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Serial changes in markers measuring coagulation, fibrinolysis, and vasoactivity in patients with ischemic stroke

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ACADEMIC DISSERTATION

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To my family

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List of original publications

This thesis is based on the following publications, referred to in the text by their Roman numerals:

I Haapaniemi E, Tatlisumak T, Hamel K, Soinne L, Lanni C, Opgenorth TJ, Kaste M. Plasma endothelin-1 levels neither increase nor correlate with neurological scores, stroke risk factors, or outcome in patients with ischemic stroke. Stroke 2000; 31: 720–725.

II Haapaniemi E, Helenius J, Soinne L, Syrjälä M, Kaste M, Tatlisumak T. Serial measurements of plasma homocysteine levels in early and late phases of ischemic stroke. Eur J Neurol 2006 (in press).

III Haapaniemi E, Soinne L, Syrjälä M, Kaste M, Tatlisumak T. Serial changes in fibrinolysis and coagulation activation markers in acute and convalescent phase of ischemic stroke. Acta Neurol Scand 2004; 110: 242–247.

IV Haapaniemi E, Tatlisumak T, Soinne L, Syrjälä M, Kaste M. Natural anticoagulants (antithrombin III, protein C, and protein S) in patients with mild to moderate ischemic stroke. Acta Neurol Scand 2002; 105: 107–114.

V Haapaniemi E, Helenius J, Jakovljević D, Syrjälä M, Kaste M, Tatlisumak T. Clinical, laboratory, radiological, and prognostic characteristics of ischemic stroke patients with heterozygous factor V Leiden mutation (submitted).

In addition, some unpublished data are presented.

Abbreviations

AF	atrial fibrillation
AH	arterial hypertension
APC	activated protein C
APCR	activated protein C resistance
AT	antithrombin
BI	Barthel Index
BMI	body mass index
CBF	cerebral blood flow
CBS	cystathione-beta-synthase
CRP	C-reactive protein
CT	computerized tomography
DM	diabetes mellitus
DVT	deep vein thrombosis
ET-1	endothelin-1
F1+2	prothrombin fragment 1+2
FVLm	factor V Leiden mutation
GCS	Glasgow Coma Scale
Hcy	homocysteine
IS	ischemic stroke
MMSE	Mini-Mental Status Examination
MRI	magnetic resonance imaging
mRS	Modified Rankin Scale
MTHFR	5,10-methylenetetrahydrofolate reductase
NIHSS	National Institutes of Health Stroke Scale
OCSP	Oxfordshire Community Stroke Project
PAI-1	plasminogen activator inhibitor type 1
PAOD	peripheral arterial occlusive disease
PC	protein C
PFO	patent foramen ovale
PS	protein S
SSS	Scandinavian Stroke Scale
TAT	thrombin-antithrombin complex
TOAST	Trial of Org 10172 in Acute Stroke Treatment
t-PA:Ag	tissue plasminogen activator antigen
UFH	unfractionated heparin
u-PA	urokinase-type plasminogen activator

Abstract

Ischemic stroke (IS) is a heterogeneous disease in which outcome is influenced by many factors. The hemostatic system is activated in association with cerebral ischemia, and markers measuring coagulation, fibrinolysis, and vasoactivity could be useful tools in clinical practice. However, previous studies have not determined these markers in a well-defined population of patients with IS, nor have studies included well-structured follow-up with repeated measurements.

The main purpose of this study was to identify changes in the activities of markers measuring coagulation, fibrinolysis, and vasoactivity in a population of patients with first-ever IS. It was investigated whether repeated measurements of these markers reveal patterns that might help in evaluating IS patients, including the early diagnosis of stroke subtypes, in estimating prognosis and risk of recurrence, and in selecting a treatment for secondary prevention of stroke.

Markers assessing coagulation, fibrinolysis, and vasoactivity were measured in 102 consecutive mild to moderate IS patients on four occasions: on admission and at 1 week, 1 month, and 3 months after stroke, but only once in the control group. All patients underwent neurological examination and blood sampling in the same session. All patients were contacted 3 years later for prognostic determination. Vasoconstrictor peptide endothelin-1 (ET-1), homocysteine (Hcy), indicators of thrombin formation and activation (prothrombin fragment 1+2/F1+2, thrombin-antithrombin complex/TAT), indicators of plasmin formation and fibrinolysis (tissue plasminogen activator/t-PA, plasminogen activator inhibitor-1/PAI-1, and D-dimer), and natural anticoagulants (antithrombin/AT, protein C/PC, and protein S/PS) were measured. Furthermore, 740 patients with clinically and radiologically confirmed IS without an obvious etiology were screened for factor V Leiden mutation (FVLm). Forty-two IS patients with proven heterozygous FVLm were evaluated in detail for specific clinical, laboratory, and radiological features.

Repeated measurements of ET-1 levels did not disclose information that could aid in the diagnostic evaluation of IS patients (I). Increased local production of ET-1 may not be correctly detected in peripheral blood because ET-1 is a local regulating factor and is higher at the interface of the endothelium than in plasma. Measurement from the cerebral blood flow (CSF) during the hyperacute phase may deliver more accurate results.

Plasma Hcy levels were significantly lower in IS patients than in control subjects in the acute stage, increasing and remaining stable during the

convalescent period (II). This marked decrease in Hcy levels upon occurrence of IS may be a protective reaction of the human body aimed at limiting brain injury or may be a nonspecific acute phase reaction.

The follow-up examination of IS patients showed that F1+2 level at 3 months after stroke had a positive correlation with recurrence of thromboembolic events and may be used as a predictive marker of subsequent cerebral events (III). These data support the idea that IS patients with sustained activation of the blood coagulation system and increased thrombin generation may have an unfavorable prognosis.

The elevated D-dimer levels on admission and 1 week after IS were strongly associated with stroke outcome and disability (III). Elevated levels of D-dimer may reflect the level of activation of both the fibrinolytic and coagulation systems, and, thus, the ongoing thrombotic process.

AT level on admission (IV) was significantly correlated with stroke severity and outcome. These results probably reflect an increased consumption of active AT due to the extent of thrombosis.

The specific analysis of IS patients with heterozygous FVLm (V) more often revealed a positive family history of thrombosis, a higher prevalence of peripheral vascular disease, and multiple infarctions in brain images, most of which were 'silent infarcts'. Presence of FVLm in IS patients did not affect stroke severity or outcome.

In summary, results of this study suggest that changes in the markers measuring coagulation, fibrinolysis, and vasoactivity could be useful for predicting prognosis of IS patients. A clear need exists for a randomized prospective study to determine whether a subgroup of IS patients with markers indicating activation of the fibrinolytic and coagulation systems might benefit from more aggressive secondary prevention of IS.

Introduction

The property of the circulation whereby blood retains its fluidity within the vasculature while the system simultaneously prevents excessive blood loss upon injury is known as hemostasis. Approximately 150 years ago, Virchow proposed that three anatomic compartments – blood flow, blood components (soluble and cellular constituents), and the blood vessel wall – are involved in a delicately orchestrated biochemical interplay which, under normal conditions, maintains the hemostatic equilibrium. Blood constituents do not normally interact with undamaged vascular endothelium. In response to vascular injury, clotting reactions are initiated to create an insoluble fibrin-platelet plug, arrest blood loss, and eventually restore vascular integrity. The arterial flow condition produces platelet-rich ("white") thrombi and static venous flow yield fibrin- and red cell-rich ("red") thrombi. Disturbances are reflected in functional changes in the endothelium, platelets, coagulation, or fibrinolysis that predispose to thrombus formation or hemorrhage, or sometimes both (35) (Figure 1).

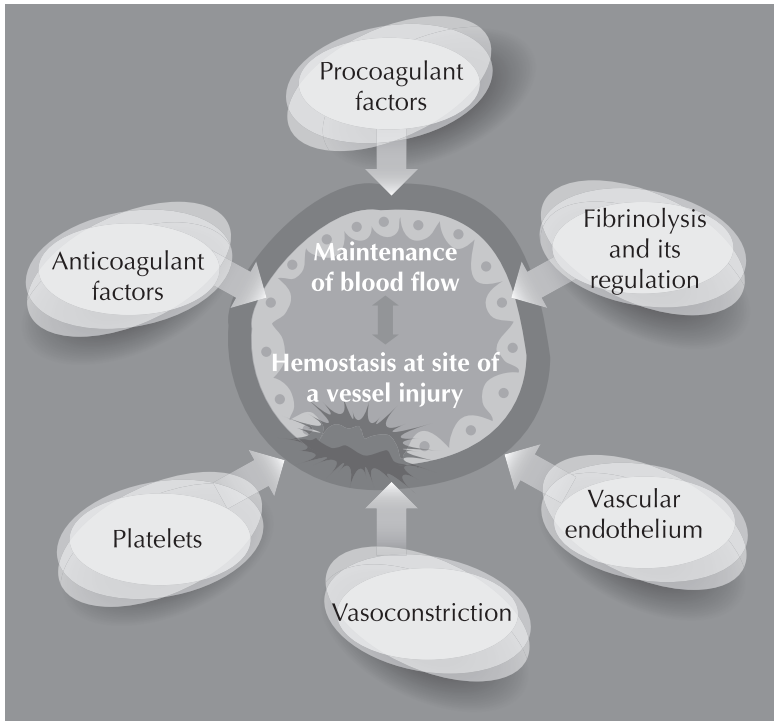

Figure 1. Normal physiology of hemostasis. Adapted from Casey: Nurs Stand, Volume 18 (7), 2003, 45–51.

A large number of epidemiological and case-control studies have provided strong evidence of an association between activation of the hemostatic system and ischemic stroke. Several hemostatic markers have been measured in the acute as well as the chronic phase of IS. Some data have been published about early involvement of the hemostatic system in arteriosclerosis. Evidence exists of increased thrombin generation and fibrin turnover, altered fibrinolytic activity, and disturbed endothelial function in IS (16, 20, 21, 164, 259). However, most previous studies have included one-time measurements of markers of coagulation and fibrinolysis or measurement of only a limited number of factors.

Shifts in the balance of the hemostatic system may play an important role in the efficacy and safety of IS treatment. Markers measuring coagulation, fibrinolysis, and vasoactivity could potentially be used as indicators of the risk-benefit ratio when patients are evaluated for thrombolytic therapy. They could also explain the subsequent response to thrombolysis (241).

This study aimed to improve our understanding of the underlying mechanism of IS and to clarify how the course of IS influences changes in hemostatic balance. To this end, serial changes in markers measuring coagulation, fibrinolysis, and vasoactivity in patients after first-ever IS were investigated. Further, we identified whether measurement of these markers discloses patterns that might yield causative, prognostic, and preventive information useful in evaluation of IS patients.

Review of the literature

Ischemic stroke

Epidemiology

Stroke is a clinically defined syndrome of rapidly developing symptoms or signs of focal loss of cerebral function with no apparent cause other than vascular origin. However, the loss of function can at times be global. Symptoms of stroke last more than 24 h or may lead to death before this (11). Stroke is the third leading cause of death in developed world after coronary artery disease and all types of cancer combined. It causes 4.4 million (9%) of the total of 50.5 million deaths each year (112). In most Western populations, 0.2% of the population (2000 persons per million) suffer a stroke each year, of whom one third die over the next year, one third remain permanently disabled, and one third make a reasonable recovery. Each year about 14 000 people suffer a stroke in Finland, 5000 of whom die either acutely or during the first year after the stroke (91).

In the past, Finland had one of the highest reported incidences of stroke in the world (17). Most studies have described a steady declining trend, but also an increase in stroke incidence during the 1960s and 1970s has been reported. Since the 1990s, however, the incidence of stroke has declined steeply (268). During a 10-year period (from 1988 to 1997) the incidence of IS in the Kuopio area fell by 5.2%/year in both men and women. The rates declined from 289 to 182 per 100 000 in men and from 158 to 99 per 100 000 in women (235). In the FINSTROKE register population, the mortality rate fell during a 15-year period (1983–1997) from 85 to 51 per 100 000 in men (–3.7%/year) and from 50 to 30 per 100 000 in women (–4.1%/year) (235).

A significant circadian rhythm has been observed for cerebrovascular disease (201). About one-third of all strokes occur in the morning between 7 a.m. and noon (99).

As the most important cause of long-term disability, stroke has a considerable impact on public health. One-third of strokes occur in working-aged people, being a more common cause of early retirement in Finland than ischemic heart disease in people under the age of 55 years. Stroke is a leading neurological reason for hospitalization, accounting for 29 000 hospitalizations and 2 million bed-days annually in Finland (214). The overall direct and indirect costs of strokes were estimated to be as high as 440 million euros in 1999, i.e. 6.1% of the total national health system budget (214).

Risk factors

Epidemiological investigations have identified numerous IS risk factors. These can be divided into two groups for practical reasons: nonmodifiable and modifiable risk factors. Age, sex, ethnicity, heredity, and family history of vascular disease have been established as nonmodifiable risk factors.

