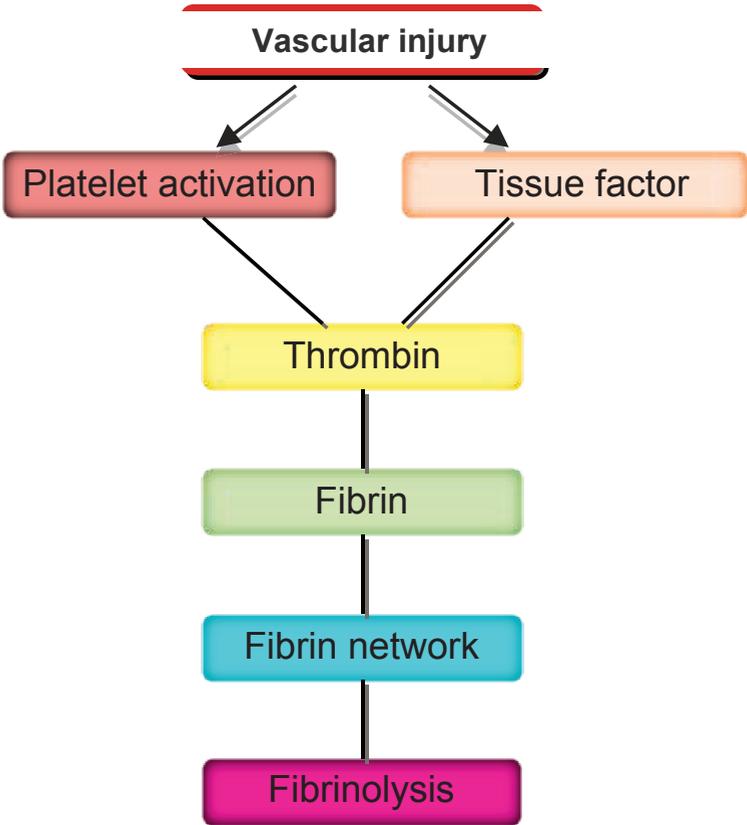
Modified from Favorolo, with permission from the publisher (Thromb Res, 2011, Suppl.2;13-16) .

2 BACKGROUND

2.1 THE HEMOSTATIC SYSTEM

Hemostasis (Greek *hai`ma* = blood, *stasis* = act or condition of stopping) is the physiological process by which the body is prevented from suffering an uncontrolled bleeding at a site of injury of a blood vessel, at the same time as the blood is kept flowing. Mechanisms are also there to prevent coagulation from progressing outside the injured site. When the vessel wall has healed underneath a thrombus, the thrombus will be dissolved by the fibrinolytic system and the vessel will resume its original function and shape. If, for some reason the thrombus is not resolved, it will eventually grow until it fills the whole lumen and blood flow will be obstructed. The area beyond the thrombus is starved of oxygen and will suffer from anoxia and cell death if blood flow is not restored. Thus, the balance between coagulation and fibrinolysis is very important and any condition that alters this balance is a potential threat to the equilibrium of hemostasis. In addition, hemostasis may also be partly involved in angiogenesis²¹, which is of great importance in wound healing and vessel injury. With Virchow’s triad in mind (Fig 1), one of the three problems concerns abnormalities in blood constituents. A simplified flow chart of the hemostatic system is presented below (Fig 2) in which some processes of importance to the present thesis are emphasized.

Fig.2. A schematic flow chart of the hemostatic system.



2.2 THROMBOSIS AND STROKE

2.2.1 History of stroke

Hippocrates was probably the first to acknowledge the stroke disease (400 B.C.) and he observed that persons suffering from apoplexy were mostly “between the ages of forty and sixty”. He noted that there were several blood vessels connected to the brain, and two of them were “stout”. It was common knowledge in Greece at that time that obstruction of these vessels could cause unconsciousness and therefore they were called the carotids, from the word *Karos* = deep sleep.

It was not until the 17th century that the anatomic and clinical nature of the apoplexy was considered to be caused by an obstruction of either the carotids or the vertebrals, by two famous physicians, Wepfer (1620–1695) and Willis (1621–1675). Wepfer described the carotid siphon and the middle cerebral artery in connection with autopsy studies of patients who had died of apoplexy/stroke. Willis published “*Cerebri anatomica*” in which the anastomotic circle of the brain vessels was mapped and he also introduced the transient ischemic attacks. Rudolf Virchow (1821–1902), the famous German pathologist, was the first to describe the phenomenon of embolization, either *in situ* or as an artery-to-artery embolus in stroke and VTE; “*The detachment of larger or smaller fragments from the end of the softening thrombus which are carried along by the current of blood and driven into remote vessels. This gives rise to the very frequent process on which I have bestowed the name of Embolia*”. After the Second World War the neurologist Fisher (born in 1913), observed that transient ischemic attacks (TIAs) frequently preceded a manifest stroke and that TIAs were often caused by obstructions of the carotid arteries. In addition, he also made careful clinical observations and coupled them to pathological findings in the brain and thereby stated the five typical lacunar syndromes (pure motor, pure sensory, sensorimotor, ataxic hemiparesis and dysarthria-clumsy hand syndrome)²².

2.2.2 Coagulation and stroke

The general term for susceptibility to more easily coagulate the blood is called *thrombophilia* and includes both hereditary and acquired forms. There are several hereditary coagulopathies associated with cerebral thrombosis especially in younger stroke patients (< 45 years of age) and in children, although the associations found are consistently weak in the studies performed²³. Good anamnesis often raises the suspicion of a genetic or an acquired coagulation disorder. A variety of coagulation factors can be measured in plasma but it is not economically feasible or even wise to analyze all of them (Table 1).

Table 1. Hereditary and acquired coagulation disorders associated with arterial thrombosis. (c=child, a=adult).

Hereditary	Mixed	Acquired
Protein S deficiency [c+a?] ^{24 25, 26 27}		APS
Protein C deficiency [c] ²⁸⁻³⁰		SLE
Factor V Leiden [c+a?] ^{24 31}	Hyperhomocysteinemia	Hyperhomocysteinemia ³²
Prothrombin G20210A [c+a] ³¹		
Antithrombin deficiency [c+a?] ^{33 34}		

2.2.2.1 Hereditary coagulation disorders

Hereditary coagulation disorders are generally considered to be of greater importance in VTE than in arterial thrombotic conditions such as ischemic stroke. Below a short overview of the factors discussed in the literature is given:

Protein S is a powerful inhibitor of the coagulation system as a cofactor to activated protein C (APC), and protein S deficiency has been reported in small case studies concerning adult ischemic stroke patients^{25 26}, of note, without control groups. However, in a recent prospective study no association between protein S-levels in plasma and stroke in middle-aged men was shown²⁷.

Protein C is a powerful inhibitor of the coagulation system and a deficiency of this factor has been shown to be associated with an increased risk of recurrent ischemic stroke in children in prospective studies²⁸. However, studies in adults have failed to prove the importance of protein C deficiency in cases of ischemic stroke^{29,30}.

Factor V is a cofactor of factor X (Fig 4, part 2.5.1) and a gene mutation which is called Factor V **Leiden** may cause APC-resistance. A weak relationship between the gene mutation and arterial thrombosis was present in a meta-analysis concerning adult stroke³¹, but the heterogenous results in the studies made the conclusion doubtful²³.

Prothrombin is the precursor of thrombin (Fig 4, part 2.5.1) and a specific mutation of **prothrombin** (G20210A) has been associated with a fairly strong stroke risk in prospective studies in adults³¹ with an odds ratio (OR) of 1.44 (1.11–1.86). This provides some support to analyse this mutation in cases of stroke in a young stroke person without any other apparent risk factor(s).

Antithrombin (AT), the major inhibitor of thrombin (formerly called antithrombin III) and a deficiency is described in case reports concerning cerebral venous thrombosis^{35 36}, but case-control studies have failed to reveal any associations between adult ischemic stroke patients and low levels of AT³⁴. In children with acute ischemic stroke, a recent meta-analysis of observational studies has established antithrombin as a contributing factor of thrombophilia³³.

In conclusion, only a minority (approximately 1–4 %) of the younger ischemic stroke and TIA patients have a genetic coagulopathy that predisposes them to thrombosis³⁷. Thus, screening should be directed towards those stroke patients who have unexplained and recurrent thrombotic conditions at an early age and/or a strong family history.

2.2.2.2 Acquired coagulation disorders

More common than the inherited disorders are the acquired coagulation disorders (table 1) where the antiphospholipid syndrome (APS) is an especially intriguing syndrome. This syndrome is characterized by the presence of autoantibodies directed towards phospholipid-protein complexes on cell-membranes. APS includes, in addition to the presence of autoantibodies, at least one episode of arterial thrombosis (stroke or MI) *or* venous thrombosis *or* an obstetric complication. The most common presentation of arterial thrombosis in APS is an ischemic stroke. Activated partial thromboplastin time (APTT) may be prolonged in APS and sometimes a mild to moderate increase of cardiolipin antibodies is found although its clinical relevance is uncertain. Full APS screening involving lupus

antibodies, cardiolipin antibodies and the more specific anti β_2 glycoprotein-1 antibody is therefore recommended, and this testing will identify patients at high risk of thrombosis³⁸. Autoantibodies should be demonstrable on two or more occasions, 12 weeks apart, preferably without anticoagulant treatment. Data are conflicting concerning the risk of stroke in APS. The results of prospective studies have proven an association²³ but the reliability of the data is uncertain because of difficult interpretation of the diagnostic methods³⁹. An overlap between APS and systemic lupus erythematosus (SLE) is present. The rare condition of (non-infectious) Libman-Sacks endocarditis found in SLE, with accumulation of immune complexes on the mitral valve is an uncommon source of cardioembolic stroke⁴⁰. APS and SLE occur more often in women than in men.

Hyperhomocysteinemia Extremely high levels of homocystine in the urine were first described in 1962 to be associated with mental retardation, visual and skeletal problems as well as arterial and venous thrombosis at an early age. The plasma levels of homocystine in the children were more than 100 $\mu\text{mol/l}$ as compared to the milder form ($>15 \mu\text{mol/l}$) that has been associated with a two-fold risk of ischemic stroke in cohort and case-control studies³². The MTHFR (methylene tetrahydrofolat reductase) genotype has been found to be the cause of this disturbance in the folate metabolism²³ and since 2010 the screening test of all newborns in Sweden includes hyperhomocysteinemia. Milder forms can still be diagnosed in adults (www.socialstyrelsen.se/ovanliga_diagnoser/homocystinuri).

The mechanism(s) behind hyperhomocysteinemia and atherosclerosis progression is(are) not fully understood but may be through direct effects on the vascular endothelium, increased platelet adhesiveness and increased oxidation of LDL⁴¹. Also direct effects on coagulation has been shown resulting in adverse effects^{42,43}. Therapeutic efforts involving B-vitamins have failed to reduce the risk of stroke in large randomized trials^{44,45}.

Other acquired coagulation disorders include polycythemia vera, systemic malignancy, myeloproliferative syndromes, thrombotic thrombocytopenic purpura (TTP), estrogen treatment, nephrotic syndrome and sickle cell anemia⁹.

2.2.2.3 Hemostasis and prognosis of ischemic stroke

Beyond acquired thrombophilia *per se*, several hemostatic markers have been identified as prognostic factors of AIS:

Fibrinogen is converted to fibrin by thrombin in the last step of the coagulation cascade (Fig 4, part 2.5.1.), and the fibrin fibrils formed are cross-linked by factor XIII into an insoluble fibrin network. This plasma protein is also involved in platelet aggregation through binding to glycoprotein (GP) IIb/IIIa receptors. Fibrinogen is an important regulator of thrombin activity in clotting blood and, paradoxically, afibrinogenemic patients develop both arterial and venous thrombosis⁴⁶. Fibrinogen concentrations in plasma have most convincingly been shown to be independent riskfactors of ischemic stroke and coronary heart disease⁴⁷ – the higher the fibrinogen levels the greater the risk.

Prothrombin fragment 1+2 (F1+2) is a prothrombotic marker and a 1:1 split product formed when prothrombin is converted to thrombin (Fig 4, part 2.5.1). Levels of F1+2 in plasma were independently associated with poor outcome after 3 months in ischemic stroke patients in the Heparin in Acute Embolic Stroke Trial (OR 1.77)⁴⁸. Also, in a study of 82 TIA patients, F1+2 plasma levels were found to predict a new cerebral or cardiovascular event when followed up for more than two years⁴⁹. Especially in the group of patients with multiple ischemic events ($n=26$), the mean F1+2 levels in plasma were significantly increased.

Furthermore, F1+2 has been shown to be persistently upregulated three months after an ischemic event⁵⁰, and in ischemic stroke patients with large aortic plaques and higher F1+2-levels in plasma, a worse outcome was observed compared to those patients who had large aortic plaques but lower F1+2 levels⁵¹. It was postulated that the increase of this marker of thrombin generation may reflect the atherosclerotic burden of the patient as endothelial damage and/or dysfunction can enhance activation of hemostasis.

The **thrombin-antithrombin complex (TAT)**, is another marker of thrombin generation and high plasma levels have been demonstrated both in cardiovascular disease^{52, 53, 54} and diabetes with complications⁵⁵. In ischemic stroke, an increase in TAT has been shown⁵⁶ but data are somewhat contradictory⁵⁰. Taken together, it seems that elevated thrombin generation is associated with more severe disease and poorer outcome in cases of ischemic stroke.

In a prospective case-control cohort-study, **VWF** and the platelet secretory protein **β -thromboglobulin** were the hemostatic factors significantly predicting long-term mortality after ischemic stroke⁵⁷.

2.3 FIBRINOLYSIS AND STROKE

In addition to hereditary or acquired disturbances of coagulation, an impaired fibrinolysis may also contribute to the pathophysiology of ischemic stroke. As a short introduction, some additional aspects will be presented of thrombus formation and the characteristics of the thrombus:

2.3.1 The properties of the clot / thrombus

Fibrin-rich thrombi with entrapped red cells (*red clots*) are considered to be formed in a low-flow system in contradistinction to platelet-rich (*white*) thrombi where the main constituents are platelets formed preferentially at high shear stress⁵⁸. According to the literature, the emboli in cardioembolic stroke are more commonly fibrin-rich and may be more readily lysed by intravenous thrombolysis with tissue plasminogen activator (tPA), as this fibrin-specific agent can persist within the thrombus for one or more days⁵⁹. Thus, one would expect a cerebral embolus e.g. from the heart is more prone to be lysed, but no such effect was seen in sub-group analyses of the NINDS trial⁶⁰. On the contrary, patients with rapid recovery has presented to a lesser extent with a cardioembolic source of stroke⁶¹.

However, the size and/or length of the thrombus are also important factors as endogenous lytic compounds like tPA and plasminogen or thrombolytic agents may not penetrate the thrombus as easily⁶²⁻⁶⁴. Unfortunately, alternative treatments such as intra-arterial thrombolysis or mechanical thrombectomy by catheter intervention may also be more complicated to perform in such cases⁶⁵.

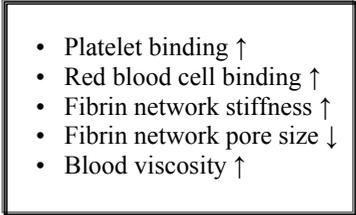
2.3.2 The fibrin network

The fibrin network structure of a clot may influence fibrinolysis. Thinner fibrin fibers are more easily lysed than thicker fibers. However, tighter fibrin networks are usually composed of thinner fibrin fibers packed closely together, resulting in smaller liquid pores between the fibers. This higher fiber density renders the network more difficult to lyse due to the fact that there are more fibrin fibers to be processed and there is restricted permeation of fibrinolytic factors into the tight network⁶⁶. The availability of fibrinogen - the "substrate" of the fibrin network - affects the structure of the fibrin network resulting in a tighter network in the

presence of higher fibrinogen concentrations^{67, 68}. Interestingly, a resistance to thrombolysis has been shown in a mouse model of experimentally induced hyperfibrinogenemia. This suggests causative connections between high fibrinogen levels, fibrin network formation and thrombotic complications⁶⁹.

Potential ways through which elevated plasma levels of fibrinogen could lead to impaired dissolution of thrombi are shown below (Fig 3):

Fig 3. Potential ways through which higher levels of fibrinogen can lead to thrombus formation and impaired dissolution of the thrombus⁶⁷.

- 
- Platelet binding ↑
 - Red blood cell binding ↑
 - Fibrin network stiffness ↑
 - Fibrin network pore size ↓
 - Blood viscosity ↑

A tighter network has been observed in heart failure⁷⁰, coronary heart disease⁷¹, diabetes mellitus⁷², nephrotic syndrome⁷³, end-stage renal disease⁷⁴, rheumatic arthritis⁷⁵ and chronic obstructive pulmonary disease⁷⁶. In ischemic stroke patients, the fibrin network permeability has recently been demonstrated to be tighter in the acute phase and a relation to neurological deficit could be seen⁷⁷. However, larger prospective clinical studies on fibrin network structure and its relationship to future thrombotic complications are still lacking.

2.3.2.1 Fibrinolysis in ischemic stroke disease

Markers of the fibrinolytic system are of great interest in research on stroke, as in all other arterial thrombotic disorders. The most studied factor is **plasminogen activator inhibitor-1** (PAI-1). PAI-1 is the main endogenous inhibitor of tPA. The latter compound is important in fibrinolysis as it converts plasminogen to plasmin which in turn degrades fibrin.

Elevation of plasma PAI-1 concentrations is present in various risk populations such as those with diabetes type 2⁷⁸, obesity⁷⁹, cardiovascular disease⁸⁰ and ischemic stroke⁵⁰. In addition, the 4G/5G polymorphism in the PAI-1-gene has been associated with ischemic stroke⁸¹.

D-dimer, a split product from the fibrin molecule, is usually considered to be a marker of “fibrin turnover” more than a marker of the fibrinolytic process itself. Levels of d-dimer in plasma have been shown to be elevated more than a month after an ischemic stroke⁸² as a marker of an activated hemostasis.

Thrombin activatable fibrinolysis inhibitor (TAFI), is a factor that attenuates fibrinolysis (for further information see Fig.8 part 4.2.1) and it is generated from circulating pro-enzyme of TAFI (pro-TAFI) by thrombin. In a study by Montaner *et al.* (2003) on 30 patients with ischemic stroke, TAFI antigen levels were found to be increased in the acute phase⁸³. Leebeek *et al.* (2005) demonstrated higher levels of functional TAFI in a 1:1 case-control study of more than 100 first-ischemic stroke patients at 7–14 days after the stroke⁸⁴. In prospective studies in cardiovascular disease patients, i.e. patients with similar risk profiles as ischemic stroke patients, high plasma levels of TAFI have been associated with increased cardiovascular mortality⁸⁵. In addition, in the ATTAC study, which involved a group of younger patients with first ever ischemic stroke and coronary heart disease, significantly

higher inactive TAFI levels were reported for patients compared with controls⁸⁶. In summary, TAFI seems to be an interesting factor in the context of arterial thrombotic conditions.

2.3.2.2 Thrombolytic treatment and fibrinolytic markers

Responders to thrombolytic treatment with tPA in acute ischemic stroke have been reported to have higher **d-dimer** peak-levels in plasma than the non-responders⁸⁷. Non-responders, in contrast, have been found to have higher **PAI-1** concentrations in plasma at admission^{88,89}. In a small study of patients undergoing thrombolytic treatment higher TAFI_{max} levels were present in those who did not recanalise or who suffered neurological deterioration⁹⁰ although data are contradictory in this matter^{89,91}. The development of TAFI inhibitors as profibrinolytic agents is promising⁹² and as they are effective in animal thrombosis models⁹³, trials in patients are awaited.

2.3.2.3 Platelets and ischemic stroke

Hemostasis involves platelet activation which includes platelet adhesion, aggregation and secretion of various biologically active factors e.g. ADP, β -thromboglobulin, thromboxane and various coagulation factors which together promotes further platelet activation and thrombin generation. The platelets are considered to be mainly involved in arterial thrombosis especially under high shear stress. Platelets interacts and cross-talks with many other cells e.g. leukocytes and endothelial cells. The significance of platelets in ischemic stroke disease is undebated and antiplatelet agents are widely used as secondary prophylactic treatment, although their relative risk reduction is modest (only about 13%)².

2.4 SUBTYPES OF ISCHEMIC STROKE

Ischemic stroke is a heterogeneous disease with various manifestations and plausible causes. The classifications available today take into account both the area of brain tissue damage and results of clinical investigations.

2.4.1 In research

2.4.1.1 TOAST/CCS

The most widely used type of classification in research of stroke is the TOAST classification (Trial of Org. 10172 in Acute Stroke Treatment) system, originally created in a study of low-molecular-weight-heparin in acute ischemic stroke⁹⁴. The original study failed to show a favorable outcome⁹⁵, but the subtyping of stroke etiology was a useful contribution to the scientific community and the classification has since then been widely used in clinical studies in ischemic stroke.

The subtypes included in the TOAST classification are *large vessel disease*, *small vessel disease*, *cardioembolic stroke* and *other determined or undetermined/mixed cause*.

The major weakness of the TOAST classification is the fairly large proportion of patients classified as undetermined or mixed stroke etiology (commonly around 30 - 40%) even after extensive investigations. Yet another problem, though not specific to the TOAST classification, is the difficulty of detecting “silent atrial fibrillation”. This probably leads to underestimation of the prevalence of the cardioembolic stroke subtype. A further development into a computerized algorithm evaluation system, the TOAST-Causative Classification System (CCS)⁹⁶, has been performed to facilitate the classification procedure e.g. in large multicenter trials (CCS available at <http://ccs.martinos.org>).

Agreement between TOAST and CCS ranges from good to excellent⁹⁷ but investigator bias is present in all types classifications, reliability being higher as regards CCS than in TOAST⁹⁸.

2.4.1.2 Bamford classification

Bamford classification (also called the Oxford Community Stroke Project [OCSP] classification) of ischemic stroke is based on the patient's neurological presentation assessed before any of the investigations into etiology have been performed eg. duplex imaging of the carotids or ECG. The syndromes are dependent on the affected ischemic area of the brain and are divided into *Total Anterior Circulation Infarcts (TACI)*, *Partial Anterior Circulation Infarcts (PACI)*, *Posterior Circulation Infarcts (POCI)* and *Lacunar Circulation Infarcts (LACI)*⁹⁹ (see Table 2 below):

Table 2. Bamford classification.

Area affected	<i>Total anterior circulation (TACI)</i>	<i>Partial anterior circulation (PACI)</i>	<i>Posterior circulation (POCI)</i>	<i>Lacunar circulation (LACI)</i>
Signs	All of: motor or sensory; higher cortical dysfunction eg. aphasia, neglect; hemianopia	2 of the following: motor or sensory deficit; higher cortical dysfunction; hemianopia	Isolated hemianopia; brain stem signs; cerebellar ataxia	Motor or sensory deficit only

This classification is excellent as regards to epidemiological research and research into pathophysiology, but in a clinical setting it is of limited use, as the treatment of ischemic stroke and TIA is the same (as yet) regardless of the area affected. It is sometimes hard to discriminate between small vessel disease in the posterior circulation and a “pure” posterior circulation syndrome. Occasionally, a patient's symptoms do not allow clear classification, e.g. “tendency to fall to one side”, but that is often due to poor anamnesis and/or inadequate neurological examination. Investigator bias is high, and thus a reliability problem is present. Correlation with CT or MRI findings is poorly validated, although the correlation seems to be best in cases of anterior circulation syndrome and non-lacunar stroke¹⁰⁰.

2.4.1.3 A-S-C-O

The latest contribution to ischemic stroke etiological subtyping is the A-S-C-O-classification system published in 2009¹⁰¹. This classification better takes into consideration the different levels of evidence (grades 1-3, 1 stands for high evidence) regarding A=atherosclerosis, S=small vessel, C=cardiac source and O=other causes of ischemic stroke (eg. A2S0C1O0). It may be good in very large epidemiological or in genetic studies, but due to the large number of possible categories this system is not suitable for studies with relatively small sample sizes.

2.4.2 In clinical practice

In clinical evaluation of a specific stroke patient it is most important to discriminate between cardioembolic and non-cardioembolic stroke, because efficient treatment with oral anticoagulants is available if an atrial fibrillation or an other high risk cardioembolic factor is found. Otherwise, an antiplatelet agent is considered sufficient as secondary prophylaxis. The risk of cardioembolic stroke in relation to clinical findings or heart disease is summarized in table 3.

Table 3. Type of heart disease and risk of cardioembolic stroke.

High risk	Medium/low risk
Atrial fibrillation/flutter	Calcification of mitral valve ring
Acute myocardial infarction < 6 weeks	Patent foramen ovale
Mechanical valve prosthesis	Atrial septum aneurysm and PFO
Mitralis stenosis of rheumatic origin	Calcified aortic stenosis
Atrial or ventricular thrombi	Bioprosthetic valve
Atrial myxoma	Mitral valve prolapse
Infectious/noninfectious endocarditis	Spontaneous echo contrast
Complex aortic arch atheromatosis	Sick sinus syndrome
Dilated cardiomyopathy	
Patent foramen ovale and systemic embolism	

Modified from TOAST-CSS classification and ¹⁰².