After age, arterial hypertension (AH) is the most powerful stroke risk factor. The incidence of stroke rises in proportion with blood pressure. In the Framingham study, the relative risk of stroke for a 10 mm Hg increase in systolic blood pressure was 1.9 for men and 1.7 for women. A systematic overview of 14 prospective studies indicated that a decrease in diastolic blood pressure of 5–6 mm Hg reduces the risk of stroke by 42% (59). Cardiac disease is an important precursor of IS, particularly coronary artery disease, atrial fibrillation (AF), valvular heart disease, myocardial infarction, congestive heart disease, and left ventricular hypertrophy. AF is associated with one of every six IS. In patients with AF, the annual risk of stroke is 1.3–6%. Stroke risk nearly doubles in those with coronary artery disease and quadruples in subjects with cardiac failure (219). Stroke affects 0.7–4.7% of patients after acute myocardial infarction (279).

Diabetes mellitus (DM) constitutes an independent risk factor for IS, with a relative risk ranging from 1.8 to 3.0 depending on type and severity. Data from the Framingham study suggested that subjects with glucose intolerance have an increased risk of IS by a factor of two as compared with nondiabetics (219). In the Honolulu Heart Study, the risk for thromboembolic stroke was lowest in persons with low-normal fasting glucose and increased with high-normal fasting glucose, asymptomatic high blood glucose, and DM, the latter of which was associated with the highest stroke risk (48).

The link between serum cholesterol level and the incidence of stroke is not fully established. A report from the Atherosclerosis Risk in Communities (ARIC) Study found only a weak and inconsistent association between IS and each of five lipid factors in the 305 subjects experiencing IS after 10 years of prospective investigation (231). In a meta-analysis of 45 prospective epidemiological studies comprising 450 000 subjects among whom 13 000 strokes occurred, no significant association between total serum cholesterol and total stroke incidence was seen (7). Despite the lack of association of blood lipids with incidence of IS, a significant reduction in stroke incidence has been shown in a series of trials of statins in patients with coronary heart disease (5, 9, 207).

Obesity has been associated with higher levels of blood pressure, blood glucose, and atherogenic serum lipids (219). Obesity, as reflected in body mass index (BMI), and waist circumference were related to stroke incidence (271). Smoking has been clearly linked to IS. Smoking cessation markedly reduces the increased risk of IS in two years, and the risk is nonexistent after five years (144). The role of alcohol as a stroke risk factor depends on stroke subtype and the amount of alcohol consumed (51).

Pathophysiology

The brain is supplied by two major arterial systems. Much of the cerebral hemispheres are supplied by the carotid arterial system, whereas the entire posterior fossa, occipital lobes, and portions of the temporal lobes are supplied by the vertebrobasilar system. A series of anastomotic channels, including the circle of Willis located at the base of the brain, interconnect these two systems. Half of the brain weight and volume comprises gray matter and the other half white matter (275).

Cerebral blood flow (CBF) of the gray matter is about 80 mL and of the white matter about 20 mL/min/100 g brain tissue. CBF is normally maintained at a relatively constant rate by a process of autoregulation and can thereby compensate for a wide range of fluctuations in perfusion pressure and cerebrovascular resistance. Ischemic strokes result from occlusion of an artery, preventing CBF to a specific area of the brain (45). The residual CBF depends on the degree of obstruction and the availability of collateral flow. Ultimately, the amount of injury is proportional to the duration and severity of the ischemia. As the amount of flow diminishes, the brain compensates by local vasodilatation, opening of collaterals, and increasing the fraction of extracted oxygen and glucose. When CBF is reduced below 18 mL/min/100 g tissue, synaptic transmission stops and electrical silence ensues, thereby decreasing cerebral energy use by 50%. After a further decline in CBF to below 8 mL/min/100 g, a series of events unfold ("ischemic cascade"). The process of metabolic failure leading to cell death involves insufficient blood flow, loss of energy, and neuronal depolarization (45). The resultant release of glutamate, entry of Ca^{2+} into cells, generation of oxygen free radicals and nitric oxide, and activation of proteases and lipases all contribute to pathologic changes in neuronal function and structure.

If the normal protective mechanisms are insufficient to compensate for this deprivation, death of tissue, i.e. infarction, develops (31). The primary injury results in an initial ischemic core. The CBF in the core is usually below 25% of normal. Surrounding this core is an area of decreased blood flow ("ischemic penumbra") that may be salvaged if blood flow is reestablished promptly. The CBF in the penumbra is approximately 25–50% of normal, which is sufficient to preserve energy metabolism and maintain tissue viability for a period of hours. Since autoregulation is lost in the area of ischemia and the ischemic region is perfusion-dependent, any decrease in systemic arterial blood pressure can extend the area of ischemia and infarction. If the state of low CBF continues, the penumbra zone or part of it may proceed to irreversible cell death (31).

Classification

Approximately 80% of all strokes are ischemic, 10% are due to intracerebral hemorrhage, and the rest are due to subarachnoid hemorrhage. In IS, the most common pathophysiological bases in the majority of cases are atherosclerosis

of extra- and intracranial arteries (14–40% of all IS), cardiogenic embolism (15–30%), and penetrating small-artery disease (15–30%) (221). Stroke prognosis, recurrence risk, and management choices are influenced by IS subtype. The stroke subtype diagnosis guides treatment decisions and may be important for understanding differences in the impact of a given intervention in a clinical trial setting. There is no universally accepted classification of IS subtypes. The Trial of Org 10172 (danaparoid sodium, low molecular weight heparinoid) in Acute Stroke Treatment (TOAST) (13) classification grades IS patients with regard to pathophysiology and etiology; it has been widely used in both prospective clinical trials and retrospective studies of patterns of care and stroke-related outcomes, and it provides information relevant to secondary prevention. There are five major categories of IS in the TOAST classification: (1) large-artery atherosclerosis (LAA), including large-artery thrombosis and artery-to-artery embolism; (2) cardioembolism (CE); (3) small-artery occlusion (SAO); (4) stroke of other determined cause (OC); and (5) stroke of undetermined cause (UND). Strokes of undetermined origin required fulfilling one of the following two criteria: (1) no cause was found despite an extensive evaluation or (2) a most likely cause could not be determined because more than one plausible cause was found. The subtype definitions were based on risk factor profiles, clinical features, and results of diagnostic tests. The latter included computerized tomography (CT) scan, magnetic resonance imaging (MRI), vascular imaging (carotid duplex, transcranial Doppler), electrocardiography (ECG), Holter recording, echocardiography (transesophageal or transthoracic), assessment of prothrombotic syndromes, and postmortem examination. In a recent study (146), the distribution of subtypes in a European population was as follows: LAA 13%, CE 27%, SAO 23%, OC 2%, and UND 35%. Frequency of infarct subtypes may differ among populations depending on patient selection and geographic region. The Oxfordshire Community Stroke Project (OCSP) criteria categorize subtypes of IS primarily on the basis of vascular territory into four groups: total anterior circulation infarction (TACI), partial anterior circulation infarction (PACI), lacunar infarction (LACI), and posterior circulation infarction (POCI) (27). This classification is reasonably predictive of etiology, prognosis, and the size and site of cerebral infarction with CT scan. Distribution of the IS subtypes among the patients in the OCSP emerged as follows: 17% had TACI (both cortical and subcortical involvement), 34% PACI, 25% LACI, and 24% POCI.

Clinical aspects

The main clinical feature of stroke is its acute onset. The symptoms produced reflect the location and nature of the pathological process. Lesions involving the carotid system may alter function in the distribution of any of its three clinically important branches: opthalmic artery, anterior cerebral artery, and middle cerebral artery. Therefore, the various combinations of hemiparesis,

hemisensory deficit, monocular visual loss, and aphasia are suggestive of the lesion in this system. Lesions involving the vertebrobasilar system may alter function in the region of the cerebellum, the brainstem, and the occipital and temporal lobes via the posterior cerebral artery. The various combinations of diplopia, dysarthria, dysphagia, and disequilibrium associated with hemiparesis, hemisensory deficit, or homonymous hemianopia are suggestive of a lesion in the vertebrobasilar system (255).

Clinical grading scales are important in the evaluation and management of patients with acute neurological disorders. In addition, these assessments are needed to identify those stroke patients at higher risk for early death, and to provide patients and their caregivers with more accurate prognostic information. Several scales have been developed to measure recovery and disability after acute stroke. Some of the most widely used scales are the Glasgow Coma Scale (GCS) (254), the National Institutes of Health Stroke Scale (NIHSS) (44), the Scandinavian Stroke Scale (SSS) (10), the Barthel Index (BI) (178), the modified Rankin Scale (mRS) (274), and Mini-Mental Status Examination (MMSE) (92). Data about the neurological scores mentioned above are summarized in Table 1.

Table 1. Neurological scales.

Clinical grading scale	Measures	Evaluates	Points
Glasgow Coma Scale	level of consciousness	speech, eye movements, motor reactions	0–15
National Institutes of Health Stroke Scale	severity of stroke symptoms	level of consciousness, extraocular movements, speech, language, visual field deficits, facial muscle function, motor and sensory functions, coordination, hemi-inattention	0 – 36
Scandinavian Stroke Scale	severity of stroke symptoms	level of consciousness, motor function, disorders of cranial nerves, cutaneous sensitivity, gait, speech	0–58
Barthel Index	self-care and mobility	bowel function, bladder function, grooming, toilet use, feeding, transfer, mobility, dressing, bathing	0–100
Modified Rankin Scale	functional independence	no symptoms – need for supervision or assistance with activities of daily life – severe disability or bedridden – death	0–6
Mini-Mental Status Examination	cognition	orientation, registration, attention, calculation, recall, language, visuospatial construction	0–30

No single outcome measure can describe or predict all dimensions of recovery and disability after stroke, and each scale has a potential role in patient care and research.

Management

Pharmacotherapy

Therapy for acute IS relies on the presence of brain tissue that is viable and salvageable. The infarct core requires almost immediate reperfusion if it is to be saved. The penumbra, which maintains viability for a longer time, is a natural target for medical intervention. Recent advances in thrombolytic therapy have made it possible to reopen occluded vessels. To date, only four modalities, intravenous t-PA, intraarterial prourokinase (proUK), ancrod (an intravenous defibrinogenating agent), and NXY-059 (disodium 2,4-disulfophenyl-N-tert-butylnitrone, a nitrone with free radical trapping properties), have been shown in large prospective randomized studies to improve the outcome after acute IS (6, 97, 160, 232). These agents must be administered within a very narrow time window after stroke onset (3 h for intravenous t-PA and ancrod, 6 h for intraarterial proUK and NXY-059), and only t-PA has been approved for use in this indication by the Food and Drug Administration (FDA) and the European Medical Association (EMA) (3). The option of thrombolytic therapy is available to a limited number of well-selected patients. Neuroprotection can be offered to all stroke patients. Neuroprotective agents have the potential to reduce ischemic injury and improve stroke outcome. Despite several studies with various pharmacological agents, including N-methyl-d-aspartate antagonists, Ca^{2+} channel blockers, anti-inflammatory antibodies, and intravenous magnesium, no neuroprotective agent has yet been demonstrated to have a conclusive benefit in large prospective randomized efficacy trials on patients with acute IS, except NXY in one trial (43, 69). NXY-059 reduces the size of the infarct and preserves brain functioning in animal models of acute IS (152). A recent Stroke-Acute Ischemic NXY Treatment (SAINT I) study for acute IS showed a reduction in disability (160). The administration of NXY-059 within 6 h of the onset of acute IS significantly improved outcome as measured by the modified Rankin score at 90 days, but it did not significantly improve other outcome measures, including neurologic functioning measured by the NIHS scale.

Anticoagulation in acute IS is a point of great controversy. Although the rationale for anticoagulation to prevent progression or recurrence of stroke is appealing, no study or meta-analysis has provided conclusive proof of a benefit with heparin in acute IS. Despite this, heparin is commonly used in patients with cardioembolic stroke, large vessel stroke progression, and basilar artery thrombosis, and to prevent deep vein thrombosis (DVT) and pulmonary embolism (3).

Stroke unit

Acute stroke patients should be treated in a stroke unit by a multidisciplinary team. General management of acute stroke includes monitoring and support of conditions that need to be stabilized (respiratory and cardiac care, fluid and metabolic management, arterial blood pressure control, glycemic control, and treatment of elevated intracranial pressure) as well as prevention of DVT and pulmonary embolism and treatment of infections and seizures (3). Rehabilitation begins at the acute phase of stroke and continues after discharge (141). A meta-analysis by the Stroke Unit Collaboration (3) showed an 18% relative reduction in mortality and a reduction in death and need for institutional care when patients were treated in a stroke unit as compared with a general medical ward. All types of patients with stroke benefit from treatment and rehabilitation in stroke units: males and females, the young and elderly, and those with mild, moderate, and severe stroke.

Future of stroke care

Future directions in the critical care management of acute IS are anticipated to encompass five main areas: reperfusion (thrombolysis and manipulation of systemic circulation to augment cerebral blood flow), neuroprotection, their combination, hypothermia, and decompressive surgery. At the moment, no specific neuroprotective strategy is in routine use, but several are under development. Multimodal approaches, such as magnesium administration, which has a wider safety margin, are also being investigated. Moreover, interest has increased in hemispheric decompression and hypothermia in large hemispheric stroke. This strategy may allow patient survival but it was often associated with substantial neurological deficit. Recently, emphasis has been placed on the development of combined acute and rehabilitation stroke units, which seems promising in terms of reduced mortality and disability (3).