2.4.3 Hemostatic disturbances in different subtypes of stroke

Based on presumed pathophysiology, clinical manifestations and efficacy of different antithrombotic regimes, there is reason to believe that disturbances of the hemostatic system could be different in the different subtypes of ischemic stroke and TIA.

2.4.3.1 Small-vessel disease

Small-vessel disease (lacunar stroke) accounts for 20-25% of all cerebral infarcts ⁹ and is currently regarded as a matter of microscopic (lipohyaline) changes of the vessel wall with subsequent occlusion of the nutritional blood flow and a plausible “starvation” at the end artery area. In late stages of the “lacunar disease” a microthrombus is believed to be formed secondary to stagnation of blood flow ¹⁰³. In cohorts of patients with small vessel stroke disease a reduced degree of hemostatic activation has been seen (Table 4), which fits with the presented pathophysiological model.

2.4.3.2 Large-vessel disease

Large-vessel (artery) disease accounts for about 5-10% of all cerebral infarcts and is mainly a result of a stenosis/atherosclerotic plaque in the internal carotid or vertebral arteries as a result of atherosclerosis. The ruptured arterial plaque, similar to that in myocardial infarction, and the turbulence of blood flow created over the stenosis leads to development of embolizing thrombi consisting mainly of platelet aggregates but also involving coagulation and fibrin formation. In severe cases the entire lumen of the carotid or vertebral artery is occluded and a large stroke will develop if the thrombus is not dissolved. Intracranial stenotic lesions are more common in Asians and Africans but very rare in Caucasians ¹⁰⁴. Several studies have shown activation of hemostasis in patients with large-artery disease (Table 4).

2.4.3.3 Cardioembolic stroke

Cardioembolic strokes accounts for about 25% of all cerebral infarcts ¹⁰² and are most commonly due to embolization of a thrombus formed in the atrial appendage of the fibrillating left atrium. Emboli into the cerebral circulation follow the bloodstream and often end up in larger arteries (eg media circulation) where they occlude the vessel and generate strokes with more severe neurological deficits and subsequent worse prognoses ¹⁰². In cardioembolic stroke the suggested thrombotic mechanism is similar to that in venous

thromboembolism, and oral anticoagulating agents are effective in the prevention of future embolic stroke^{105 106 107}. In agreement with this idea, immunohistochemical studies have shown that the embolized thrombus is relatively rich in fibrin¹⁰⁸. Increased thrombin generation as measured by elevated plasma levels of the thrombin-antithrombin complex (TAT) and prothrombin fragments (F 1+2) have recently been found in patients after conversion of atrial fibrillation¹⁰⁹. A low d-dimer concentration in plasma in the acute phase has been suggested to make a cardioembolic stroke more unlikely. Thus, it has been postulated that this marker may be useful as a tool in the decisions regarding what clinical investigations should be performed¹¹⁰. Cardioembolic stroke is regarded by many as the subtype with the most convincing evidence of hemostatic activation (Table 4).

2.4.3.4 Undetermined stroke

Undetermined (or cryptogenic) strokes accounts for about 30% of all cerebral infarcts and are often not presented in clinical studies of hemostasis, probably because of the heterogeneity of the patients and the lack of a clear-cut etiology. Nevertheless, this group is interesting because these patients can be regarded as possible “cardioembolic patients” (cryptogenic embolism), as a complete investigation can reveal e.g. a patent foramen ovale (PFO) or a paroxysmal (silent) atrial fibrillation¹¹¹. To our knowledge, only a few larger studies have concerned this group in the context of hemostatic evaluation (Table 4) if studies of hereditary coagulation disturbances are excluded (part 2.2.2.1).

Table 4. Hemostatic disturbances in different ischemic stroke subtypes.

Subtype	Population	Main findings	Year
Small vessel	N=30*	d-dimer ↑, TAFI ↑	2010 ¹¹²
	N=38	PF4 ↑ F1+2 → d-dimer →	2001 ¹¹³
	N=58	FpA →, d-dimer → Fibrinogen →, TAT → ATIII →, FDP →	2000 ⁵⁶
	N=33	PAI-1 ↑, tPA ↑	1996 ¹¹⁴
	N=12 (<7d) N=15 (8-28d) N= 35 (>29d)	vWF →, PF4 → fVIIIc →, TF → Fibrinogen →	1993 ⁸²
Large vessel	N=170	F1+2 ↑	2008 ⁵¹
	N=10*	d-dimer ↑, TF ↑	2003 ¹¹⁵
	N=41	FpA ↑, d-dimer ↑ Fibrinogen ↑, TAT ↑ ATIII →, FDP ↑	2000 ⁵⁶
	N=10 (<7d) N=9 (8-28d) N=20 (>29d)	d-dimer ↑	1993 ⁸²
	Cardioembolic	N=26	F1+2 ↑ Fibrinogen ↑

cont. Table 4. Hemostatic disturbances in different ischemic stroke subtypes.

	N=38	FpA ↑, d-dimer ↑ Fibrinogen ↑, TAT ↑ ATIII ↓, FDP ↑	2000 ⁵⁶
	N=23 (<7d) N=17 (8-28d) N=20 (>29d) } N=20	d-dimer ↑ FPA ↑, TAT ↑ protein C ↓	1993 ⁸²
	N=20	vWF ↑, fVIIIc ↑ Fibrinogen ↑, d-dimer ↑ β-thromboglobulin ↑, PF4 ↑	1990 ¹¹⁷

Undetermined/ cryptogenic	N=89	Ks ↓	2009 ¹¹⁸
	N=162	TAFI ↑	2007 ¹¹⁹
	N=56	F1+2 →	2004 ¹²⁰

*No other subgroup of ischemic stroke was included for comparison.

Ks: fibrin network permeability coefficient, FpA: fibrinopeptide A, TAT: thrombin-antithrombin complex, FDP: fibrin degradation products, PF4: platelet factor 4.

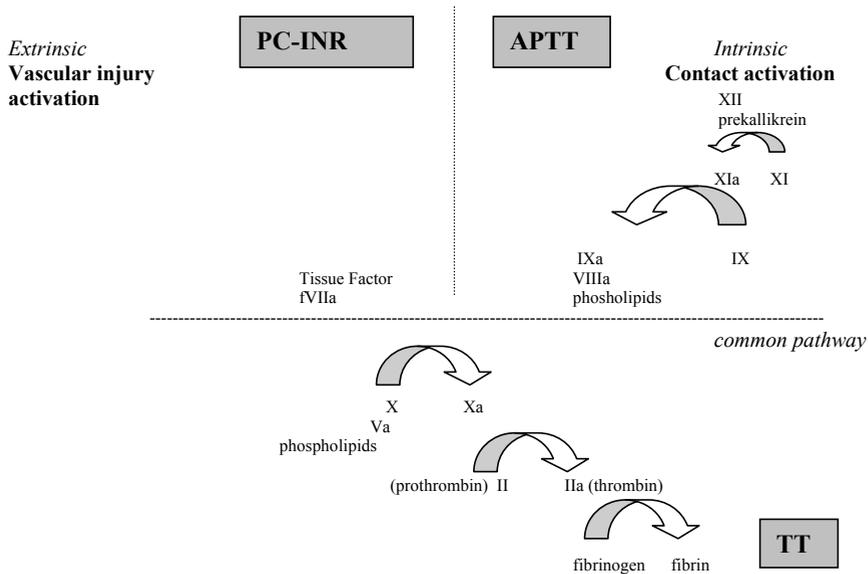
2.5 GLOBAL METHODS IN HEMOSTASIS

From a clinical point of view, there is a desire to find a method which assesses a patient's individual risk of recurrence of either stroke or myocardial infarction in a more functional way. Evaluation of more global functions of coagulation or fibrinolysis in the patient rather than measurement of single factors, would allow more confidence in the decision regarding prophylactic antithrombotic treatment. Here follows a brief overview of coagulation and fibrinolysis screening tests available:

2.5.1 Historical perspective

In 1964, two independent groups introduced a cascade or a “waterfall” model of coagulation composed of a series of steps in which activation of one clotting factor led to activation of another, finally leading to a burst of thrombin generation ^{121 122}. This model was originally applied for laboratory purposes but was also widely spread as the reigning dogma of the coagulation system *in vivo*. The system is divided into “*extrinsic*” and “*intrinsic*” pathways according to the type of activation (Fig 4):

Fig 4. The coagulation cascade with the traditional clotting assays: Prothrombin complex – International Normalized Ratio (PC-INR), activated partial thrombin time (APTT) and thrombin time (TT).



Today a cell-based model is often used to describe the coagulation cascade. This model includes platelets, leukocytes and even red blood cells as important contributing components with which the coagulation co/factors interact¹²³.

2.5.2 Screening assays of hemostasis

2.5.2.1 Prothrombin Time (PT)

As a screening assay for the vascular injury (*extrinsic*) activation pathway group of coagulation factors we have the PT (or PC) measurement available in all clinical laboratories. This is used mainly for monitoring of vitamin-K-dependent anticoagulants such as warfarin. The assay was introduced in the 1930s by Quick *et al.*¹²⁴. The coagulation factors II, VII, IX and X are called the prothrombin complex (PC), and with the exception of factor IX, these are evaluated by the PT assay (Fig 4). The electromechanical change of the sample is measured by a sensor that monitors a ball rotating on the bottom of the cuvette. When the clot is created the ball is stopped and the clotting time is obtained (normally within 11–15 seconds). Nowadays many different methods of assessing PC-INR are used all over the world, but in Scandinavia we use a reagent consisting of TF-rich thromboplastins together with bovine plasma to start the coagulation process in the plasma sample¹²⁵. In an attempt to overcome the differences in the various reagents used in different laboratories around the world, the International Normalized Ratio (INR) is applied.

2.5.2.2 Activated Partial Thrombin Time (APTT)

Measurement of APTT (also known as Partial Thromboplastin Time, PTT) is a screening method for the contact activation (*intrinsic*) pathway and a majority of the coagulation factors are screened, except for factor VII (Fig 4). The method was developed in 1953¹²⁶ and estimates the time in seconds for clotting to occur in a plasma sample, i.e. time to detectable fibrin formation (normal range commonly 28–40 seconds). The reagents used today are

calcium chloride, phospholipids (synthetic or from rabbit brain tissue) and a negatively charged contact activator (e.g. kaolin) as the triggers of clotting. The expression “partial” means that the reagent does not contain TF as in the PT assay and the result is always compared against a control plasma sample. In the antiphospholipid syndrome the APTT is paradoxically prolonged due to interaction with antibodies and phospholipids (lupus anticoagulans), even though the clinical consequence is venous or arterial thrombosis.

2.5.2.3 Thrombin Time (TT)

Measurement of TT, also called Thrombin Clotting Time (TCT), is a simple clotting test to evaluate the conversion of fibrinogen to fibrin by adding thrombin to citrated plasma. A slightly modified version of this method, i.e. diluted thrombin time, is considered to be useful to monitor treatment with the new direct thrombin inhibitors¹²⁷. TT is strongly dependent on the different reagents used, thus results between different labs can not be directly compared. The normal value of TT is usually around 15 seconds.

2.5.3 Global assays of hemostasis

As screening tests for bleeding disorders the clotting assays are excellent tools, but as for detection of hypercoagulable disorders they have been claimed not to be sensitive enough²⁰. Other methods to investigate hemostatic activation are therefore needed and new assays have emerged in the search for more global techniques in order to improve clinical decision-making¹²⁸. Here follows an overview of the most common global assays of hemostasis:

2.5.3.1 Thrombelastography (TEG)

Thrombelastography was developed in 1948 by Hartert¹²⁹ and has many advantages as it is a bedside test in whole blood. Thus it can be used in critical situations such as sepsis, various types of surgery and following severe trauma. Using TEG, hemostasis under low shear stress conditions is monitored in the presence of all blood cells, thus allowing cell-cell interactions and cell-based coagulation. There is a mechanical detection system which allows us to visualize different typical graphical patterns giving information about the time until detectable fibrin formation, the kinetics of thrombus generation, the maximal amplitude (i.e. the strength and stability of the clot), and lysis time¹³⁰. Originally, no trigger of coagulation was applied in TEG except for calcium, but during recent years a modified version of the assay is often employed which uses minimal amounts of TF to trigger coagulation¹³¹.

New techniques have been developed where a rotating pin instead of a fixed piston (as in TEG) is used. Today the ROTEG[®] (Rotation Thrombelastography)¹³² or ROTEM (Rotation Thrombelastometry) systems¹³³ are perhaps more widely used techniques than TEG. The major drawbacks of all these methods are the limited time between blood collection and analysis (samples must be analyzed within 8 hours), and the fact that frozen-thawed samples can not be used.