Prevention of stroke recurrence

The risk of recurrent stroke after a first IS is high and depends on the etiology. Recurrent stroke risk from AF has been reported to be 12% per year (2), and among patients with intracranial atherosclerosis may be as high as 10% per year (57). There are many strategies for lowering this risk. These include management of such risk factors as high blood pressure, diabetes mellitus, dyslipidemia, medical treatment with anticoagulation or antiplatelet agents, and surgical or endovascular intervention. The choice of therapy should be tailored to the specific stroke subtype, its etiology, and the condition of the patient.

Antithrombotic treatment

Certain drugs can inhibit the basic processes of platelet aggregation and release reaction. Platelet-vessel wall interaction is influenced by the selective

oxygenation of arachidonic acid in both the platelets and the vascular endothelium. In the platelets, thromboxane synthase converts prostaglandin H_2 to thromboxane A_2, which is a potent aggregator of platelets as well as a constrictor of arterial conductance vessels. The vascular endothelium, however, metabolizes prostaglandin H_2 to prostacyclin, which antagonizes platelet aggregation and dilates blood vessels. These observations suggest that pharmacological agents that selectively inhibit thromboxane synthase or facilitate the biosynthesis of prostacyclin might be beneficial in preventing the thrombotic complications of atherosclerosis.

The use of aspirin, clopidogrel, and dipyridamole in various combinations in treatment of IS patients has been evaluated in large trials(1, 75, 76). While there appears to be no major differences between these three drugs, the ESPS-II trial verified the superiority of the combination of aspirin with extended release dipyridamole over aspirin alone (76). Combined aspirin and clopidogrel slightly reduces the risk of IS recurrence but increases bleeding risk over clopidogrel alone (75). In the absence of contraindications, anticoagulation should be planned for patients with high risk of cardioembolism in stroke prevention (3).

Invasive treatment

Carotid endarterectomy has been shown to be superior to medical treatment for the secondary prevention of IS in patients with high-grade carotid artery stenosis. A recent systematic review by the Cochrane Collaboration showed carotid endarterectomy to be the most effective treatment measure in terms of reduction of stroke recurrence or death in patients with symptomatic stenosis >50% and surgical complications <6% (58). Angioplasty and stenting therapy constitute promising new options in the management of patients with carotid atherosclerotic disease (140).

Outcome following

Development of novel strategies of IS treatment and prevention requires elucidation of stroke outcome, including death and stroke recurrence. The greatest risk of poor outcome and death for patients with IS occurs in the first 30 days, with case-fatality rates ranging from 8% to 20% (278). After one month, cardiovascular disorders, stroke, and diseases resulting from stroke were the causes of death in up to 80% of patients. The one-year mortality ranges from 20% to 35% (168). In the Framingham Study, the five-year mortality rates after atherothrombotic brain infarction were 44% for men and 36% for women (222). Poor outcome may still occur because IS is a heterogeneous disease in which outcome is influenced by many factors, including concomitant diseases. The great variability in outcome in stroke patients has led to interest in identifying predictors of outcome through the use of prediction models. Many predictors have been reported to have a relationship with outcome. Nonmodifiable factors, such as age, gender,

and race-ethnicity, have been identified as potential determinants of stroke outcome. Older patients are less likely to recover than young patients with similar-sized infarcts (120). A study of a large cohort of patients with IS treated with thrombolytic agents identified significant correlations between age, outcome, and bleeding complications. Older IS patients were less likely to have a good outcome even when receiving intravenous t-PA (71).

The two main cerebrovascular risk factors that influence outcome are AF and DM. Strokes in patients with AF are usually more severe, more disabling, and associated with a higher mortality (68, 134). IS patients with DM have a higher risk of death and disability after stroke (135, 186). Among clinical findings, severity of stroke has been found to be one of the major predictors of poor outcome in many studies (191). The baseline NIHSS score strongly predicted mortality and functional outcome in the TOAST trial; one additional point on the baseline NIHSS score decreased by 24% the likelihood of survival and excellent outcome at 7 days and by 17% at 3 months (14). Markers of initial stroke severity, such as decreased level of consciousness, gaze deviation, headache, nausea, or vomiting, may predict poor outcome in clinically severe strokes (148). Stroke subtype also seems to predict outcome. Small-vessel disease is more frequently associated with favorable outcome than IS of cardioembolic and large- vessel atherosclerotic cause. In these categories, 63%, 47%, and 29% of patients, respectively, have a favorable outcome (8). Some markers of tissue injury or inflammation have been shown to predict a poor outcome after stroke. High glutamate levels strongly correlated with large infarct size and severe neurological deficit (55). Increased serum levels of S-100 also correlate with neurological outcome (12). CRP elevation within 12–72 hours of stroke is associated with a high risk of death. Patients with CRP levels above 10.1 mg/L had significantly worse likelihood of survival (190).

Recurrent stroke is a major cause of morbidity and mortality among stroke survivors. Large prospective studies indicated that the risk of recurrence after stroke varies from 1.7% to 4% in the first 30 days, from 6% to 13% in the first year, and from 5% to 8% per year for the next 2–5 years, culminating in a 5-year cumulative risk of stroke recurrence of 19–42% (49, 116). Prognostic factors for recurrent stroke are clinically important because they may help to identify patients at high recurrence risk and provide insights into ways of improving outcome. IS subtype has been found to be associated with stroke recurrence. In the Rochester Epidemiology Project, IS due to large-vessel atherosclerosis with stenosis had a higher risk of early recurrence. More than 18% of patients with this stroke subtype had a recurrent stroke within 30 days of the first stroke (206). Stroke risk factors (age, AH, cardiac disease, and DM) have a great effect on risk of recurrence (217). Evidence for the relationship between IS recurrence and inflammatory and hemostatic markers is also accumulating (241). Possible biochemical predictors of recurrent thromboembolic events include elevated C-reactive protein (CRP),

fibrinogen, and homocysteine (Hcy) levels (73).

Although the risk of poor outcome and new vascular events after first-ever stroke is high, prediction of death or recurrence of vascular events is difficult on an individual level. Discrepancies in identifying predictors of stroke outcome and recurrence have been observed. These may result from the patient population selection, including age, comorbidity, and stroke subtype, the definition of the predictors used in the studies, and the duration of follow-up. Most likely, our ability to predict stroke outcome and recurrence will improve with further developments in technology and laboratory methods.

Hemostasis and thrombogenesis

Hemostasis is a physiological process initiated when damage occurs to a blood vessel wall and culminates in the formation of a stable clot that prevents the escape of further blood from the vessel. This occurs in three stages: vasoconstriction, platelet response, and blood coagulation. A fourth stage occurs when the clot is dissolved following repair of the blood vessel (54) (Figure 1). In vasoconstriction, injury to the blood vessel wall triggers a reflex contraction of the circular layers of smooth muscle surrounding the endothelium. This action compresses the blood vessel to minimize blood loss and may continue for several minutes to some hours, and is enhanced by the release of platelet factors. This is not a permanent solution so the other phases of hemostasis are required to complete the process and to initiate effective blood vessel repair (54). Formation of an initial platelet plug is a complex series of responses by platelets, leading to generation of a clot at the site of the vessel wall defect. Platelet membrane glycoproteins are activated by contact with collagen in the wall of the damaged vessel. The process of binding to extravascular matrix proteins activates platelets. This activation leads to changes in the shape of the platelets, from a disc form to a compact sphere, and to the release of the various substances and proteins needed in platelet aggregation and initiation of coagulation reactions. After primary hemostasis, i.e. instant vasoconstriction and formation of initial platelet plug, activation of the coagulation system is needed to stabilize the loose accumulation of platelets by cross-linked fibrin (121).

Endothelial functions and endothelin-1

Endothelium constitutes the structural and functional interface between blood and the vascular wall. The anatomic location of endothelium, its expansive interactive surface area, and the magnitude of endothelial tissue mass contribute to its functional roles as a selectively permeable, blood-compatible, secretory membrane that regulates inflammatory, vasomotor, growth factor, and hormonal responses. An important function of intact

endothelium is limitation of thrombus extension through the inactivation of thrombin activity, the reduction in thrombin generation, and the production of antithrombotic and vasodilating factors such as prostacyclin and nitric oxide. Endothelial cell activation results from stimulation of endothelial cells by excessive shear stress, injury, increased turbulence, or inflammation. Mechanisms that maintain the thromboresistance properties of the endothelial surface may then be lost (179).

The endothelium produces potent vasoconstrictors, endothelins (ETs). Their powerful and long-lasting vasoconstrictive action has been documented in several species (286). There are three structurally and pharmacologically separate ET isopeptides: ET-1, ET-2, and ET-3. All contain 21 amino acids and two sulfide bonds, but ET-1 differs from ET-2 by two and from ET-3 by six amino acids (125). ET-1 is produced by vascular endothelial cells, ET-2 is present in the kidney and jejunum, and ET-3 occurs in the adrenal glands, jejunum, and kidney (105). ETs act through specific receptors with at least two distinct types: ET_A and ET_B. ET receptors occur in many different organs, including the central nervous system (216). Within the brain, large variations exist in receptor concentration, with high concentrations occuring in the brainstem and cerebellum (247). ET_A receptors are present on smooth muscle cells and in the heart, whereas ET_B receptors are located on endothelial cells and in the central nervous system (30). ET-1 is derived from peptide precursor, preproendothelin-1, which is subsequently cleaved to "big" ET and then to active ET-1 (286). Experiments with smooth muscle cells suggest that ET-1 acts on the ET_A, causing increases in Ca2+ concentration, which leads to contraction of smooth muscle cells and vasoconstriction.

ETs have many important physiological functions and are probably involved in the pathophysiology of numerous disease processes. ET-1 is likely the most relevant form in cardiovascular disease, whereas ET-3 is a neuropeptide; the role of ET-2 remains unclear (243). Due to its vasoconstrictor capacity, the potential role of ET-1 in the pathogenesis of AH has been discussed. Patients with hypertension show elevated levels of ET-1 in the cerebrospinal fluid and plasma (62, 196), although perfectly normal ET-1 levels have also been observed (226). Secretion of ET-1 is stimulated by insulin in human endothelial cells (90). Some studies have reported a positive, strong association of ET-1 level with DM (78, 204), but other studies have found no correlation (137). Dyslipidemia as well as obesity, sleep apnea, and smoking have been associated with elevated ET-1 levels, proposed to reflect damage to endothelial cells and endothelial dysfunction (107, 180, 204, 218).

The ability of ET-1 to overwhelm homeostatic mechanisms that maintain CBF has attracted interest in its role in the cascade leading to IS. In addition to its effects on vascular tonus, ET-1 increases blood-brain barrier permeability (242). A recent study by Matsuo et al. (184) demonstrated that administration of an ET_A receptor antagonist during reperfusion after

transient middle cerebral artery occlusion significantly attenuated edema formation and mortality of animals after cerebral ischemia. This supports the involvement of ET-1 in the pathophysiology of acute IS. Local application of ET-1 has been shown to induce neuronal damage (177). Intraventricular administration of ET-1 in rats reduced CBF and led to the development of brain infarction (280). In normal conditions, circulating ET-1 levels are very low (105). An increase in plasma ET-1 levels occurs in various animal models of global and focal ischemia (30). Several clinical studies have measured plasma and cerebrospinal fluid ET-1 levels in various subtypes and phases of IS (20, 23, 86, 155, 289). At the acute stage, ET-1 levels were reported to be elevated (86, 289) or normal (20, 155). In the chronic stage, ET-1 levels were increased (23).

The causes of the stroke-associated increase in plasma ET-1 levels remain unclear. A variety of factors have been proposed to cause the increased ET-1 levels in IS. One of these may represent a nonspecific enhancement of ET-1 production by systemic vascular endothelium in response to the stress associated with acute ischemia (280). The increased ET-1 production may be attributable to stress-induced release of acute-phase reactants (38). A second explanation is that cerebral ischemia is associated with hypercoagulability and platelet hyperactivity. Activated platelets accelerate preproendothelin production via local accumulation factors such as thrombin (40). A third possible explanation is that the source of increased ET-1 levels may be IS itself. Bian et al. (34) demonstrated elevated ET-1 levels in the ischemic brain region and in plasma after middle cerebral artery occlusion in rabbits. Ischemic damage may increase the leakage of ET-1 from injured endothelial cells of the involved cerebral microvessels (174). However, the relationships between plasma ET-1 levels, stroke pathogenesis, recurrence of thromboembolic events, and prognosis remain obscure.

Homocysteine

Hcy is a sulfur-containing amino acid formed during the metabolism of methionine that is further metabolized by one of two main routes, methylation and trans-sulfuration (114). The trans-sulfuration pathway involves the enzyme cystathione-beta-synthase (CBS), the action of which results in the condensation of Hcy with serine to form cystathionine using vitamin B_6 as a cofactor (114). The remethylation of Hcy to methionine by the enzymes methionine synthase and methionine synthase reductase requires vitamin B_{12} as a cofactor and 5-methyltetrahydrofolate as a cosubstrate (formed from 5,10-methylenetetrahydrofolate by the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR)) (114). Human plasma contains reduced and oxidized Hcy. The reduced form is called homocysteine, and the oxidized form homocystine. The oxidized form comprises 98–99% of total human plasma Hcy, 80–90% of which is bound to protein (16).

A great number of environmental factors have been found to play a

determinant role in Hcy levels (126). Lussier-Cacan et al. (175) determined that Hcy level in women is 21% lower than in men. Hcy levels increase with age, as has also been found in previous studies (197, 260). Race or ethnic origin is another determining factor. Ubbink et al. (269) demonstrated that black South Africans had lower Hcy levels than white South Africans, although their diets were similar. It is likely that this difference is confined to genetic differences within groups. The most common genetic alteration that results in severe homocysteinuria is reduced CBS activity (74), and the most common genetic defect associated with mild homocysteinemia is a MTHFR gene mutation (70). Diet deficiencies and folic acid, vitamin B_6, or vitamin B_{12} malabsorption have been linked with hyperhomocysteinemia (270). A rise in Hcy level is associated with smoking, alcohol consumption, and high intake of coffee (197). High Hcy levels exist in the following conditions: DM, AH, dyslipidemia, and sleep apnea (158, 202, 205, 209).

The hypothesis that high levels of Hcy acts as an atherogenic and thrombogenic risk factor leading to atherosclerosis was presented more than 30 years ago (185). There probably is a multifactorial effect, the main consequences of which are endothelial and vascular wall damage and changes in procoagulant substances. Hcy can cause endothelial cell damage by promoting peroxide formation and impending its activation (118). Tawakol et al. (253) described defective endothelium-dependent vasodilatation in mildly hyperhomocysteinemic subjects. Hcy also stimulates the proliferation of vascular smoothmuscle cells, presumably thereby contributing to neointimal thickening (266).

Many studies have shown that an elevated Hcy level is an independent risk factor for cardiovascular disease (42, 83). It remains to be determined whether the relationship is causal or whether it is confounded by factors associated with high Hcy, such as current smoking, elevated arterial blood pressure, cysteine deficiency, or even acute vascular events themselves, whereby the tissue damage may temporarily increase plasma Hcy levels (113). The link between Hcy and vascular diseases has been speculated to be mediated in part by activation of coagulation (106).

A correlation between elevated Hcy levels and risk of IS has also been proposed, but studies have given conflicting results (19, 122, 138, 149, 163, 188). The risk of IS associated with Hcy level was significantly weaker in prospective studies than in retrospective studies, which may reflect bias in the latter because of difficulties in selecting controls or the effect of changes in treatment, renal function, or other factors after the onset of disease (4). Results of several large prospective trials have shown no preventive benefit of the use of folic acid and vitamin B_{12} in patients with established vascular disease and mild hyperhomocysteinemia (4, 39, 261).

Platelets

Platelets are an essential part of the physiologic hemostasis. They are derived from stem cells in the bone marrow by fragmentation of megakaryocyte cytoplasm. Thrombopoietin is the dominant hormone controlling megakaryopoiesis and thrombopoiesis (143). Platelets are anucleate cells with diameters of 2–4 μm. Each day, an adult human produces approximately 1×10^{11} platelets. A normal platelet count, $150–350 \times 10^9$/L, is essential for hemostasis. Platelets survive for about 10 days on average; younger platelets have greater functional ability. The spleen continually sequesters circulating platelets. The liver has also been suggested to be an important site of platelet destruction (100).

Under normal conditions, platelets are at rest and flow through blood vessels without interacting with any other cells. They respond to vessel wall injury, alternations in blood flow, or chemical stimuli with the activation of a functional triad (adhesion, secretion, and aggregation) that leads to formation of a rapidly enlarging platelet thrombus (Figure 2). Vessel injury exposes subendothelial von Willebrand factor (vWF) and collagen to the circulating blood. Platelets have a range of surface membrane receptors that interact with these constituents, helping platelets to attach to target sites (283). Platelets adhere to the site of injury via a glycoprotein (GP) Ib/IX/V and Ia/IIa complex. Adherent platelets become activated. These in turn cause activation of various receptors and release of two potent platelet agonists, thromboxane A2 (TXA2) and adenosine diphosphate (ADP), which bind to specific platelet receptors, resulting in a change in form, from the normal disc shape to a compact sphere with irregular extending pseudopodi, secretion of granular contents, and promotion of platelet-platelet interaction (aggregation) (283). Aggregation is mediated by GP IIb/IIIa receptors on the platelet surface that bind various adhesion proteins. One of the most important adhesion proteins is fibrinogen. Binding of fibrinogen and VWF to GP IIb/IIIa receptors results in a thrombin-mediated conversion into fibrin and thrombus formation. This process can be attenuated by endothelial or platelet release of nitric oxide (NO). The morphologic changes are accompanied by redistribution of the phospholipids in the platelet membrane, building a surface for coagulant protein molecules (94).

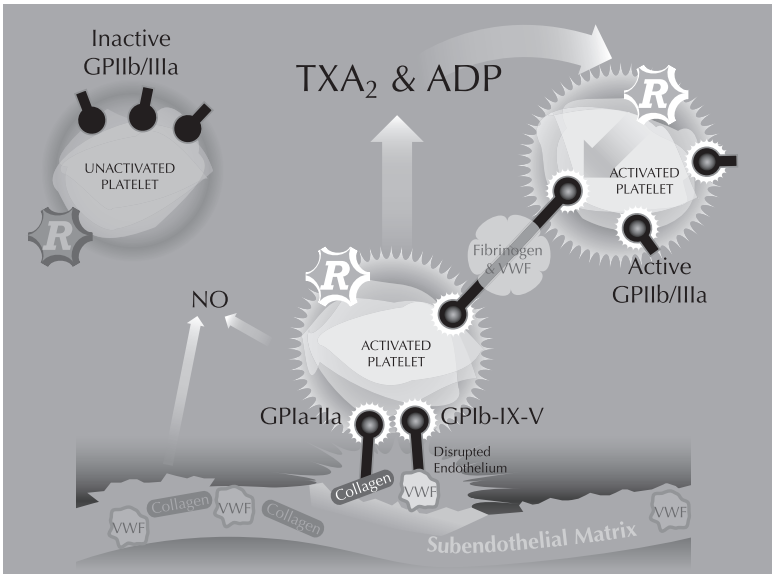

Figure 2. Platelet adhesion and aggregation. Damage of vascular wall exposes subendothelial von Willebrand factor (VWF) and collagen to the blood. Platelets adhere to the site of injury when glycoprotein (GP) Ib/IX/V and Ia/IIa complexes expressed on the platelet surface bind to VWF and collagen, respectively. This event triggers the synthesis and release of thromboxane A2 (TXA2) and adenosine diphosphate (ADP) and causes activation of various receptors (R). Conformational changes in GP IIb/IIIa follow, enabling high-affinity binding of fibrinogen and VWF, resulting in thrombus formation. This process can be attenuated by endothelial or platelet release of nitric oxide (NO). Adapted from Freedman: Circulation, Volume 112, 2005, 2725–2734.

Coagulation system

The coagulation process is referred to as a cascade because each factor in the process acts on many molecules in the next stage of the process (Figure 3). The blood coagulation system is composed of inactive precursors of coagulation enzymes and cofactors, which become activated and then activate the next enzyme in the chain. Even though an initial stimulus may be small, a large quantity of the final factor, fibrin, is produced (54). Coagulation at sites of disruption of the vessel wall occurs in three overlapping phases.

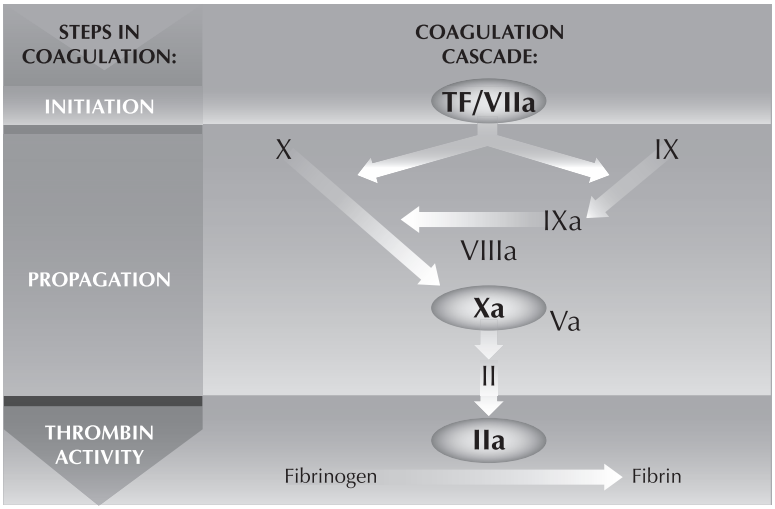

Figure 3. Steps in the coagulation cascade. The initiation of coagulation is triggered by the tissue factor/factor VIIa complex (TF/VIIa), which activates factors IX and X. Activated factor IX (IXa) propagates coagulation by activating factor X in a reaction that utilizes activated factor VIII (VIIIa) as a cofactor. Activated factor X (Xa) with activated factor V (Va) as a cofactor converts prothrombin (II) to thrombin (IIa). Thrombin then converts fibrinogen to fibrin. Adapted from Weitz: Chest, Volume 126 (3), Supplement, 2004, 265S–286S.

The process is initiated by exposed tissue factor (TF), which forms a complex with activated factor VII (FVIIa) (165). Low concentrations of FVIIIa circulate in the plasma so that the system is primed pending TF exposure. The FVIIa-TF complex then activates factor IX (FIXa) and factor X (FXa), but FX activation is more efficient. FXa cleaves small amounts of prothrombin to generate thrombin. A low concentration of thrombin enhances adhesion of platelets at the site of injury and activation of factor V (FVa), factor VIII (FVIIIa), and factor XI (FXIa). After the platelets have been activated and activated FV and FVIII have bound to their surfaces, propagation and large-scale thrombin generation begin. In the presence of calcium, FIXa binds to FVIIIa on the surface of activated platelets, thereby forming intrinsic tenase, the complex that activates FX (FXa). FXa then binds to FVa on the activated platelet surface to form the prothrombinase complex. This complex activates prothrombin (FII) on the phospholipid membrane through two sequential cleavages, yielding thrombin and prothrombin fragment 1+2 (F1+2) (Figure 4).

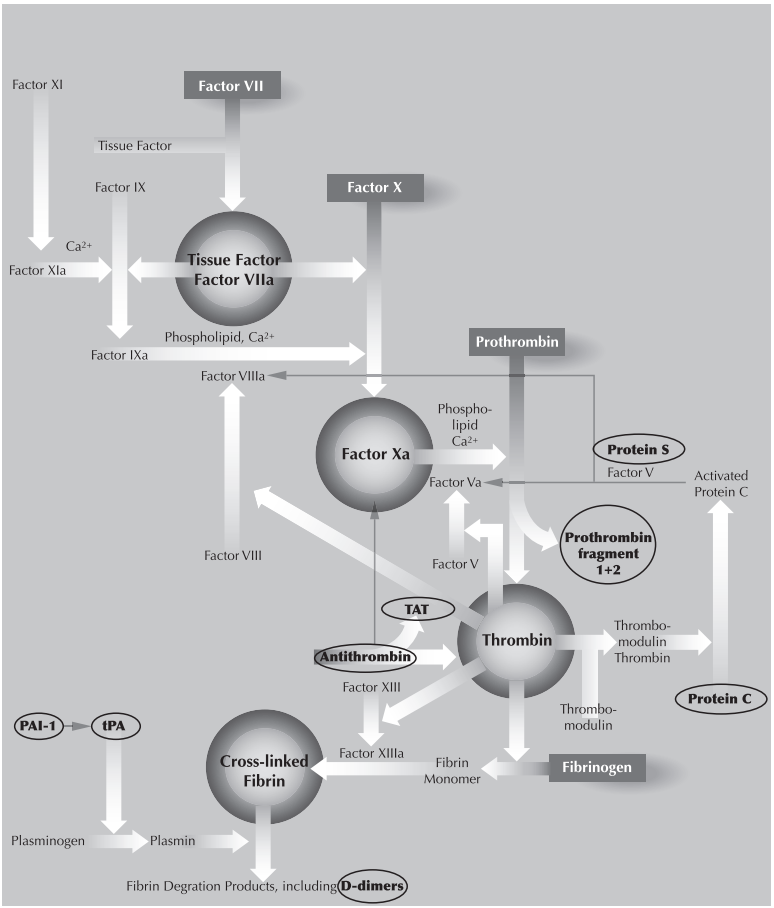

Figure 4. Factors of the coagulation and fibrinolysis systems and their interactions. Thin lines indicate inhibition/inactivation. Measured markers assessing coagulation and fibrinolysis are marked as ovals. Adapted from Godsland: Drugs, Volume 60 (4), 2000, 721–869.

Thrombin then dissociates from the platelet surface and converts fibrinogen to fibrin, the final step in the coagulation process. Thrombin also activates factor XIII (FXIIIa), which stabilizes the platelet/fibrin thrombus by cross-linking the fibrin network, thereby rendering it more resistant to lysis (165).

F1+2 is a marker of thrombin generation. For each molecule of thrombin produced, one F1+2 molecule is cleaved from prothrombin (129). Thus, F1+2 reflects *in vivo* thrombin formation and may provide an important diagnostic tool in the evaluation of hypercoagulable states and in the diagnosis of different clinical conditions related to thrombotic phenomena. In previous studies, increased F1+2 levels have been associated with conventional vascular risk factors such as advanced age, AH, DM, smoking, increased body mass index, sedentary lifestyle, family history of ischemic heart disease,

and dyslipidemia (173, 193, 282). A cohort study performed in Sweden demonstrated that F1+2 levels predict coronary morbidity in hypertensive patients (15). Because thrombin, a central enzyme in the coagulation cascade, plays a role in atherosclerosis progression, measurement of plasma levels of F1+2 might be of value in identifying of patients at high vascular risk who may benefit from prophylactic antithrombotic strategies.