2.5.3.2 Overall Hemostatic Potential (OHP)

Measurement of OHP was developed by He *et al.*¹³⁴ and further modified^{135 136} to be applicable in clinical bleeding and thrombotic conditions²⁰. By adding triggers of both coagulation and fibrinolysis, changes in turbidity are measured by spectrophotometry as fibrin is formed and lysed in the plasma sample. The OHP method was later further developed into the Overall Hemostasis Index assay (OH-index)¹³⁷ and these global methods have been used in the present work and are further described in the Methods section (part 4.2.2.).

2.5.3.3 Other turbidimetric assays

In the turbidimetric assay by Grant the clotting is triggered by the addition of small amounts of thrombin in combination with calcium¹³⁸ (similar to OHP). The method gives information about the functional aspects of fibrin formation, but not the more precise structure of the fibrin network. By measurements of absorbance (e.g. every 18 seconds for one hour) one evaluates how turbid the plasma gets when it clots and a graphical curve is plotted using a special software. Within the turbidimetric assay, the clot *lysis* is evaluated separately from the clot *formation*. This aspect of the assay is performed by adding tiny amounts of tPA before the addition of the coagulation trigger. Lysis is typically complete after one hour but readings may sometimes continue for up to nine hours depending on what plasmas that are investigated. The assay is quick and easy to perform, but it does not give the same precise information about the fibrin network properties as the fibrin network permeability assay does (see below). The CV is normally less than 10%¹³⁸ but as with all hemostatic methods it may be user dependent.

2.5.3.4 Thrombin generation assays (TGAs)

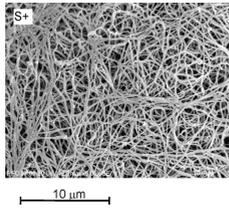
Interest in thrombin (factor IIa), responsible for the conversion of fibrinogen to fibrin, has resulted in various global assays in which thrombin generation is measured in a test tube with clotting triggers added. Thrombin generation assays have been used since the 1950's to study coagulation in patients with hemorrhagic diseases or venous thromboembolism. At first TGAs were very time-consuming, but they were improved by Hemker *et al.* who replaced the manual work with automated continuous chromogenic measurements. Hemker also limited the assay to measure thrombin generation only, excluding the last stage, i.e. the fibrin formation. This was done by removing fibrinogen from the plasma before analysis¹³⁹. Originally, a chromogenic substrate was used but nowadays a fluorogenic substrate is used in some assays instead of the chromogenic one. In the fluorogenic method, thus, defibrination of plasma is unnecessary. The principle of the method is to measure the optical density, thus the appearance of thrombin generated in the plasma sample¹⁴⁰.

The major part of thrombin is generated after the lag phase has terminated (i.e. after clotting time), thus only approximately 5% of total thrombin generated is measured with the traditional clotting assays APTT and PT¹⁴¹. Thus, TGAs give additional information also on the kinetics of the *total* amount of thrombin generated in plasma. The coagulation process is initiated by different 'triggers', i.e. tissue factor, phospholipids together with calcium. The exact concentration of the triggers can influence the data¹⁴² and there is some evidence of a better discrimination between "disease and health" using a lower TF-concentration¹⁴³. The Calibrated Automated Thrombogram (CAT), the Thrombin Generation Test (TGT) and Technothrombin TGA, are three commercially available TGAs¹⁴⁴. The CAT-assay is used in the present work and further explained in Methods section (part 4.2.4.).

2.5.3.5 Fibrin network assay

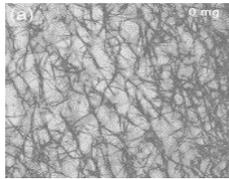
Assessment of the last step of coagulation beyond thrombin generation, i.e. the formation of the fibrin network, can also be viewed upon as a global method. The fibrin network can be visualized in different ways e.g. through its morphology using light-scattering techniques¹⁴⁵, scanning electron¹¹⁸ or confocal microscopy¹⁴⁶ (Fig 5). An evaluation of the functional properties of the fibrin network is feasible by way of a liquid permeability test¹⁴⁷ or by a gel turbidity assay. In this work the fibrin network permeability technique developed by Blombäck *et al.* has been used and is further described in Methods section (part 4.2.3).

Fig 5. Fibrin network morphology.



Scanning electron microscopy

From Undas et al. with permission from publisher (Stroke, 2009; 40:1499-1501)



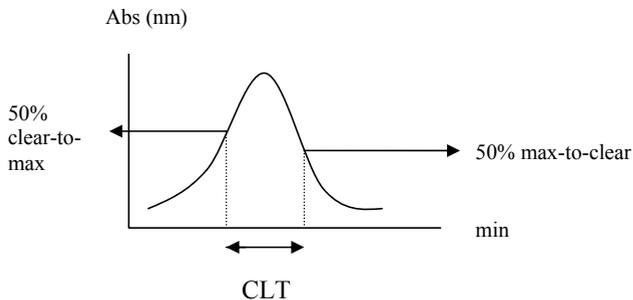
Confocal 3D laser scanning microscopy

Antovic et al. with permission from publisher (Thromb Res, 2005; 116:509-517)

2.5.3.6 Clot lysis Time (CLT)

The CLT assay is a global method of fibrinolysis in which triggers of both coagulation (TF and phospholipids and calcium) and fibrinolysis (tPA) are added to plasma and changes in absorbance are measured during clot formation and lysis. The coefficients of variation has been reported to be low (interassay 4%, intraassay 3%)¹⁴⁸. A typical CLT curve is shown below in Fig 6.

Fig 6. Definition of Clot Lysis Time (CLT) estimated as the time from 50%-clear-to-max to 50% max-to-clear turbidity.



In this work we have used a slightly different CLT assay (performed as part of the OHP-method) (**Study I**) but the principals are the same. This method has so far been used for research purposes only.

3 AIMS

Overall aims:

- To investigate hemostasis in acute ischemic stroke (IS) through descriptive studies with emphasis on global methods
- Try to identify subgroups of acute IS patients with more activated hemostasis, thus being at potential risk of cerebral thromboembolic complications

Specific aims:

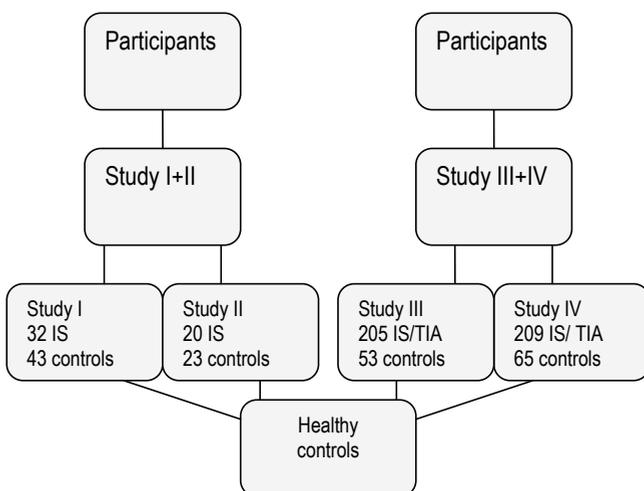
- To study platelet-derived microparticles in acute IS
- To study thrombin generation in acute IS
- To study fibrin formation, fibrin network permeability and fibrinolytic capacity in acute IS

4 MATERIAL AND METHODS

4.1 PATIENTS

All patients included in this work were recruited from Stroke Units at three hospitals in Stockholm, i.e. Danderyd Hospital (n=177), Southern Hospital (n=95) and Karolinska University Hospital, Solna (n=9). The criteria for ischemic stroke were those according to the WHO¹⁴⁹, i.e. sudden onset of neurological deficit and/or signs of focal loss of cerebral functions with duration of more than 24 hours. A neuroradiological assessment, most often by computer tomography (CT) but on some occasions with additional MRI, excluded hemorrhagic stroke or other conditions such as tumors. The new definition of TIA was used, i.e. a transient episode of neurologic dysfunction caused by focal brain, spinal cord or retinal ischemia *without* evidence of acute infarction¹⁵⁰. Thus, neurologic events occurring for < 24 hours with evidence of brain infarction were diagnosed as ischemic stroke, regardless of time of recovery.

Fig 7. Distribution of participants in Studies I-IV.



4.2 METHODS

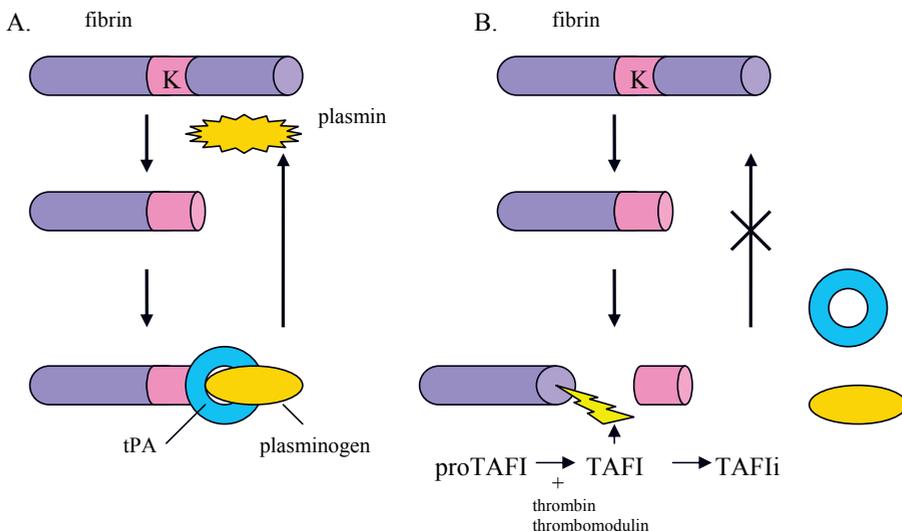
In **Studies I and II** the patients were recruited within 24 hours of symptom onset and a follow-up examination was performed at 60 days. In **Studies III and IV** the patients were recruited within two weeks of stroke onset and a follow-up examination was performed after approximately 30 days. Blood samples were taken in a fasting condition and after ten minutes of rest. The Stroke Units at Danderyd Hospital and at Södersjukhuset were responsible for sampling and were instructed to undertake atraumatic venous puncture without stasis. If stasis was necessary, a blood pressure cuff was used and a first extra “slush” tube was taken. At Karolinska Hospital a specially trained research nurse took the blood samples and delivered

them to the lab. Blood samples were all handled in the same way with platelet poor plasma prepared from citrated (trisodium-citrate) whole blood (ratio 1+9) and K₃-EDTA whole blood by immediate centrifugation (within 30 min) at room temperature at 2000 × g for 20 minutes. The plasma was thereafter aliquoted and frozen at -80°C until tested. Centrifugation and sample handling was performed at the Clinical Chemistry labs at the hospitals involved.

4.2.1 TAFI

In **Study I** we analyzed TAFI antigen concentrations by using a commercially available ELISA-method. TAFI is the short name for thrombin activatable fibrinolysis inhibitor, also called Carboxypeptidase U. The TAFI molecule was discovered in the late 80's by two independent research groups^{151, 152} and defined further to be an attenuator of fibrinolysis¹⁵³. By its action as a carboxypeptidase it cleaves off the binding site for plasminogen to fibrin and thereby inhibits binding of plasminogen to the fibrin molecule. Thus less plasminogen will be bound to fibrin and less plasminogen will be converted to active plasmin by fibrin-bound tPA (Fig 8). The target antibody used in the present study was directed towards the three different forms of TAFI antigens; pro-TAFI, active TAFI (TAFI) and inactive TAFI (TAFIi) and the method is described in more detail in **Paper I**. Thrombin (together with the cofactor thrombomodulin) is considered to be the most important physiological activator⁹² with conversion from pro-TAFI to TAFI (Fig 8). The nomenclature of TAFI can differ somewhat between studies.

Fig 8. A. Degradation of fibrin by plasmin activated by tissue plasminogen activator (tPA). B. TAFI inhibits the activation of plasminogen to plasmin through cleavage of a carboxy-terminal lysine residue (K) i.e. the binding site for plasminogen, from partially degraded fibrin.



Modified from Bouma et al. with permission from publisher (Thrombosis Res, 2001;101:329-54).