Several studies have found elevated plasma levels of F1+2 to be associated with the presence and severity of atherosclerotic disease (101, 200). However, a prospective study reported no association between F1+2 and risk of future cardiovascular events (263). Whether F1+2 levels are associated with increased IS risk has not been established (61, 96, 240). Studies in AF, a model of thromboembolism, provide some insights. F1+2 levels are higher in AF patients than in non-AF stroke patients or control subjects with a sinus rhythm (239). The etiology of increased thrombin generation in patients with cerebrovascular disease remains unclear. Thrombin generation is directly related to thrombus formation, and thus, reflects secondary hemostatic activation due to inflammation and endothelial injury associated with atherosclerosis.

In the final process of thrombus formation, thrombin acts with fibrinogen to form an insoluble fibrin clot, a step which may be inhibited by natural anticoagulant antithrombin. Antithrombin binds to thrombin (Figure 4) to form an irreversible thrombin-antithrombin complex (TAT) that reflects, similar to F1+2, generation of thrombin *in vivo*. Data concerning the relationship between vascular risk factors and TAT levels are controversial (52, 102, 172, 282). TAT levels have been proposed as a marker of atherothrombotic risk (145). Some evidence indicates that TAT levels increase in peripheral arterial occlusive disease (PAOD), particularly in patients with recent symptom onset, which may represent an embolus rather than a thrombosis (203). Lassila et al. (156) found that TAT levels were associated with the extent of PAOD, possibly reflecting coagulation activation and fibrinolysis.

Both elevated and normal TAT levels have been observed in IS patients (272). In IS patients with atherothrombotic or cardioembolic infarct, TAT levels have been reported to be increased (142, 195, 262), while no elevation of TAT levels was noted in patients suffering from lacunar infarcts (248, 249). No relationship between TAT levels and recurrence of IS has been found (47). However, Soncini et al. did show higher mortality in IS patients with increased TAT levels (240). Elevated TAT levels in IS might reflect the presence of ongoing thrombosis within cerebral vessels or may be a marker of systemic hypercoagulability (142).

Regulation of coagulation

Coagulation is regulated via three inhibitory systems. Tissue factor pathway inhibitor (TFPI) targets the initiation of coagulation, antithrombin blocks

thrombin generation and thrombin activity, and protein C inhibits the propagation of coagulation.

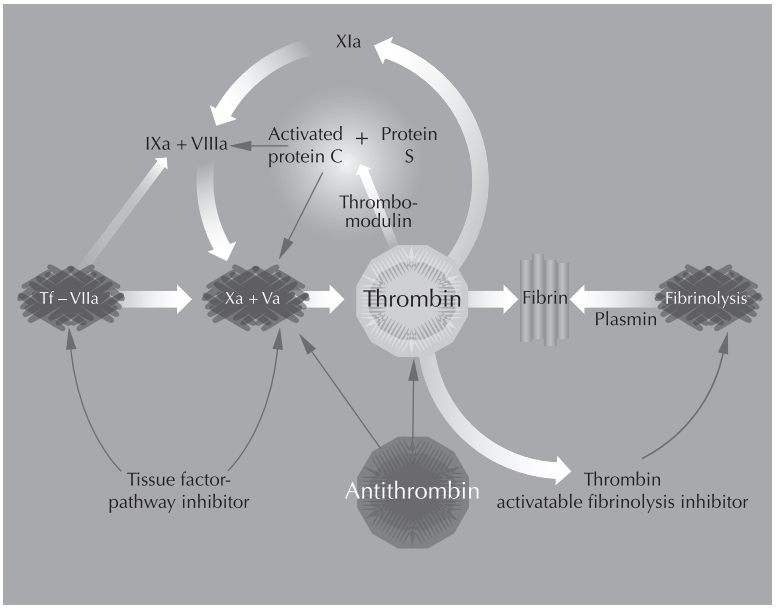

Figure 5. Schematic diagram of regulation of coagulation. Formation of a clot is highly regulated by natural anticoagulant mechanisms that confine the hemostatic process to the site of injury on the vessel. Thin lines indicate inhibition/inactivation. Adapted from Nesheim: Chest, Volume 124 (3), Supplement, 2003, 33S–39S.

Tissue factor pathway inhibitor (TFPI)

Tissue factor pathway inhibitor (TFPI) blocks FVIIa in a two-step fashion. TFPI first binds and inactivates FXa, and the TFPI/FXa complex then inactivates FVIIa bound to tissue factor. Circulating levels of TFPI are low, suggesting that the system is designed to block uncontrolled activation of coagulation by the FVIIa/tissue factor (TF) complex, while allowing propagation of coagulation by intrinsic tenase (Figure 5). Propagation of coagulation is ensured because thrombin activates FXI on the platelet surface, where it is poised to activate platelet-bound FIX (129).

Antithrombin

The most important direct inhibition mechanism of coagulation is neutralization of thrombin and factor Xa by antithrombin (AT) (Figure 5). AT is synthesized in the liver. It was described as a protein required for antithrombotic activity of plasma (229). AT inhibits all coagulation proteinases (thrombin, FIXa, FXa, FXIa, and FXIIa), but not FVIIa and FVa. Arginine-rich centers in AT react irreversibly with the serine center of serine proteases. Complex formation between AT and its target enzymes is enhanced by heparin, which binds to lysyl residues on AT, making the critical arginine

residue more available for interaction with thrombin. Bound by heparin, the heparin-AT complex inhibits activated serine proteases with >1000-fold greater efficacy than AT alone (36). Egeberg et al. (80) first established an association between AT deficiency and recurrent venous thrombosis in a Norwegian family. Patients with AT deficiency have a high risk of developing venous thrombosis, and the risk increases with age (256).

Protein C pathway

Protein C (PC) is a vitamin K-dependent protein that is synthesized primarily in the liver, kidneys, and male reproductive organs (85). Designed to regulate thrombin generation, the PC pathway is initiated when thrombin binds to thrombomodulin, its receptor on the endothelial cell surface. This forms the thrombin/thrombomodulin complex, which then activates PC by cleaving a single arginine residue. The binding of thrombin to thrombomodulin results in a ~1000-fold increase in the activation of protein C to activated protein C (APC). Activated protein C functions as an anticoagulant by proteolytically degrading and inactivating FVa and FVIIIa (Figure 5), thereby attenuating thrombin generation (85). The inactivation of FVa by APC has been reported to be enhanced by heparin and inhibited by prothrombin and Hcy (234).

Degradation of FVa by APC is markedly enhanced by protein S (PS). PS, a vitamin K-dependent single-chain glycoprotein, circulates in the blood as an active free protein and in nonconvalent association with a large, multisubunit protein of the complement system, C4bBP, in an inactive state (117). PS is expressed in many tissues, including the liver, endothelium, testis, brain, and megakaryocytes. The principal anticoagulant activity of PS is the inhibition of thrombin generation by acting as a cofactor to APC in the degradation of FVa and FVIIIa (Figure 5) and independently inhibiting the prothrombinase- and FX- activating complexes. PS may also play a role in vascular injury repair and in bone development (234). PS specifically enhances the rate of APC cleavage at Arg306 by inducing a conformational change and reducing the distance of the APC active site from the phospholipid surface. In the absence of PS, APC inactivation of FVa on the phospholipid surface is biphasic, with the first and most rapid cleavage occurring at Arg506, and slow proteolysis at Arg306, resulting in complete loss of activity (215).

To date, several factors correlated with levels of natural anticoagulants have been identified: age, gender, race, body mass index, DM, AH, dyslipidemia, smoking, and sleep apnea (213, 273, 282). Although deficiencies in natural anticoagulants are responsible for up to 20% of nontraumatic venous thrombosis (267), arterial thrombosis has not been prominently associated with deficiencies in these factors (251), except in single case reports or small series of young patients with cerebral infarctions of unknown origin (29, 182). Studies of the relationship between levels of natural anticoagulants and risk of cardiovascular disease have yielded contradictory. Low, normal, and high levels of natural anticoagulants have been found in coronary artery

disease patients (60, 157, 282). Only a few studies have evaluated natural anticoagulants in different phases of IS, with conflicting results (21, 248, 249, 258, 285).

Factor V Leiden mutation (FVLm)

In 1993, Dahlbäck described a novel mechanism for familial thrombophilia (66). He identified individuals with unexplained personal and familial histories of venous thromboembolism whose plasmas exhibited a poor response to APC. He called this phenomenon APC resistance (APCR). It was soon shown that the cause of APCR is most often a defect involving the substitution of arginine 506 with glutamine at nucleotide 1691 (Q506 Leiden) in factor V cDNA (33). This is the site at which APC cleaves FVa, and this sequence alteration makes the mutant FVa molecule biochemically resistant to inactivation by the enzyme. The Arg506Gln substitution was found to be the cause in more than 90% of Dutch patients with APCR, but the mutation was also present in 2–4% of healthy Dutch control subjects (33). The Physicians's Health Study has provided valuable data regarding FVLm as a risk factor for venous as well as arterial thrombosis. In a retrospective case-control study of 14 916 healthy men aged 40 years or more, with a mean follow-up period of 8.6 years, heterozygosity for FVLm was identified in 12% of subjects with a first episode of deep venous thrombosis or pulmonary embolism and in 6% of controls (211).

There has been considerable interest in determining whether FVLm leads to an increased risk for arterial thrombosis. Large case-control studies (110, 147, 154, 211, 252) have failed to find an elevated frequency of FVLm among IS patients. Both case-control and prospective studies from the Copenhagen City Heart Study argue against an association between FVL and risk of adult IS. This conclusion is supported by the findings of a meta-analysis (133).

However, some data indicate that FVLm may increase stroke occurrence in younger persons (18, 181). Moreover, several case reports have suggested that epistatic (gene-gene) interactions increase the risk of arterial thrombosis. An association between FVLm and migraine with aura has been proposed (63, 147), but no relationship between FVLm and the major IS risk factors has been found (154). Although it is not absolutely clear whether FVLm is a risk factor for IS itself, many young patients without an obvious cause and no common artheriosclerotic risk factors undergo testing for APCR and FVLm. Only a few studies exist on the clinical and radiological characteristics of IS patients with FVLm (115, 147, 154, 290). Almost all studies concerning the relationship between IS patients and FVLm were hampered by the small number of such patients. Since FVLm is found only in a small fraction of the population, most studies aimed at clarifying its role have not been conclusive.

Fibrinolysis

Endothelial control of fibrin deposition is exerted via the fibrinolytic system. The main function of this system is to limit clot formation and degrade the fibrin deposits formed (Figure 6). The key enzyme responsible for doing this is plasmin. Plasmin cleaves fibrin into soluble fragments, but also has other important functions, such as increasing the activity of matrix metalloproteinases, resulting in enhancement of its proteolytic activity. Active plasmin is formed from plasminogen by the specific proteolytic action of tissue-type plasminogen activator (t-PA). Plasminogen can also be activated by urokinase-type plasminogen activator (u-PA), which is only marginally involved in clot lysis. The activities of both t-PA and u-PA are controlled by their specific inhibitors, plasminogen activator inhibitors (PAIs), mainly PAI-1, PAI-2, and PAI-3, PAI-1 being the most important (150). PAI-1 binds to fibrin and forms a stabile complex with t-PA or u-PA, inhibiting plasmin activation and fibrinolysis.

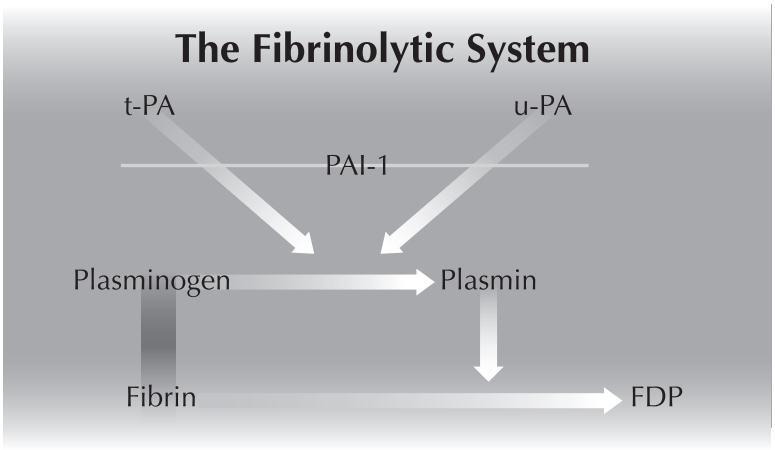

Figure 6. Plasmin-induced degradation of fibrin. Fibrin localizes plasminogen to the surface of the thrombus, where plasminogen is activated to plasmin by t-PA and u-PA. These enzymes are regulated by PAI-1. Plasmin is responsible for the degradation of fibrin into fibrin degradation products (FDPs). The thin line indicates inhibition. Adapted from Lee: Arch Intern Med, Volume 163 (19), 2003, 2368–2392.

Endothelial cells of the normal arterial wall secrete t-PA, but little or no u-PA. t-PA expression is particularly high in the brain (284). t-PA expression has been shown to respond to a few modulators, including endotoxin, bradykinin, endothelin, tumor necrosis factor (TNF), and thrombin (109). Expression of u-PA is highest in the kidney. u-PA provides pericellular proteolysis during cell migration and tissue remodeling and does not bind specifically to fibrin (162). PAI-1 is found in the circulation, largely in association with platelets. It is also secreted by endothelial cells (150). PAI-1 expression is minimal in normal arteries (223). Inflammatory cytokines, TNF, and lipoproteins are important factors in regulating vascular PAI-1

expression (109). Both t-PA and PAI-1 show circadian rhythm, with the highest PAI-1 activity and the lowest t-PA activity in the early morning (22). The morning hypercoagulability may partly be related to catecholamine and cortisol excretion (281).

The concept that impaired fibrinolysis is a risk factor for future vascular disease is strongly supported by numerous studies. PAI-1 activity is regulated by age; older populations tend to have lower PAI-1 levels (287). PAI-1 gene expression has been demonstrated to be increased in atherosclerotic plaque (223), and the increased local expression of PAI-1 at the site of atherosclerotic lesion formation could disturb the natural balance between coagulation, anticoagulation, and fibrinolysis, leading to a local prothrombotic condition (228). Several of the well-defined risk factors for atherosclerosis are associated with excessive PAI-1 activity (166, 282, 287). Two of the most common disorders leading to the development of atherosclerosis, AH and DM, alter PAI-1 and t-PA production unfavorably. A significant correlation exists between plasma PAI-1 activity and the plasma renin-angiotensin/ system (46). Low t-PA levels have been reported in patients with AH (128). Plasma insulin has been suggested to be a major physiological regulator of PAI-1 activity and t-PA levels in plasma (132, 187). Recent data indicate that adipose tissue is an important source of PAI-1 (169). Two clinical studies demonstrated that patients with coronary syndrome had higher levels of t-PA:Ag and PAI-1 activity than controls (127, 224, 257). Activity of the fibrinolytic system in IS patients has also been investigated widely (53, 123, 130, 176), but the results are conflicting. Two prospective studies have shown that a high concentration of t-PA:Ag among healthy men was associated with an increased risk of future IS, especially thromboembolic stroke (212, 237). Elevated PAI-1 activity in stroke patients has also been reported (56, 164), and this might reflect an endogenous predisposition to thrombogenesis (130).

After coagulation is initiated, the reaction must be localized to the site of vascular damage and tightly regulated to maintain intravascular fluidity. The fibrinolytic system removes fibrin-rich thrombi by plasmin-mediated proteolytical degradation of fibrin clots into soluble fragments. Plasmin-induced degradation of fibrin is a stepwise process that results in the formation of many fragments (98). Fibrin fibrils are degraded to X-oligomers that consist of D- and E-fragments of fibrin in various combinations. The fragments decrease in size as the fibrinolytic process continues. The oligomers are further degraded to DDE-fragments and eventually to a D-dimer (79).

Elevated levels of D-dimer have been suggested to be a sensitive marker of intravascular thrombin or plasmin generation. It also may reflect the extent and severity of underlying atherosclerosis (119, 159, 210). DeSouza et al. (72) found a significant influence of age on plasma D-dimer levels. Prospective studies have reported elevated D-dimer levels in hypertensive patients (166, 276), but this observation was not confirmed in a later study

(264). The correlation between D-dimer and dyslipidemia also seems to be weak (167). There is some evidence that D-dimer correlates with diabetes mellitus as well as obesity (26). Modestly elevated circulating D-dimer values might reflect minor increases in blood coagulation and turnover of cross-linked intravascular fibrin (which is partly intra-arterial in origin), and thus, may be relevant to coronary heart disease (171). D-dimer has been associated with the risk of future coronary events (67) and the severity of coronary artery disease (250). Lassila et al. (156) found the severity of atherosclerosis to be associated with increased D-dimer levels in PAOD patients.

Some studies have demonstrated elevation of D-dimer levels in patients with acute IS (21, 259) and a correlation of D-dimer levels with stroke severity (32). With regard to stroke etiology, results have been inconsistent (64, 87, 103, 142, 183). Several previous studies have identified D-dimer as an independent predictor of future stroke in healthy subjects (170, 236). Elevated D-dimer levels can predict progression of stroke (28), stroke mortality (87), and a high risk of recurrent stroke (248). Elevated D-dimer levels may reflect an acute-phase response (28), an activation of coagulation secondary to tissue damage, or a complication in the course of stroke (infection or venous thromboembolism) (32).

Aims of the study

Serial changes in markers measuring coagulation, fibrinolysis, and vasoactivity in defined IS patients were investigated. Also of interest was to determine whether these markers could predict short- (3 months) and long-term (3 years) outcome, including recurrent IS, in this patient population. We selected mildly or moderately affected IS patients to maximize the likelihood of survival over the study period and the possibility of follow-up, including both serial clinical and laboratory evaluation.

Specific aims were to shed light on the following questions regarding markers of coagulation, fibrinolysis, and vasoactivity:

1. How do markers measuring coagulation, fibrinolysis, and vasoactivity change in IS patients compared with control subjects free of vascular diseases (Studies I–IV)?

2. How do these markers vary over time following IS (Studies I–IV)?

3. Are these markers associated with IS risk factors or stroke etiology (Studies I–IV)?

4. Do any changes in the markers measuring coagulation, fibrinolysis, and vasoactivity emerge as clinically useful in predicting the prognosis or recurrence of vascular events in IS patients (Studies I–IV)?

5. Is the presence of FVLm associated with specific clinical, laboratory, radiological, or other characteristics (Study V)?

Subjects and methods

Subject characteristics

All study protocols were approved by the Ethics Committee of the Department of Neurology, University of Helsinki, and were carried out according to the principles of the Declaration of Helsinki. Informed consent was obtained from all participants. IS diagnosis was based on both clinical evidence and radiological findings. Risk factors for IS were elicited from patient history, physical examination, previous hospital records, laboratory tests, and relatives' reports in accordance with general criteria (104). All patients received standard stroke care and secondary prevention consistent with generally accepted recommendations (25) to minimize their risk of recurrence of thrombosis. Patients unable to give informed consent or refusing to participate, patients with a decreased level of consciousness, and patients suffering severe strokes, thus likely to remain bedridden or to not survive survive the follow-up period, were excluded. Probability of survival was decided based on clinical findings (NIHSS < 20) (220).

One hundred and two consecutive likely-to-survive patients with clinically and radiologically proven first-ever IS admitted to the Department of Neurology, Helsinki University Central Hospital, were included (Studies I, II). Blood samples from one patient were missed before analysis in Study I (n=101). Similar numbers of sex- and age-matched control subjects, free from atherothrombotic or hematological disease, were selected from patients referred to the Outpatient Clinic of the Department of Neurology and from investigators' relatives. None of the patients or controls had any concomitant medication or disease that could alter Hcy levels (126). In Studies III and IV, 55 consecutive patients of the 102 patients described above were chosed based on their order of admission to hospital. The study protocol was similar to the one described above. An equal number of sex- and age-matched controls (n=55) were selected from the 102 control subjects described above. None of the control subjects received any medication that might affect coagulation or the fibrinolytic system.

In Study V, another 42 patients with clinically and radiologically confirmed IS and documented FVLm admitted to the Department of Neurology, Helsinki University Central Hospital, were selected from 740 patients with IS without obvious etiology who were tested for FVLm. An equal number of FVLm-negative IS patients matched for sex and age were selected from the same population. The decision for FVLm testing, based on the written protocol of our department, was made by the physicians responsible for the patients.

Brain imaging and infarction classification

All patients underwent CT or MRI of the brain. Infarct volumes were calculated using the following formula: AxBxC/2, as previously described (199). Classification of IS was according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria (13) (Studies I–IV). In Study V, the subtypes of ischemic stroke were categorized according to the Oxfordshire Community Stroke Project (OCSP) classification (27). The presence and degree of leukoaraiosis (focal or diffuse hypodensities in the periventricular or deep white matter, not involving the cortex) were evaluated with the leukoaraiosis Visual Rating Scale (37). The location and number of infarcts were determined by careful visual evaluations of brain images using either CT or MRI. The patients were classified into four subgroups according to radiological features. Group 1 had small-vessel occlusion (one or more hemispheric or brainstem infarctions with diameter < 1.5 cm), Group 2 large-vessel occlusion (cortical/cerebellar/brainstem infarctions with diameter ≥ 1.5 cm), Group 3 subcortical hemispheric infarctions, and Group 4 mixed vascular pathology (246).

Assessment of neurological status and outcome

Patients underwent neurological examination on admission and at 1 week (7 ± 1 days), 1 month (30 ± 3 days), and 3 months (90 ± 7 days). National Institute of Health Stroke Score (NIHSS), Scandinavian Stroke Scale (SSS), and Glasgow Coma Scale (GCS) were performed at each time point, and the Barthel Index (BI), Modified Rankin Score (mRS), and Mini-Mental State Examination (MMSE) once at 3 months. A 3-year follow-up was performed by telephone, and in case of IS recurrence, hospital records were reviewed (Studies I–V). No patients were lost to follow-up, but two patients died in the acute stage of stroke because of a large brain infarction. Both FVLm-positive and FVLm-negative IS patients underwent neurological examination on admission, at discharge from the hospital, and at 3 months. NIHSS and SSS were performed at each time point, and BI and mRS at 3 months and at the end of the follow-up period (Study V).

Blood sampling

Fasting venous blood samples were collected without venous stasis from an antecubital vein via a 19-gauge scalp needle at the time of the neurological examinations on admission (within 2 days of stroke) and at 1 week (7 ± 1 days), 1 month (30 ± 3 days), and 3 months (90 ± 7 days). Sex- and age-matched controls underwent blood sampling once. All blood samples were

collected between 8 and 10 a.m. to eliminate possible circadian effects. After discharging the first 2–3 mL, blood was collected into a sterile 3-mL vacuum tube containing 10 mL sterile 3.2% sodium citrate (Venoject, Terumo Europe N.V., Leuven, Belgium). Plasma was separated by centrifugation immediately at 2 000g for 20 min at 4°C. Plasma was quickly frozen and stored at –70°C until analyzed.

Plasma ET-1 (pg/mL) concentration was quantified using a commercially available sandwich-enzyme immunoassay kit (R and D System) after extraction (244) (Study I).

Plasma Hcy was measured using a standard competitive immunoassay method (Immulite 2000, Diagnostic Product Corporation, Los Angeles, California, USA). Serum B12 vitamin, serum folic acid, and CRP concentrations on admission were determined with standard laboratory methods (Study II).

AT and PC activities were measured with chromogenic assays (Coamatic AT 400 and Coamatic Protein C, Chromogenix AB, Mölndal, Sweden) on an automated coagulameter (Thrombolyzer, Behnk Elektronik GMBH & Co., Norderstedt, Germany). Free PS activity was measured on the same instrument after polyethylene glycol treatment with an immunological method (Liatest Protein S, Diagnostica Stago, Asnieres, France). Percentage (of activity/amount of a reference plasma or pooled normal plasma) is an established way to express the amount of clotting factors in plasma samples. AT and PC levels are expressed as percentage of activity of normal reference plasma. PS levels were measured as free PS antigen, and the results are expressed as percentage of free PS antigen in pooled normal plasma (Study III).

In Study IV, F1+2 (nmol/L) and TAT (mg/L) concentrations were measured with commercial reagents (Enzygnost F1+2 micro and Enzygnost TAT micro, Behringwerke AG, Marburg, Germany). t-PA levels (ng/mL) were measured as antigen concentration with a commercially available enzyme-linked immunosorbent assay (ELISA) method (Coaliza t-PA, Chromogenix AB) according to the manufacturer's instructions. PAI-1 activity (AU/mL) was determined with a chromogenic method (Coatest PAI; Chromogenix AB, Mölndal, Sweden) according to the manufacturer's instructions. D-dimer concentration was measured with a commercial method (Asserachrom D-Di, Diagnostica Stago, Asnieres, France), and the results are expressed as fibrinogen equivalent units (FEU, mikrog/L).

In Study V, all patients were tested for both APCR and FVL. The laboratory technique for testing APCR was to define the ratio between activated partial thromboplastin time before and after adding APC to the sample. The cut-off value was 2.0. We used minisequencing complementary DNA to detect the FVL mutation, as described by Bertina et al. (33). In addition to the APCR and FVLm tests, all patients were screened for the presence of any concomitant thrombophilic coagulation disorder, including deficiencies in

AT, PC, and PS, presence of lupus anticoagulant or cardiolipin antibodies, and plasma homocysteine concentration according to standard laboratory methods. Blood glucose and serum lipid levels were determined in serum 3 months after stroke with standard laboratory methods (Studies I–V).

Statistical methods

The main results are given as median and mean ± SD. Because of positively skewed distributions, the comparisons were made with logarithmically transformed data. The distributions of the parameters were plotted and tested with Shapiro-Wilk's test. The data for hemostatic markers for patients at different time points and for controls were compared by Wilcoxon's matched-pairs signed-rank sum test and corrected for multiple comparison (Bonferroni). Patients with and without a particular IS risk factor as well as patients with and without a recurrent ischemic stroke were compared with Mann-Whitney U-test for continuous variables, and chi-square test or Fisher's test, as appropriate, for dichotomous variables. Correlation between two continuous variables was calculated with Spearman's rank correlation coefficient. Stratified by different etiologies of stroke, the parameters were studied with Kruskal-Wallis analysis of variance. Correlations between hemostatic markers and neurological scores were calculated by Spearman's rank correlation coefficient. The predictive value for recurrence or outcome was determined with fixed and stepwise logistic regression. A two-tailed p-value < 0.05 was considered significant.