4.2.2 OHP and OH index

In **Study I** the Overall Hemostatic Potential (OHP) method was used as described by He *et al.* (2001) (see part 2.5.3.2.), a functional method to assess fibrin formation and lysis, performed in a 96-well microplate. The OHP curve is an absorbance curve formed and based on repeated spectrophotometric measurement after triggers of coagulation (calcium and thrombin) and fibrinolysis (tPA) have been added to the plasma sample in the well. The OHP curve is formed when both triggers of coagulation and fibrinolysis are present in the plasma sample. The Overall Coagulation Potential (OCP) is a curve formed when only triggers of coagulation are added. The Overall Fibrinolytic Potential (OFP) represents the difference between the areas under the OCP and OHP curves and is calculated according to the formula below:

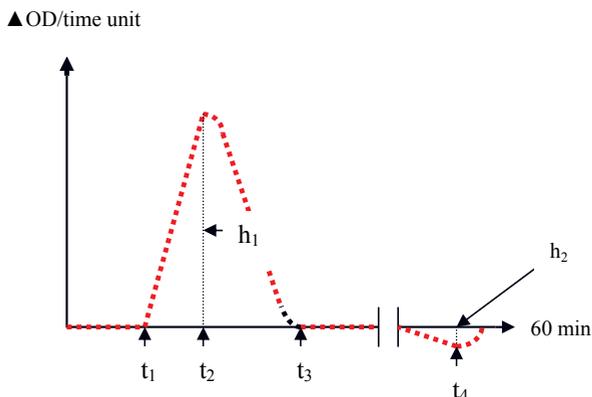
$$\text{OFP} = \frac{(\text{OCP}-\text{OHP})}{\text{OCP}} \times 100\%$$

The OHP method is simple to perform. If a spectrophotometric reader is available (which usually is the case in a clinical chemistry laboratory) about 30 patient plasma samples can be analyzed within one hour. The calculations are carried out by using a simple Microsoft Excel software. OHP and OCP variables are expressed as Abs-sum (summation of absorbance) and OFP values are expressed as percentages. The method is described in more detail in **Paper I**.

A further development of the OHP method, the Overall hemostatic index (OH index), was used in **Study II**¹³⁷. The principles are the same as in OHP, with triggers of coagulation and fibrinolysis added to the plasma sample, but the OH index assay takes into consideration both *time* and *rate* of coagulation and fibrinolysis (Fig. 9) in a more complex formula:

$\text{Coagulation profile (Cp)} = (t_1)^{-1} \times \frac{ h_1 }{(t_2 - t_1)}$	$\text{Fibrinolysis profile (Fp)} = t_1 \times \frac{ h_2 }{(t_4 - t_3)}$
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Fig 9. The difference in optical density per time unit forms a curve from which the different variables of the OH index formula can be estimated. The formula stated above includes the different variables obtained from a curve of optical density changes per time unit.

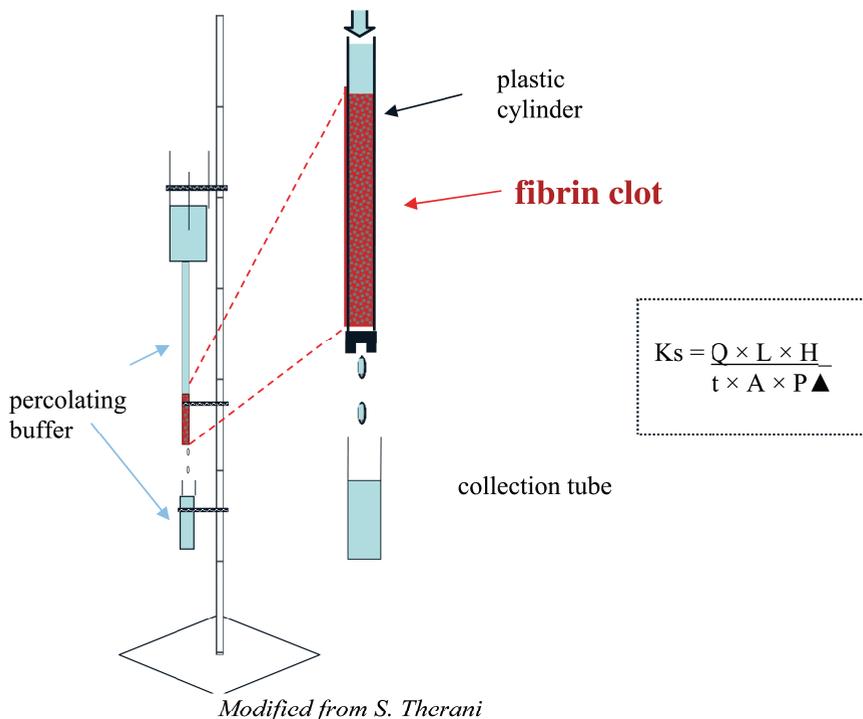


In this method low (picomolar) concentrations of tissue factor (TF) are used instead of thrombin in order to start “higher up” in the coagulation cascade and thus this method relies more on several different components present in patient plasma. Together with TF, washed frozen-thawed platelets and CaCl_2 are added. Spectrophotometric data gathered in the same manner as in the OHP method. The raw data obtained are put into a software and the C_p and F_p are calculated (see formula above). The ratio of C_p to F_p is called the Overall hemostatic index (Oh-index) and is suggested to reflect the balance between coagulation and fibrinolysis in the plasma sample.

4.2.3 Fibrin network

The fibrin network assay was originally developed by Blombäck *et al.*¹⁴⁷ and further modified by He *et al.*¹⁵⁴. The method is based on the principle that fibrin formation in a plasma sample is triggered by addition of thrombin (or TF) and calcium. The fibrin network is left to mature into a gel in a plastic cylinder overnight. As can be seen below in Fig.11, buffer passes through the fibrin gel to evaluate its permeability. The volume of the eluate is collected under different hydrostatic pressures and the permeability constant (Ks) is calculated according to a formula (Fig 10) stated by Carr *et al.*¹⁵⁵. Information about the fibre mass/length ratio (μ) can also be provided. The major advantage of the modified assay is that it requires a considerably smaller plasma volume compared to the original method (250 μl instead of 3000 μl). In addition, the modified assay has been shown to have an even better reproducibility than the older version¹⁵⁶. The method is both labor- and time-consuming and approximately 8-10 samples can be analyzed in one day.

Fig 10. Experimental set-up of the fibrin network permeability assay. Q = eluate volume, t = percolation time, H = viscosity, L = gel length, A = internal area of cylinder, P ▲ = difference in hydrostatic pressure.



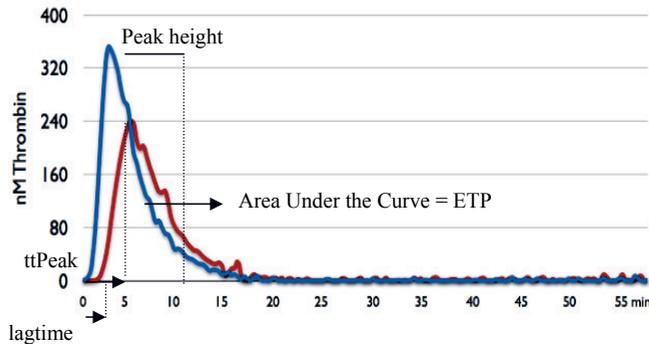
4.2.4 CAT assay

The calibrated automated thrombogram (CAT) assay used in **Study III** is a method of thrombin generation now used in several laboratories around the world. So far it has mainly been used for research purposes, although it may have the potential to be used in clinical investigations of bleeding disorders and/or thrombophilias.

The method was developed by Hemker *et al.* as described previously (part 2.5.3.4.) and involves a fluorogenic substrate for thrombin and its formation can subsequently be measured by fluorometry. As in many other global methods, measurements are performed repeatedly (here every 30 seconds) in order to obtain a temporal profile (curve) of thrombin generation. A calibrator is used for each sample and the samples are all analyzed in triplicate and mean values together with standard deviation presented when transformed into a Microsoft Excel

format after calculation using a commercially available Thrombinoscope software®. The variables *lag time*, *time to peak*, *peak height* (i.e. peak thrombin concentration attained) and *Endogenous Thrombin Potential (ETP; area under the curve)*, are obtained via the software (Fig 11).

Fig 11. The Thrombogram curve of the CAT-assay.
Blue = stroke patient. Red = healthy control. ttPeak = time to Peak.

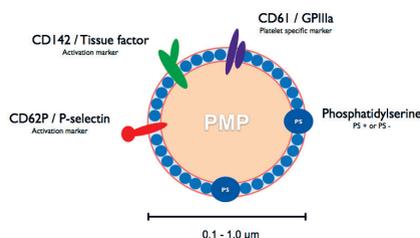


This method has acceptable reproducibility, with an interassay coefficient of variation of less than 5%. The CAT assay is convenient to perform and it takes only one hour for 32 samples to be run in a microplate.

4.2.5 Microparticles

Microparticles (MPs) are small vesicles “budding off” from apoptotic or activated cells such as platelets, endothelial cells and white and red blood cells. MPs have generally been considered to have procoagulant activity through the expression of negatively charged phospholipids (mainly phosphatidylserine; PS) (Fig 12). However, some studies suggest that MPs also may have a multitude of other biological effects, depending on the molecules they carry¹⁵⁷.

Fig 12. A platelet-derived microparticle exposing various markers on its surface.



Formation of MPs has been associated with various diseases such as multi-infarction dementia¹⁵⁸, multiple sclerosis¹⁵⁹, antiphospholipid syndrome^{160, 161}, thrombotic thrombocytopenic purpura (TTP)¹⁶², and ischemic stroke^{158, 163 164}. In diabetes type 2¹⁶⁵ and in acute coronary syndromes¹⁶⁶, conditions that often coexist with stroke, MPs may also

play an important role. Platelet derived MPs (PMPs) are of interest in ischemic stroke both as markers of platelet activation and through possible biological effects e.g. procoagulant effects through expression of TF. As regards methodology, various assays have been used in previous stroke studies (Table 4).

Table 4. Studies on platelet derived microparticles (PMPs) in ischemic stroke.

Condition	Effect	Number of patients	Method	Year
small vessel+ large "	PMP (count) ↑ - small vessel ↑ than large v.	52	flowcyt (GPIb)	1993 ¹⁵⁸
acute cerebral infarction	PMP (Abs) ↑ - large vessel ↑ + "other inf" ↑ ↓ at 1 month	252	ELISA (CD42a/b capture Ab)	2009 ¹⁶³
acute cerebral infarction	PMP (Abs) ↑	110	ELISA (GPIb human capture Ab)	2010 ¹⁶⁴

Abs; absorbance. Ab; antibody.

The opportunity arose to use a method developed in our laboratory ^{167 168} for analysis of PMPs. The method is described in more detail in **Paper IV** but in brief, this is a flow cytometric assay where the particles are gated according to size of the MPs (<1 μm). The flow cytometer is calibrated through the use of beads of different sizes. Fresh platelets are also used to check gating and settings. The cellular source of the MPs is determined through fluorescently labelled antibodies directed against cell-specific molecules may be used, such as CD41 or CD61 for platelets, CD144 for endothelial cells and CD45 for leukocytes. Phosphatidylserine (i.e. PS, mentioned above) can be measured by lactadherin binding ¹⁶⁹ and various other markers exposed on MPs can be measured as well (e.g. tissue factor or P-selectin). This method makes it possible to assess platelet function in frozen plasma samples.

4.2.6 Other laboratory methods

Commercially available kits and calibrators were used to measure the standard inflammatory and hemostatic markers used in this study:

4.2.6.1 CRP

C-reactive protein (high-sensitivity assay) was determined using immunonephelometry (BN Systems; Dade Behring GmbH, Marburg, Germany).

4.2.6.2 VWF

For Von Willebrand factor antigen determination, the Liatest VWF (Diagnostica STAGO, Taverny, France) was used.

4.2.6.3 Fibrinogen

Fibrinogen was determined using immunonephelometry (BN Systems; Dade Behring GmbH, Marburg, Germany).

4.2.6.4 Prothrombin fragment 1+2

Prothrombin fragment 1+2 (F1+2) was determined with an ELISA kit (Dade Behring) using a Multiscan MCC/340 (Flowlab, North Ryde, Australia).

4.2.6.5 D-dimer

Fibrin d-dimers were analyzed with latex fortified turbidometry (using a Sysmex CA-1500 and reagents from Dade Behring).

4.2.6.6 PAI-1

Plasminogen activator inhibitor-1 antigen concentrations were analysed in plasma with an ELISA kit (Dade Behring) using a Multiscan MCC/340 (Flowlab, North Ryde, Australia).

4.2.6.7 Routine analyses

Concentrations of plasma lipids, plasma glucose, platelet counts and serum creatinine were all analysed by routine laboratory techniques at the Clinical chemistry laboratories at Danderyd Hospital, Södersjukhuset and Karolinska University Hospital, Solna, respectively.

5 STATISTICAL ANALYSES

Here is an overview of the statistical analyses used in this work. For further details see Paper I–IV, respectively:

- Students t-test – used between groups with normally distributed variables (**Paper II**).
- Wilcoxon’s signed rank test and the Mann-Whitney *U*-test – used to determine if differences are present between groups of dependent or non-dependent skewed variables (**Paper I-IV**).
- Spearman’s correlation coefficient – used to estimate if associations were present between continuous variables, expressed as either *r* values or r^2 values (**Paper I-III**).
- The Kruskal Wallis test – a form of one-way ANOVA for non-parametric continuous variables, used for comparison between three or more groups. Dunn’s *post hoc* analysis is performed if the overall p-value is < 0.05 (**Paper III, IV**).
- ANCOVA-analysis – a multivariate regression analysis of covariance between groups, and used to test for interactions of factors decided according to their possibility of influencing the dependent variable, e.g. age (**Paper III**).
- Mixed model analysis – used for compensation for samples lost by chance. A one-way repeated measures analysis of variance (ANOVA) using the procedure Mixed in SAS[®] (System 9.1) is performed. Skewed data is log-transformed preceding the analysis. (**Paper I**).

For in-house statistical calculations Graph Pad Prism[®] 3.02 or Statistica Statsoft[®] were used. Values of $p < 0.05$ were considered statistically significant.

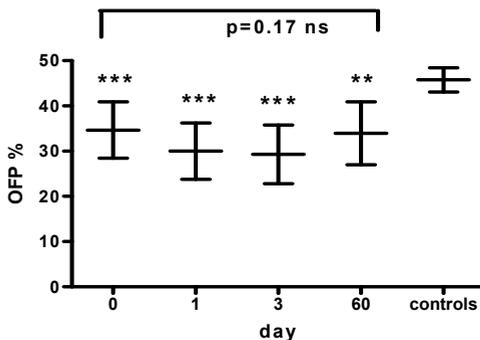
6 RESULTS AND DISCUSSION

6.1 PAPER I

Acute ischemic stroke – a hypofibrinolytic condition?

The TAFI antigen concentrations in plasma were found to be elevated in the acute stage of IS but they were decreased 60 days after the event. Fibrinolysis, as measured by the global marker of fibrinolysis OFP, was also depressed and this hypofibrinolytic condition remained also at the 60 day measurement (Fig 13).

Fig 13. Values of Overall Fibrinolytic Potential (OFP) in patients with ischemic stroke in the acute (day 0,1,3) and convalescent phase (day 60). ** $p < 0.01$, *** $p < 0.001$ vs. controls. Data presented as mean \pm 95% CI. No significant change over time including all four time points (one-way ANOVA).



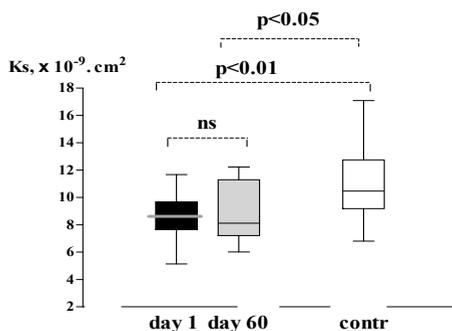
Thus, the main finding in this study was the observation of a decreased fibrinolytic capacity in IS. This was shown by way of two different methods, i.e. a *global* method of assessing overall coagulation and fibrinolytic capacity and through measurements of a *single* factor that attenuates fibrinolysis, i.e. TAFI. There were also relationships between inflammatory markers (CRP and fibrinogen) and fibrinolysis (OFP and to some extent TAFI), supporting the idea that hemostasis and inflammation interact in the pathophysiology of ischemic stroke.

6.2 PAPER II

Is a tighter fibrin network present in acute ischemic stroke?

The fibrin network formed was found to be tighter in the acute phase of IS as compared to controls. Of note, this finding persisted after two months post-stroke despite enhanced treatment with antiplatelet drugs and statins (Fig 14), drugs known to make the fibrin network more porous^{170 171 68}.

Fig 14. Fibrin network permeability (Ks) in patients with ischemic stroke and in healthy controls. Patients were investigated in the acute phase (day 1) and after two months (day 60). Data presented as medians (boxes: 25-75th percentiles, bars: min-max).



This new finding was confirmed by another research group almost at the same time¹⁷². However, our finding that the fibrin network was persistently tighter after the acute event was not reported in the study by Undas *et al.* Also, the additional information on decreased fibrinolytic capacity (decreased Fp) in the same population (Table 6) added an extra angle to our study as commented upon in a small editorial concerning our paper¹⁷. Taken together, these findings were in line with the results in **Study I**, supporting the idea that ischemic stroke represents a hypofibrinolytic condition in which fibrinolysis has to “work against” a tighter fibrin network.

Table 6. The Coagulation potential (Cp) and Fibrinolysis potential (Fp) in the acute (day 1) and convalescent (day 60) phases of ischemic stroke, and in controls. Data presented as median-values (with 25-75% percentiles). Wilcoxon’s matched pairs test was used in intraindividual analysis. Mann-Whitney *U* test was used in the comparison with controls.

	Patients day 1 n=18	Patients day 60 n=14	Controls n=23
Cp	3.4 (1.9 – 7.1)*	2.6 (1.8 – 4.1) ^{ns}	2.0 (1.1 – 3.2)
Fp	0.9 (0.5 – 1.3)***	0.9 (0.4 – 1.6)***	2.3 (1.9 – 3.6)

*** p<0.001 and * p<0.05, as compared with controls.

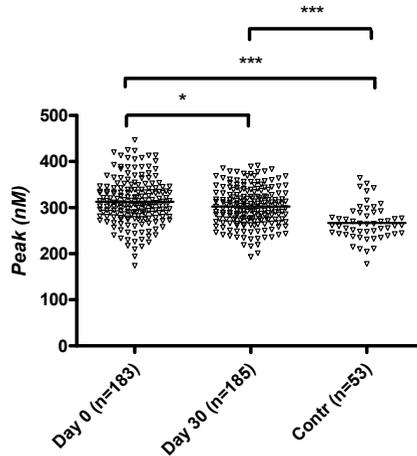
6.3 PAPER III

Is thrombin generation elevated in acute ischemic stroke?

In **Study III**, we used a thrombin generation test (CAT) and observed a clear-cut increase in thrombin generation in a representative acute IS population (Fig 15). To our knowledge, this information has not been published before with this new kind of thrombin generation test in which the potential of the patient’s plasma to generate thrombin is evaluated (coagulation is started with the addition of TF). No difference in thrombin generation was observed in the various subtypes (TOAST, Bamford; data not shown), apart from the fact that a trend toward

a higher thrombin generation seemed to be present in patients with a cardioembolic stroke due to a previously unknown paroxysmal atrial fibrillation ($p=0.08$) (Fig 2B, Paper III).

Fig 15. Thrombin generation measured as peak thrombin concentrations during the acute phase of stroke and after 1 month, vs. healthy controls. *** $p<0.0001$, * $p<0.05$. Wilcoxon's matched pairs test was used for intraindividual analyses. Mann-Whitney U -test was used for comparisons between patients and controls. Data presented as median values.



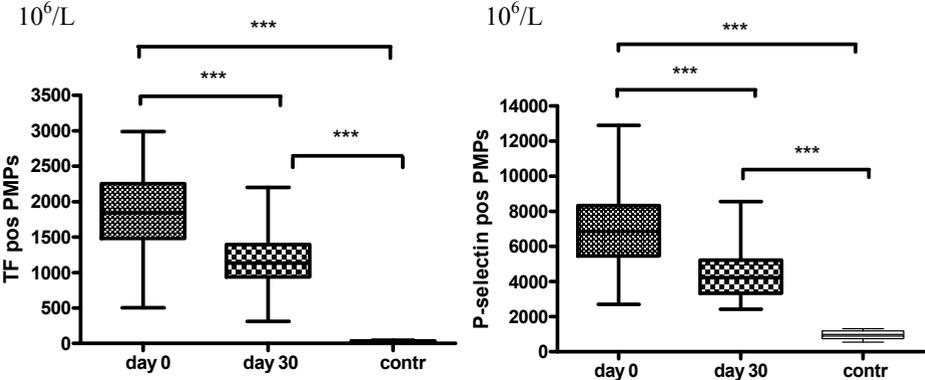
As can be seen in Fig 15 there was great inter-individual variability with respect to thrombin generation, with data from a bulk of patients lying well within the range of thrombin generation observed in healthy controls. It is clearly still too early to introduce this method in screening for hypercoagulability in stroke patients, even though use of the thrombogram is very easy and quick to perform. However, our study is of interest in the light of the results of a recent prospective study showing an increased risk of a future acute ischemic stroke (especially of the cardioembolic subtype) in patients with increased thrombin generation as measured by CAT¹⁷³.

6.4 PAPER IV

Are circulating PMPs elevated in acute ischemic stroke?

The results in **Study IV** showed an increased number of circulating platelet-derived microparticles (PMPs) in IS, both acutely and after one month. This was in congruence with other studies in the field (Table 4, part 4.2.5). The particular novelty of our study was the marked elevation of PMPs that expose tissue factor and P-selectin (Fig 16), findings which are interpreted to reflect platelet activation.

Fig 16. Tissue factor positive (CD142) and P-selectin positive (CD62P) platelet derived microparticles (PMPs) in ischemic stroke and TIA patients in the acute (day 0) and subacute (day30) phase, vs. healthy controls. PMPs presented as absolute numbers. Data expressed as median (with 25-75th percentiles). (***) $p < 0.0001$, Non-parametric Mann-Whitney *U* test and Wilcoxon's signed rank test were used.



A significant increase was present in all ischemic stroke subtypes (TOAST, Bamford; data not shown) regarding TF-expression. No significant differences in TF-expression were detected between the subtypes (TOAST and Bamford; data not shown).

7 GENERAL DISCUSSION

As a clinician one is highly interested in the more functional aspects of the hemostasis, since such an understanding could make it possible to get an overall picture of the patients risk for thrombosis or bleeding. Thus, it would be convenient to be able to assess the balance between coagulation and fibrinolysis and from such data understand how to treat the patient in the best possible way. Measurements of single hemostatic factors involved in the process of coagulation or fibrinolysis may on the other hand be of great value in the understanding of pathophysiological mechanisms, but there is not always a good correlation between the levels of factors and the severity of symptoms present. Considering antithrombotic treatment there is also a great inter-individual variability in the response to antiplatelet therapy¹⁷⁴ and anticoagulants due to factors that can either be genetical¹⁷⁵ or due to differences in the inter-individual hemostatic response.

Stroke is a more heterogenous disease than the cardiovascular disease in terms of etiology¹⁰⁴ and less is known about the more functional and global aspects of the hemostasis in the different subtypes of ischemic stroke than in cardiovascular disease^{71, 176, 177}. Also in diabetes patients, global methods of hemostasis have been used^{72, 178, 179}, but only a few studies have been performed in IS populations^{180 181} and even fewer in acute IS. We could find no prospective studies with global hemostatic markers in the literature when this thesis was planned. Hopefully, this work have added some information about the pathophysiology of hemostasis in acute IS to enlighten what should be focused upon in future clinical and experimental studies.

Hemostatic disturbances in acute ischemic stroke

We found that hemostatic disturbances in patients with acute IS include an activated coagulation and in parallel an impaired fibrinolysis and a proneness to form a tighter fibrin network. In addition we observed signs of platelet activation as measured by elevated numbers of PMPs in plasma. These disturbances may be dependent on very different mechanisms such as endothelial dysfunction, inflammation and progression of atherosclerosis. Local factors like stasis with ensuing activation of coagulation may also contribute, especially if the patient suffers from atrial fibrillation.

Microparticles

We detected an increased numbers of platelet-derived microparticles (PMPs), and slightly less than 10% of PMPs exposed TF (these PMPs also exposed phosphatidylserine). The presence of PMPs in plasma is interpreted to reflect platelet activation. Notably, PMPs may interact with coagulation through exposure of procoagulant phosphatidylserine and TF on their surface, both of which are measured in the flowcytometry assay used in the present thesis. There has been some controversies as to whether TF exposed on MPs (especially PMPs) is functional or not, and if it really has any impact on thrombogenicity^{182 183} or is merely an “activation marker”. A recent study by Steppich *et al.* in patients with acute coronary artery disease reported, however, that circulating microparticles represent the major source of TF activity in plasma. It is therefore tempting to speculate that there may be a connection between our data on increased number of TF and phosphatidylserine positive PMPs (study IV) and elevated thrombin generation (study III). However, correlation analyses did not support this idea. In addition, the CAT analysis performed uses addition of TF (in our study 5 pmol/l) and phospholipids at rather high concentrations and this makes it less likely that MPs present in the plasma sample have important influence on the thrombin generated in the test tube. Perhaps more likely is that the plasma composition, i.e. alterations in the concentrations of

various coagulation factors, influence thrombin generation and explains our findings as has been discussed in acute myocardial infarction¹⁴⁵. We have, however, no data available in our present study to support this assumption.