Results

General information about IS patients

Clinical and demographic features of study participants (Studies I–IV) are shown in Table 2. No patients had a recurrent IS during the first 3 months after stroke (Studies I–IV). Thirty recurrent IS occurred in 29 patients (Studies I, II), and 12 IS occurred in 12 patients (Studies III, IV) during the 3-year follow-up.

Table 2. Clinical and demographic features of study participants.

Stroke risk factors		Study I (n=101)	Study II (n=102)	Studies III, IV (n=55)
Sex (male/female)		64/37	65/37	40/15
Age (years)		64.3 ± 12.3	63.1 ± 12.2	60.2 ± 11.4
Body mass index (kg/m2)	< 27/≥ 27	52/49	53/49	26/29
Hypertension	yes/no	53/48	54/48	27/28
Diabetes mellitus	yes/no	20/81	19/83	7/48
Smoking	yes/no	54/47	53/49	34/21
Cholesterol (mmol/L)	< 6.0/≥ 6.0	63/38	63/39	32/23
Triglycerides (mmol/L)	< 1.75/≥ 1.75	72/29	73/29	37/18
LDL cholesterol (mmol/L)	< 4.0/≥ 4.0	58/43	59/43	27/28
HDL cholesterol (mmol/L)	< 1.12/≥ 1.12	49/52	50/52	24/21
Sleep apnea	yes/no	29/72	28/74	21/34
TOAST group				
large-artery atherosclerosis		28	29	16
cardioembolism		21	21	9
small-artery occlusion		24	24	11
stroke of other determined cause		5	5	3
stroke of undetermined cause		23	23	16

Of study patients, 27–36% were on anticoagulation therapy (Table 3).

Table 3. Patients receiving anticoagulant treatment at different time points.

Study	Admission (unfractionated heparin)	1 week (warfarin)	1 month (warfarin)	3 months (warfarin)
Study I (n=101) anticoagulation ongoing/no	42/59	33/68	29/72	27/74
Study II (n=102) anticoagulation ongoing/no	42/60	33/69	29/73	27/75
Studies III, IV (n=55) anticoagulation ongoing/no	20/35	18/37	16/39	15/40

Clinical and demographic features of Study V participants are presented in Table 4. We observed 253 patient-years among IS patients with FVLm and 262 patient-years among patients without FVLm. The mean follow-up period was 6.0 ± 2.9 years for FVLm-positive patients and 6.2 ± 2.8 years for FVLm-negative patients. No patients from either group had recurrent IS during the first 3 months after stroke, but 14 FVLm-positive patients and 11 FVLm-negative patients had a recurrent thromboembolic event during the follow-up (6.0 ± 2.9 years for FVLm-positive patients and 6.2 ± 2.8 years for FVLm-negative patients).

Table 4. Baseline characteristics of factor Leiden mutation-positive and factor Leiden mutation-negative patients.

Characteristics	FVLm-positive patients	FVLm-negative patients
Age (years)	49.5 (50.7±10.5)	49.0 (50.1±10.6)
Sex (male/female)	23/19	23/19
Hypertension	22	23
Diabetes mellitus type I	1	1
Diabetes mellitus type II	2	2
History of myocardial infarction	2	1
Body mass index (kg/m2)	24.6 (25.1±3.1)	24.0 (24.8±3.1)
Smoking	19	16
Heavy alcohol > 200 g/week	1	2
Paroxysmal atrial fibrillation	4	2
Peripheral vascular disease	4*	0
Coronary heart disease	6	3
Previous transient ischemic attack	10	8
Previous ischemic stroke	5	2
Previous venous thromboembolism	4	1
Migraine with aura	12	13
Family history of venous thromboembolism	4*	0
Family history of coronary disease	19*	8
Oral contraceptive use	2	0
Hormonal replacement therapy	4*	0
Total cholesterol level (mmol/L)	5.3 (5.6±1.4)	4.9 (5.0±1.1)
Triglyceride level (mmol/L)	1.5 (1.9±1.8)	1.3 (1.4±0.8)
HDL cholesterol level (mmol/L)	1.2 (1.3±0.4)	1.3 (1.3±0.4)
LDL cholesterol level (mmol/L)	3.1 (3.5±1.2)	3.0 (3.1±1.0)

All values are median (mean ± SD). * $p < 0.05$

Markers measuring coagulation, fibrinolysis, and vasoactivity in patient subgroups

Table 5. Markers measuring coagulation, fibrinolysis, and vasoactivity in different phases of ischemic stroke.

	Acute phase (unfractionated heparin)	1 week (warfarin)	1 month (warfarin)	3 months (warfarin)	Controls
ET-1 (pg/mL)					
All patients	2.6 (2.4±1.3)	2.4 (2.2±1.4)	2.4 (2.1±1.4)	2.3 (2.1±1.2	2.0 (2.2±0.9)
AC patients	2.9 (2.7±1.2)	2.6 (2.4±1.5)	2.7 (2.6±1.3) *	2.5 (2.5±1.2) *	
Hcy (mmol/L)					
All patients	9.6 (11.4±6.5) *	11.0 (12.3±6.7)	11.1 (11.9±4.2)	11.6 (12.5±5.1)	11.6 (13.7±7.1)
AC patients	9.5 (11.3±6.8)	11.0 (12.4±6.9)	11.2 (11.8±4.4)	11.5 (12.4±5.1)	
AT (%)					
All patients	101.6 (102.8±13.6) *	109.7 (108.3±14.9) *	112.0 (113.3±14.8) *	114.0 (113.1±12.6) *	129.0 (138.5±37.4)
AC patients	97.0 (96.8±14.2) *	102.0 (104.3±12.7)	110.0 (115.0±20.1)	116.5 (115.8±10.7)	
PC (%)					
All patients	116.8 (117.9±28.7) *	116.0 (113.1±43.0)	112.0 (112.0±38.3)	109.0 (109.8±42.4)	107.0 (105.3±13.4)
AC patients	119.0 (120.0±31.3)	63.6 (82.6±43.7) *	62.5 (73.1±33.3) *	59.6 (79.0±36.9) *	
PS (%)					
All patients	98.3 (99.6±22.1)	88.0 (83.6±31.2) *	90.4 (84.1±29.5) *	91.5 (86.4±31.3) *	100.0 (101.3±17.1)
AC patients	102.8 (103.1±25.0)	65.0 (69.2±33.4) *	51.4 (53.9±25.9) *	52.0 (57.0±23.9) *	
F 1+2 (nmol/L)					
All patients	1.5 (1.7±0.9)	1.5 (1.7±1.1)	1.4 (1.4±0.8)	1.5 (1.6±1.4)	1.4 (1.7±0.8)
AC patients	1.4 (1.4±0.5)	1.1 (1.7±1.6) *	0.4 (0.6±0.9) *	0.4 (0.6±0.7) *	
TAT (mg/L)					
All patients	4.4 (7.9±9.1)	3.2 (6.6±8.6)	3.5 (4.5±3.3)	4.3 (6.0±6.2)	3.6 (5.0±5.2)
AC patients	3.6 (5.1±5.2)	2.2 (9.8±13.3)	1.7 (2.1±1.1) *	2.5 (3.8±4.5) *	
t-PA:Ag (ng/mL)					
All patients	7.7 (8.8±4.2)	8.0 (8.4±3.4)	7.7 (8.3±3.2)	7.8 (8.7±4.3)	7.4 (7.3±2.9)
AC patients	11.0 (11.2±4.9) *	7.9 (9.0±4.2)	7.7 (8.9±4.0)	7.8 (8.2±2.6)	
PAI-1 (AU/mL)					
All patients	16.3 (17.2±7.8) *	9.1 (11.2±9.2)	14.2 (14.4±7.9)	16.3 (15.8±8.4) *	10.3 (11.8±9.5)
AC patients	19.9 (20.9±8.8) *	8.9 (10.5±9.3)	16.8 (15.8±8.4)	15.6 (18.0±7.8)	
D-dimer (mkg/L)					
All patients	508.1 (661.6±585.2)	486.0 (1606.3±3184.2) *	465.0 (786.3±890.7) *	454.1 (491.1±423.4)	414.0 (608.8±890.9)
AC patients	510.8 (608.7±373.4)	396.1 (2175.7±4024.0)	313.6 (491.1±423.4)	212.6 (386.0±300.2) *	

AC, anticoagulated. All values are median (mean ± SD). * $p < 0.05$

ET-1 (Study I)

ET-1 levels in IS patients did not significantly ($p > 0.05$) differ from those of controls at any time point (Table 5). Patients receiving warfarin had significantly higher ET-1 levels at 1 and 3 months than patients receiving antiplatelet drugs ($p = 0.009$ and 0.03, respectively) (Table 5).

Among the factors that have the potential to affect ET-1 levels, including gender, age, BMI, AH, DM, dyslipidemia, smoking, and sleep apnea, only BMI and HDL-cholesterol showed a positive correlation with ET-1 levels ($p < 0.05$).

Hcy (Study II)

Hcy levels were significantly lower in stroke patients than in controls ($p < 0.001$) on admission, and were similar at later time points, as they increased at 1 week and remained at the same level up to 3 months (Table 5). Hcy levels among patients receiving anticoagulant treatment did not differ from nonanticoagulated patients (Table 5).

There were no differences between patients and control subjects in serum B12 (384.3 ± 176.3 ng/L vs. 379.8 ± 166.1 ng/L, $p = 0.6$) or folic acid levels (14.7 ± 5.8 nmol/L vs. 17.3 ± 10.0 nmol/L, $p = 0.5$). Serum B12 and folic acid levels showed a significant inverse correlation with Hcy levels in both groups ($p < 0.01$). Presence or absence of such IS risk factors as sex, BMI, AH, DM, dyslipidemia, smoking, and sleep apnea did not affect Hcy levels. Age showed a positive correlation with Hcy levels at all time points ($p < 0.01$) in stroke patients, but not in controls. Mean CRP concentration on admission was 6.0 ± 5.2 mg/L. No correlation was identified between admission CRP concentration and Hcy levels at any time point.

Markers measuring activity of coagulation and fibrinolysis (F1+2, TAT, t-PA:Ag, PAI-1, and D-dimer, Study III)

F1+2, TAT, and t-PA:Ag levels of patients did not differ from those of controls at any time point (Table 5). PAI-1 activity of patients was significantly higher than that of controls on admission ($p = 0.02$) and at 3 months ($p = 0.003$), but not at 1 week or at 1 month (Figure 7).

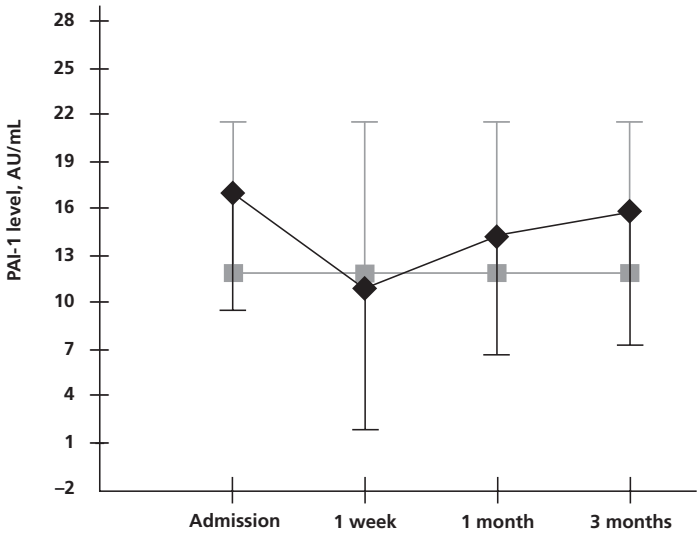

Figure 7. PAI-1 activity at various stage of IS. IS patients (◆) and controls (■). Standard deviation indicated by whiskers.

Plasma levels of D-dimer of patients at 1 week and 1 month were significantly higher than those of controls ($p < 0.005$ and $p < 0.05$, respectively) (Figure 8).

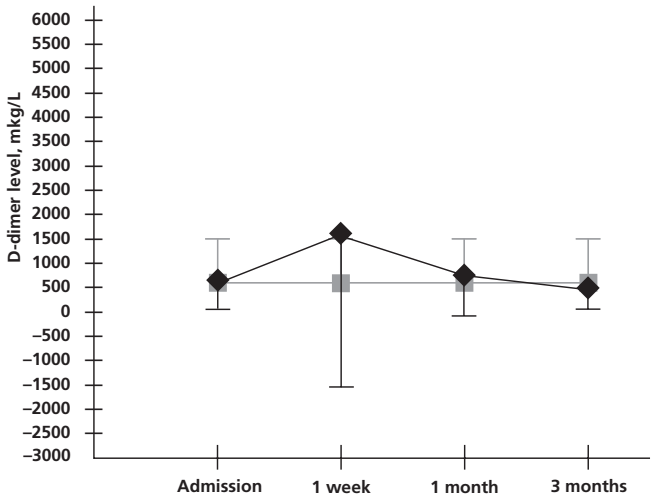

Figure 8. D-dimer levels at various stage of IS. IS patients (◆) and controls (■). Standard deviation indicated by whiskers.