MPs may also influence the fibrin network, as previously reported by our group¹⁷⁰ and others¹⁸⁴. A tighter fibrin network would be expected if plasma contains a high number of MPs expressing phosphatidylserine and exposing TF, as this would enhance coagulation and thrombin generation. However, we did not adapt the fibrin network permeability assay to detect the possible effect of MPs as we added frozen-thawed platelets and TF to the plasma samples investigated. Thus, the tighter fibrin network formed in stroke patients as presented in study II, is unlikely to be due to elevated MPs and/or PMPs exposing TF. Further research investigating the potential “thrombogenicity” of the MPs in our stroke population is clearly needed. Experiments investigating the effect of these MPs can indeed be performed in the fibrin permeability assay as well as in the CAT analysis.

Thrombin generation and fibrin formation

We investigated two aspects of coagulation, i.e. thrombin generation and fibrin formation. Thrombin generation was investigated with the CAT assay (study III). The OHP-method (study I) and the OH-index method (study II) were used to study fibrin formation. Increased thrombin generation was observed in study III, and increased fibrin formation was observed in study I and II. The OHP method and the OH-index method differ however somewhat as regards the triggers of coagulation; in the OHP method thrombin was used and in the OH-index method (study II), TF and frozed-thawed platelets were used. Thus slightly different approaches to evaluate fibrin formation were taken. However, both methods showed virtually the same thing, i.e. patients with ischemic stroke have an increased fibrin formation capacity. In addition, the CAT data indicate that this, at least in part, may be due to an increased thrombin generation. The global approach of the methods, i.e. their responses are dependent on several factors present in the plasma sample, makes it impossible to specifically determine the mechanisms. At present, the CAT analysis seems to be the most promising method of the global assays for coagulation used in this thesis if one is interested in prognostic studies, given the positive data recently published in the French case-cohort study mentioned previously (part 6.3)¹⁷³ and the interestingly wide variability in thrombin generation found in study III.

Fibrin network

The tighter fibrin network formed by stroke patients as compared to healthy controls is clearly a new and interesting finding. Mechanisms behind this observation are not known although plausible causes may include higher abundance of substrate (fibrinogen), increased thrombin generation and increased numbers of microparticles.

The patients had higher plasma concentrations of fibrinogen which may influence the tightness of the fibrin network, but we observed a significant correlation between fibrin network permeability (Ks) and plasma fibrinogen concentrations only in healthy controls, but not in patients (study II). It is therefore likely that other factors also may be involved.

One possible mechanism for the tighter network may be the increased thrombin generation observed in study III as it is well known that an increased thrombin generation leads to the formation of a tighter and more dense fibrin network. Another plausible cause of the obtained fibrin network data in study II is that there are higher numbers of MPs present in the sample. However this mechanism is not very likely, because the effects afforded by the frozed thawed

platelets and TF added, as mentioned above, probably overshadow the possible effects of MPs present in the plasma sample.

Among the IS patients in study II there were also some subjects with diabetes (22%). As diabetes through glycosylation of the fibrinogen molecule may influence the fibrin network towards a more tight structure, it may be that such mechanisms also contribute to the differences observed between stroke patients and healthy controls. Indeed, higher glucose levels in the acute stage of stroke is a known factor of poor prognosis of stroke ¹⁸⁵ and a recent study on more than 100 patients treated with i.v. thrombolysis found an eight-fold increase in poor outcome (measured by the modified Rankin scale) in patients of the upper tertile of insulin resistance ¹⁸⁶. Whether this mechanistically involves the structure and composition of the fibrin network would be of great interest to elucidate in future studies.

Fibrinolysis

We assessed fibrinolysis through measurements of two endogenous fibrinolysis inhibitors (i.e. TAFI and PAI-1) and by employing three global methods, the overall fibrinolytic potential (OFP), the fibrinolysis profile (Fp) (these two assessments are included in the OHP and the OH-index assays, respectively), and the clot lysis time (CLT; study I). In addition, we also measured d-dimer in plasma.

Our results all point towards the same direction - that ischemic stroke is a hypofibrinolytic condition. We found this to be the case both in the acute phase as well as in the convalescence of ischemic stroke. Thus, CLT was prolonged and OFP was reduced (study I) as was the fibrinolytic capacity (“fibrinolysis profile”; study II). Some correlations were also found between the single fibrinolytic markers and global fibrinolysis; TAFI correlated to CLT and was inversely related to OFP (study I). D-dimer levels were slightly but continuously elevated. This reflects ongoing fibrin formation and degradation (“fibrin turnover”), but is not a direct measurement of fibrinolysis.

The mechanisms behind impaired fibrinolysis is likely to be at least in part connected to increased inflammation and in support of this idea we observed some relationships between inflammatory markers, and TAFI as well as OFP. It thus seems that ischemic stroke is characterized by an impaired fibrinolysis. No previous studies of the more global aspects of fibrinolysis in acute IS populations have been performed to confirm our results, but interestingly Anzej *et al.* found a decrease in OFP in a population of younger (< 45 years of age) IS patients (n=44) median five years after the event ¹⁸¹. This, together with a proneness to form a tight fibrin network as shown in study II, may indeed be of pathophysiological importance with respect to thrombus formation and dissolution in IS.

Ischemic stroke subtypes

The small vessel (or lacunar) stroke did not differ from the other subtypes in any of the hemostatic markers measured in this thesis, in line with some studies ^{112, 114} but in contradiction to others ^{56, 113 82}. This work does not support the idea of treating the small vessel group differently from an antithrombotic point of view.

On the other hand, the cardioembolic subgroup due to AF seemed to have an elevated thrombin potential in line with reports from other research groups ^{56, 116, 117}. They are therefore at increased risk of cerebral thromboembolic complications, justifying treatment with oral anticoagulants.

TIA versus stroke

No significant differences in neither thrombin generation (measured by CAT) nor in microparticle activation could be detected between TIA and manifest IS patients. No study has, to our knowledge explicitly compared TIA and IS patients separately in studies of hemostasis. Lee *et al.* (Table 4, part 4.2.5) included the TIA group in the small vessel entity¹⁵⁸ which is not quite true because a TIA can be seen in patients with a small and a large vessel or in a patient with a cardioembolic source.

Objections may be raised against the fact that we have classified the TIA-patients with TOAST and Bamford in study III and IV, since these classifications have only been validated in populations with manifest stroke^{94,99}. One argument justifying this way of action is that nowadays the border between TIA and manifest stroke is not as clear-cut due to the fact that approximately one-third of the TIA-patients present with an ischemic lesion on MRI¹⁵⁰. In addition, patients often have experienced both kinds of events in their medical history and TIA/stroke also share the same riskfactors and treatment strategies. Finally, a TIA-episode could be re-evaluated as a manifest stroke when the patient comes for a follow-up visit and tells about some of the neurological deficits experienced in connection with the “TIA-episode” still to be present.

In summary, the fact that we found no significant changes between TIA and manifest stroke in any of the hemostatic variables measured, supports the intention to have the same treatment strategies for TIA and ischemic stroke.

How can these hemostatic disturbances be modulated?

Life style aspects

Hemostatic factors may be influenced by some changes in lifestyle. An increase in physical activity has been shown to have a positive effect on thrombin generation as measured by F1+2 in plasma¹⁸⁷ and obesity (both in adults¹⁸⁸ and in children¹⁸⁹) is associated with elevated thrombin generation. Thus exercise and weight reduction would be assumed to reduce thrombin generation in patients with ischemic stroke, although obesity was not a large clinical problem in the stroke population investigated in this thesis. Smoking, another well-known risk factor, affects both fibrin network tightness and clot lysis negatively¹⁹⁰. It is reasonable to assume that to stop smoking may beneficially influence these variables of which the fibrin network is, as shown in this thesis, adversely changed in patients with ischemic stroke. Only 6%, however, were smokers in study II, thus no statistical power could be reached.

Treatment aspects

The effect of **low-dose aspirin** has a positive effect on the fibrin network porosity by a postulated acetylation of lysine residues of the fibrinogen protein^{191 68}. It is conceivable that such an effect may be an additional mechanism explaining the beneficial effect of aspirin in IS and coronary artery disease. The Ks-values (reflecting network porosity) of the ischemic stroke patients studied in this thesis are almost in the same range as in diabetes patients⁷² and in patients with coronary heart patients¹⁷⁶, although comparisons must be done with some caution as slightly different methods have been used. It should be noted, however, that low-dose aspirin does not seem to be sufficient to reduce the tightness of the fibrin network found in this thesis. Thus, better treatment strategies are warranted.

Statins can be yet another way of attenuating the activated hemostasis in acute IS. As mentioned previously platelet microparticle expression of tissue factor and P-selectin are significantly reduced by statins^{170,171}. This supports the use of statins in acute IS, and is in line with what has been shown in acute coronary syndromes¹⁹². Also the fibrin network can become more porous following statin¹⁷⁰ and ACE-inhibitor treatment¹⁹³. Of note, treatment with ACE-inhibitors may also reduce thrombin generation¹⁹⁴ and this would strongly argue for increased use of ACE-inhibitors in stroke secondary prophylaxis.

Patients with paroxysmal **atrial fibrillation** had thrombin concentrations in the upper range. Prevention of AF through increased use of ACE-inhibitors and other drugs counteracting the renin-angiotensin-aldosterone system, may be yet another way to lower thrombin generation¹⁹⁴ and decrease thromboembolic complications. Previous studies of anticoagulants in acute IS have been discouraged by the increased risk of bleeding complications¹⁹⁵, yet this would be perhaps the most powerful treatment to inhibit thrombin generation¹⁹⁶ improve fibrinolysis and make the fibrin network more porous¹⁹⁷. Notably, some evidence of beneficial effect of early anticoagulation has been presented in earlier studies, with a significantly reduced risk of having a recurrent stroke within the first two weeks¹⁹⁵. This supports indirectly the results of this thesis of an activated hemostasis as a part of the acute IS pathophysiology and suggest its reversal by a early direct anticoagulating treatment.

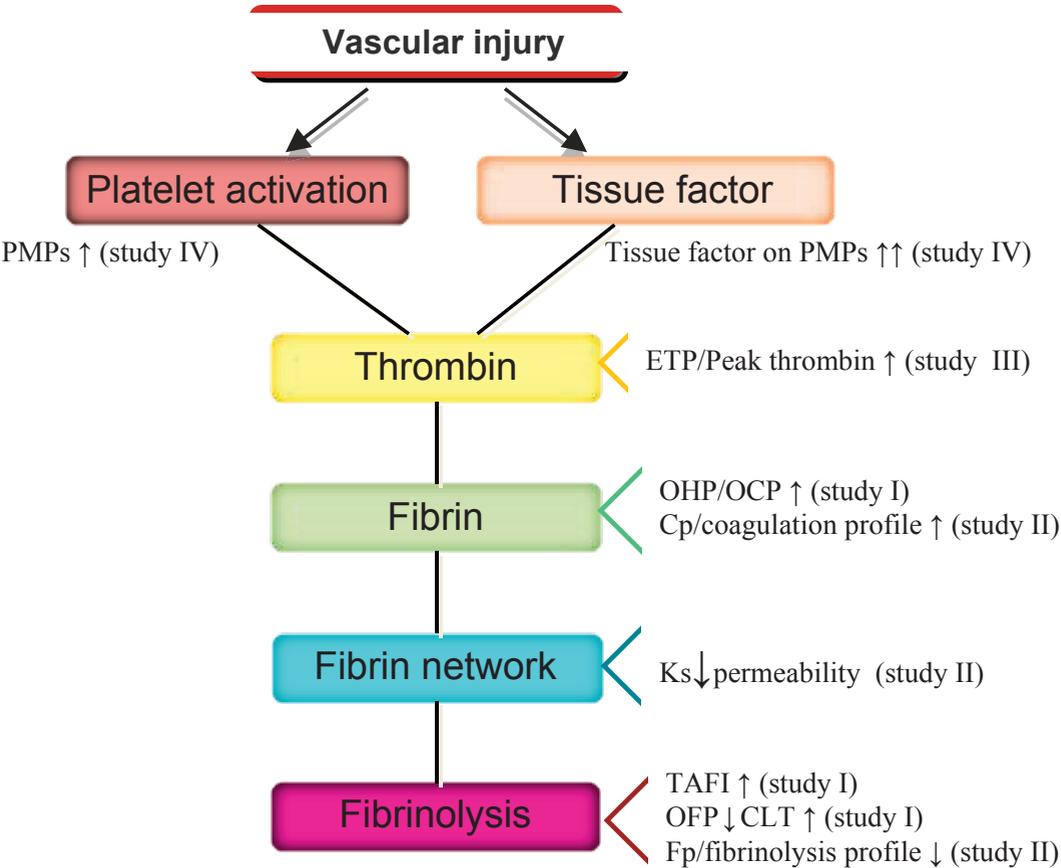
General remarks

In summary, the hemostatic disturbances of acute ischemic stroke patients involves, as shown in this thesis, indeed all three “corners” of Virchow’s triad (Fig 1). These disturbances may in part be due to atherosclerosis as the site of vascular injury and a potential “starting point” for the hemostatic activation (Fig 2 and 17). A causality between the hemostatic system and progression of atherosclerosis has recently been proposed by the research group of Ten Cate¹⁹⁸. Disturbances of hemostasis may also partly be a consequence of the stroke itself, with an acute phase reaction which declines in due course.

Regardless of etiology, global assays of hemostasis would provide a possibility to assess the hemostatic balance in ischemic stroke patients, used as a complement to conventional risk factor evaluation. There is, however no global assays ready to be introduced into the clinical point-of-care situation. At present, the Calibrated automated thrombogram is probably the strongest candidate of the methods used in this thesis.

8 CONCLUSIONS

Fig 17. A schematic flow chart of the hemostatic system and thesis results.



OVERALL CONCLUSIONS

- A disturbed hemostasis is present in patients with ischemic stroke (IS), both during the acute and convalescent phase of the disease. Disturbances involve platelet function, thrombin generation, fibrin formation and the structure of the fibrin network, as well as fibrinolysis.
- No differences in the pattern of disturbances of hemostasis could be seen when different subtypes of IS were compared, except for a tendency towards an increased thrombin generation in cardioembolic stroke due to paroxysmal atrial fibrillation.
- Large prospective studies should be carried out to determine the potential value of global methods evaluating the balance between coagulation and fibrinolysis in plasma from patients with IS. Until then, the global hemostatic methods tested in the present thesis are not ready to support the decision-making concerning antithrombotic treatment strategies.

SPECIFIC CONCLUSIONS

- Platelet-derived microparticles in plasma are significantly increased in IS, both during the acute and in the subacute phase of the disease. This reflects increased platelet activation.
- The number of platelet-derived microparticles in IS patients that expose tissue factor and P-selectin on their surface are greatly increased.
- No differences could be detected between subtypes of IS regarding the exposure of tissue factor or P-selectin on platelet-derived microparticles.
- An increased thrombin generation measured by CAT is present in plasma from patients with IS, both in the acute phase and after one month.
- The cardioembolic subgroup had a trend towards a more prominent thrombin generation measured by CAT as compared to the non-cardioembolic group.
- Plasma from IS patients is characterized by its ability to form a less permeable fibrin network. This ability is present in the acute phase and seems also to be present two months post-stroke.
- IS is associated with increased TAFI and PAI-1 antigen levels in plasma, and a decreased fibrinolysis as measured by two global fibrinolysis methods.

9 FUTURE PERSPECTIVES

It is rather difficult to tell whether the tighter fibrin network is a phenomena caused by the stroke itself or if it is a phenotype present even before the stroke occur. We did not design the study in such a fashion, but of course it would be interesting to follow a group of stroke-free subjects for a longer period and measure the fibrin network at baseline. A population with atrial fibrillation without a TIA/stroke in the history is a tempting target for a new study. Unfortunately, until a more automatized method is available, this would turn the laboratory staff crazy as this would become a very time- and labourconsuming study.

More than four years have passed since the first patient in study III and IV was included (2007 20th of June) and we now have a golden opportunity of a follow up study to evaluate both microparticles and CAT-data in a prospective manner.

Lifestyle interventions are interesting in the context of hemostasis and a fairly large randomised study in patients with impaired glucose tolerance or diabetes and ischemic stroke would be of great interest with evaluation using some of the methods presented in this thesis.

Finally, if we could be able to agree upon the best global assay of hemostasis and get it standardized with a low inter/intra-assay variability, one hopes for a tool in the future with which we can foresee the individual risk for new cardiovascular events. In that way one can be able to tailor the treatment and know when to initiate or abstain from antithrombotic treatment.

10 SVENSK SAMMANFATTNING

Stroke är den andra vanligaste orsaken till dödlighet efter hjärtkärlsjukdom. Cirka 85% av alla strokefall är orsakade av en hjärninfarkt (blodpropp i hjärnans cirkulation). Modern behandling av akut hjärninfarkt innefattar trombolys (blodproppsupplösande behandling) och blodförtunnande behandling för att förhindra nya proppbildningar. Trots denna behandling är det många patienter som drabbas av en ny stroke.

I kroppen finns ett system som gör att blodet sig hela tiden håller flytande i blodkärlen och som kallas för *hemostasen*. Detta är en finstämd balans mellan å ena sidan benägenheten att bilda blodproppar (koagulationen) och å andra sidan förmågan att lösa upp bildade proppar (fibrinolysen). Om denna balans blir störd av någon anledning kan blödningar eller blodproppar uppstå lättare. I denna studie undersöktes patienter med hjärninfarkt eller TIA (övergående syrebrist till hjärnan) där vi med tyngdpunkt på olika globala metoder som mäter balansen i hemostasen, ville se vilka som hade den största störningen i hemostasen och därmed sannolikt löper störst risk för nya stroke.

I den första pilotstudien på 32 patienter med akut hjärninfarkt (**Studie I**) mättes en faktor som dämpar fibrinolysen, den s.k. trombin aktiverade fibrinolys hämmaren (TAFI) i blodprov. Parallellt undersöktes också balansen i hemostasen med en global metod, Overall Hemostatic Potential (OHP). Resultaten visade att TAFI var förhöjt i akuta skedet efter stroke men att värdena hade normaliserats efter två månader. Den fibrinolytiska förmågan, d.v.s. förmågan att lösa upp proppar, visade sig vara nedsatt både akuta och efter 2 månader.

I nästa studie (**Studie II**) på samma grupp av patienter, analyserades genomsläppligheten hos det fibrinätverk som "armerar" blodproppen när blodet koagulerar. Fibrinätverket bildat under standardiserade former utifrån plasma från patienter med akut hjärninfarkt, visade sig vara tätare än hos motsvarande friska personer. Återigen kunde vi se att den fibrinolytiska förmågan var nedsatt både i akuta skedet och efter 1 månad, denna gång med en vidareutvecklad metod, Overall hemostatic-index (OH-index).

I en betydligt större studie med mer än 200 patienter med akut hjärninfarkt och TIA, utfördes riktade undersökningar för att hitta dolda förmaksflimmer. För att försöka hitta undergrupper av strokepatienter med ökad aktivitet i koagulationssystemet, gjordes en analys i blodet med en metod som mäter bildningen av trombin (det protein som deltar i det sista steget i koagulationen för att bilda fibrin), Calibrated automated thrombogram (CAT) (**Studie III**). Där såg man att patienterna hade en ökad trombinbildning jämfört med motsvarande friska kontroller, med en trend mot ökad bildning hos de patienter som befanns ha ett övergående förmaksflimmer. I den sista studien (**Studie IV**) mättes antalet mikropartiklar (som är kända för att aktivera koagulations-faktorerna) i blodet. Antalet mikropartiklar visade sig ligga signifikant högre hos patienter med akut hjärninfarkt och TIA.

Sammanfattningsvis är akut hjärninfarkt och TIA förenat med en störd balans med å ena sidan en ökad koagulation och å andra sidan en försämrad fibrinolys. Hur kan man då sträva efter att återställa balansen mellan koagulationen och fibrinolysen hos stroke patienterna? Med de effektiva blodförtunnande och blodfetsänkande läkemedel vi har idag kan hemostasen påverkas positivt och därför är det viktigt att diagnostisera och behandla hjärninfarkt och TIA så snabbt som möjligt. Förbättrad livstil kan sannolikt också påverka en del av de hemostasförändringar som uppdragats inom ramen för denna studie. Framtida studier får avgöra om riktade livstilsråd kan förbättra balansen mellan koagulation och fibrinolys hos stroke patienter. Den patient-nära vården är inte redo för beslut gällande ändringar av

behandling val av blodförtunnande behandling grundat på de metoder som används i denna avhandling, studier som följer patienterna över en längre tid behövs först.

11 ACKNOWLEDGEMENTS

Håkan Wallén – for the perfect mentoring you have delivered throughout the years despite your many other engagements. I have been very fortunate to work together with you as you have steered me in the right direction at all times and in a skilful manner helped me completing this thesis.

Magnus von Arbin – who has taught me the essence of what research and science really are about. I have always felt that you have had a goal of facilitating my life as a PhD-student and you have succeeded well in doing so.

Ann-Charlotte Laska – because you as a colleague and my former chief has given me your full support in everything I have done.

Margareta Blombäck – for the engaging and caring person you are; this whole research project was worth it just to get to know you!

Jovan Antovic – for your expert knowledge within the coagulation field that I needed so much.

Shu He – for valuable scientific discussions.

Jan Svensson – for valuable and wise reflections upon my research.

Jan Lundberg – my mentor, for making me realize that a PhD was the only right thing to do.

Mika Skeppholm – for all the encouragement you have given me that has pushed me forward when things were feeling tough. Without you and your “twin project” I am not sure I would have had reached this far and we have had many laughs together on the way.

Eli Westerlund – as a friend and research colleague I want you to feel the same support as I felt from you.

Joakim Bragd – thank you for all the happy moments we had with statistics and thanks for the ride! Your support during the last period of my thesis was very valuable to me.

Ingrid Dalenbring – for your ebullient energy and interest in my project although you had to bear a great load in the clinic for my sake.

Eva Lindström – for your good advice and schedule fixing so I had the opportunity to focus at last.

All my other **colleagues at the Stroke/Neuro** – for your great interest in my work, for your valuable scientific feed-back on my research and most of all your way of making such a cheerful atmosphere at our unit.

All friends and **colleagues at the Department of Internal Medicine** – I know and appreciate your hard and skilful work at the clinic while I have been off duty writing my thesis. Without your indirect support I would have had lesser possibilities of focusing as I needed to.

Fariborz Mobarrez – for always assisting me in every way with a happy mood even if you had a great deal of things to do of your own.

The **staff at Clinical Research Center North** – for your ever so positive attitude and willingness to help me at short notice.

Eva Isaksson and **Helena Kumpulainen** – without your help I would not have reached this far.

Ingrid Jacobsson – for your indispensable help in the lab. Hope to see you next time at Möja!

The **staff at the Stroke Unit** at Danderyd Hospital – for always doing the right things to support my projects, from the “leopardbox” to the thumb-ECG:s. It has been a long journey and now I will be back to work with you again.

To all the **patients and controls** – for your cooperation within the studies making this thesis possible. You have given me the opportunity to understand the stroke disease better.

Professor **Mårten Rosenqvist** – for the person of great ability to inspire me and others in the “PROPPSTOPP”-study.

Professor **Nils Wahlgren**, co-author, for good cooperation.

Associate Professor **Gun Jörneskog**, Professor **Per-Erik Lins** and Professor **Ulf Adamsson** for your scientific skills at our clinic, giving me inspiration to go on with my own work.

Erik Näslund – dean at the Department of Clinical Sciences for enabling me to join the research team at Danderyd Hospital. Associate Professor **Hans Persson** as a co-worker in the “PROPPSTOPP”-study and Professor **Thomas Kahan** for your commitment in vascular medicine.

To the **Jan Lundberg group** at the Clinical Pharmacology Department – for the fun memories of working with you all; this was the start of my first attempts in research.

Åsa Kuntze Söderqvist – who have been a dear friend of mine throughout the years, sharing the same field of interest also in our work. You have always encouraged me in what I have done and I feel lucky to have you near for pleasant walks when life is hectic.

Erik Änggård – my uncle and godfather, for introducing me to the field of research at the William Harvey Institute and for your hospitality during this very pleasant period in my life.

Anders Änggård – my father, for your true interest in my work, for your great knowledge within the scientific field, and for being such a good father to me.

Agneta Änggård – my mother, for your never-ending energy and support in both small and large matters, and for bringing little me into the world.

My brother and sister **Ulf and Ulrika** – for always believing in my ability and Ulrika for very welcome breaks during my writing work sessions.

Super – our hamster for keeping me company during long hours in front of the computer...

My loving family, **Jens, Axel and Emelie** – for your enormous support in your own personal ways during this work and for putting up with me all this time. You are the best family one can have!

These studies were supported by grants and scholarships from: Goljes Memory foundation, Stiftelsen Serafimerlasarettet, Swedish Strokefoundation, The Funds 176 and 245 of Karolinska Institutet, Magnus Bergwalls foundation, Capio Research Fund, Swedish Heart and Lung fondation and the regional Agreement on Medical Training and Clinical Research (ALF) between Stockholm County Council and the Karolinska Institutet.

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