Patients on anticoagulant treatment had higher t-PA:Ag and PAI-1 levels on admission than nonanticoagulated patients ($p < 0.01$), but not at later time points. Patients receiving anticoagulant treatment had lower F1+2, TAT, and D-dimer levels at 1 month and 3 months than nonanticoagulated patients ($p < 0.005$), but in acute and subacute phases of stroke, no difference was observed (Table 5).

Among all recorded factors that might correlate with coagulation and fibrinolysis markers (age, sex, DM, AH, serum total cholesterol, serum triglyceride, LDL-cholesterol, BMI, sleep apnea, and smoking), age showed an inverse correlation with PAI-1 activity on admission and at 1 week, 1 month, and 3 months ($p = 0.05$, 0.1, 0.04, and 0.06, respectively), and BMI showed a positive correlation with PAI-1 activity only at 3 months ($p = 0.04$). D-dimer levels were not correlated with the presence or absence of any risk factors for IS other than age on admission and at 1 week, 1 month, and 3 months (p-values of < 0.005, < 0.01, < 0.01, and < 0.001, respectively). F1+2, TAT, and t-PA:Ag levels showed no correlation with potential stroke risk factors.

Natural anticoagulants (AT, PC, and PS, Study IV)

AT levels were significantly lower at all time points in IS patients than in controls (Figure 9).

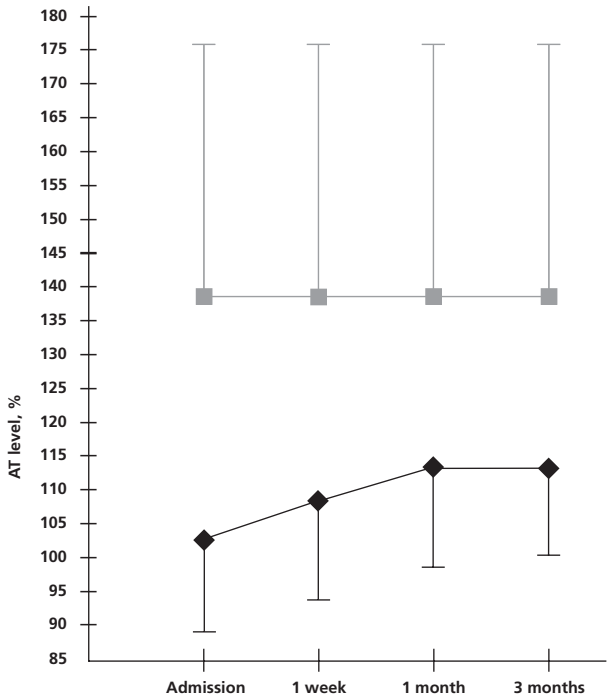

Figure 9. AT levels at various stage of IS. IS patients (◆) and controls (■). Standard deviation indicated by whiskers.

In IS patients, PC level was significantly higher on admission but not different at later time points from control subjects (Figure 10).

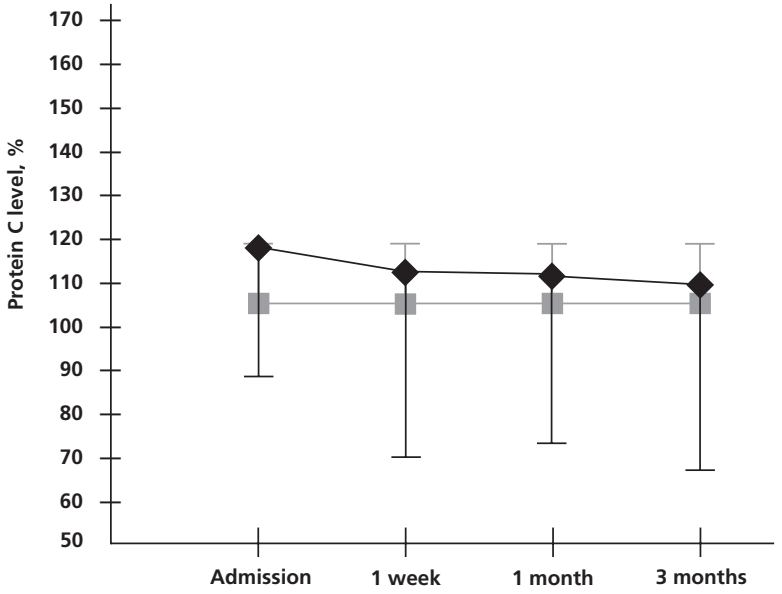

Figure 10. PC levels at various stage of IS. IS patients (◆) and controls (■). Standard deviation indicated by whiskers.

PS level was normal on admission but significantly lower at later time points over 3-month period in IS patients compared with control subjects (Figure 11).

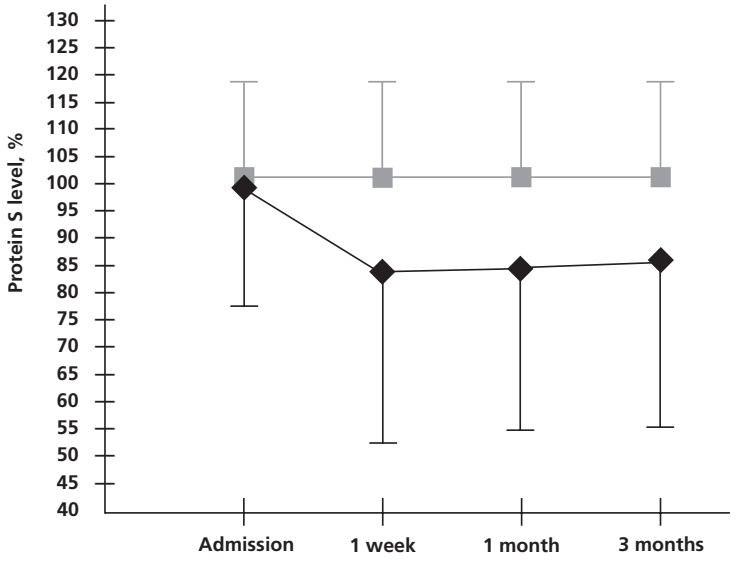

Figure 11. PS levels at various stage of IS. IS patients (◆) and controls (■). Standard deviation indicated by whiskers.

Patients receiving anticoagulant treatment had significantly lower AT levels on admission ($p = 0.01$) as well as lower PC and PS levels at 1 week, 1 month, and 3 months than patients using antiplatelet drugs (Table 5).

Age showed a significant positive correlation with AT levels at 1 week and 1 month ($p < 0.001$ and $p < 0.05$, respectively). PC level was significantly correlated with LDL-cholesterol on admission only ($p < 0.05$). Females had lower PS levels on admission and at 3 months than males. PS levels were higher in patients with sleep apnea on admission and at 1 week after stroke ($p < 0.05$). Presence or absence of the stroke risk factors of obesity, DM, smoking, and AH did not affect natural coagulant levels ($p > 0.05$ for all).

FVLm (Study V)

Baseline characteristics of FVLm-positive and FVLm-negative IS patients are given in Table 4. A few differences were seen; FVLm-positive patients more often received hormonal replacement therapy (4 vs 0, $p < 0.05$), had a higher prevalence of peripheral vascular disease (4 vs 0, $p < 0.05$), and more frequently had family histories of coronary disease (19 vs 8, $p < 0.05$) and venous thromboembolism (4 vs 0, $p < 0.05$) than FVLm-negative patients.

Markers measuring coagulation, fibrinolysis, and vasoactivity and stroke etiology, severity, outcome, and recurrence

The neurological scores of Studies I–IV are summarized in Table 6.

Table 6. Neurological scores of IS patients at different time points (Studies I–IV).

Neurological score	Admission	1 week	1 month	3 months
National Institute of Health Stroke Score				
Studies I, II (n = 102)	5 (6.8±5.4)	3 (4.6±4.4)	2 (3.1±3.7)	1 (2.6±3.3)
Studies III, IV (n = 55)	5 (6.5±5.0)	3 (4.2±4.2)	1 (2.9±2.9)	1 (2.5±3.2)
Scandinavian Stroke Scale				
Studies I, II (n = 102)	47 (44.2±11.5)	54 (49.0±10.4)	56 (52.1±8.7)	58 (53.2±7.8)
Studies III, IV (n = 55)	47 (45.0±10.8)	54 (49.7±9.9)	56 (52.7±8.0)	58 (53.8±7.0)
Barthel Index	_____	_____	_____	
Studies I, II (n = 102)				100 (91.5±16.6)
Studies III, IV (n = 55)				100 (95.2±11.6)
Modified Rankin Scale	_____	_____	_____	
Studies I, II (n = 102)				2 (1.9±1.2)
Studies III, IV (n = 55)				2 (1.8±1.0)
Mini-Mental State Examination	_____	_____	_____	
Studies I, II (n = 102)				28 (27.1±3.0)
Studies III, IV (n = 55)				29 (27.7±2.3)

All values are median (mean ± SD).

No correlation was found between ET-1 levels and stroke severity or outcome. A negative correlation was present between Hcy levels at all time points and MMSE at 3 months ($p < 0.01$). F1+2, TAT, and t-PA:Ag levels, PAI-1 activity, and PC and PS levels showed no correlation with stroke severity or outcome. AT levels on admission were positively correlated with disability and stroke severity (BI and NIHSS at 3 months, $p = 0.03$ and $p = 0.04$, respectively). D-dimer levels on admission and at 1 week were positively correlated with outcome and disability (mRS and BI at 3 months, $p < 0.05$).

The neurological scores of Study V are summarized in Table 7.

Table 7. Neurological scores of FVLm-positive and FVLm-negative IS patients at different time point (Study V).

Neurological score	FVLm-positive patients n = 42	FVLm-negative patients n = 42
on admission		
Scandinavian Stroke Scale	55.5 (52.7 ± 7.2)	52.5 (49.5 ± 10.3)
National Institute of Health Stroke Score	3.0 (3.6 ± 3.1)	4.0 (4.9 ± 3.8)
at discharge from hospital		
Scandinavian Stroke Scale	58.0 (56.3 ± 3.8)	56.0 (54.5 ± 5.0)
National Institute of Health Stroke Score	1.0 (1.7 ± 2.1)	2.0 (2.2 ± 2.3)
at 3 months		
Scandinavian Stroke Scale	58.0 (57.2 ± 2.0)	58.0 (56.6 ± 2.9)
National Institute of Health Stroke Score	0 (1 ± 1.7)	0 (0.9 ± 1.6)
Barthel Index	100 (99.0 ± 6.2)	100 (99.4 ± 2.3)
Modified Rankin Scale	1 (0.7 ± 0.8)	1 (1.3 ± 2.3)
at end of follow-up		
Barthel Index	100 (98.7 ± 6.6)	100 (99.9 ± 0.8)
Modified Rankin Scale	0.5 (0.6 ± 0.8)	1 (0.9 ± 0.8)

All values are median (mean ± SD).

No differences were observed in stroke severity, stroke outcome, or disability between FVLm-positive and FVLm-negative IS patients ($p > 0.05$).

Of all markers measuring coagulation, fibrinolysis, and vasoactivity (Studies I–IV), only F1+2 levels at 3 months showed a positive correlation

with recurrence of thromboembolic events (p = 0.02). Etiology of brain infarction was not associated with marker levels at any time of measurement (Studies I–IV). Recurrence rate of thromboembolic events showed no correlation with FVLm positivity (Study V). The annual recurrence rate in FVLm-positive and FVLm-negative groups showed no association with the treatment regimen (aspirin/warfarin, p > 0.05).

Brain imaging

In Study II, the mean infarct volume was 16.0 ± 27.5 cm^3. No correlation was found between Hcy levels and size of brain infarction.

In comparing FVLm-positive and FVLm-negative patients (Study V), no differences were observed in stroke subtype, infarction location, and size, or in presence/degree of leukoaraiosis. In the FVLm-positive group, 21 patients had more than one infarction (2 or more) compared with two patients in the FVLm-negative group (p < 0.02) (Tables 8, 9).

Table 8. Brain imaging characteristics of FVLm-positive and FVLm-negative IS patients according to CT or MRI appearance and clinical syndrome.

Characteristic	FVLm-positive patients n = 42	FVLm-negative patients n = 42
OCSP subtype		
Total Anterior Circulation Infarction (TACI)	0	0
Lacunar Infarction (LACI)	15	14
Partial Anterior Circulation Infarction (PACI)	13	10
Posterior Circulation Infarction (POCI)	14	13
Presence of leukoaraiosis: number (%)	11 (26)	18 (43)
Level of leukoaraiosis		
0	31	24
1	6	10
2	4	8
3	1	0
4	0	0
Age of patients with leukoaraiosis (years)	55.0 (60.0 ± 9.6)	49.0 (50.8 ± 8.7)

OCSP, Oxfordshire Community Stroke Project. All p-values > 0.05.

Table 9. Brain imaging characteristics of FVLm-positive and FVLm-negative IS patients.

CT/ MRI appearance	FVLm-positive patients n = 42	FVLm-negative patients n = 42
Group 1: small-vessel disease	18	13
Group 2: large-vessel occlusion	10	14
Group 3: subcortical hemispheric infarctions	6	9
Group 4: mixed vascular pathology	7	6
Multiple infarctions (2 or more)	21	2
No visible infarction	1	0

All p-values > 0.05, except for multiple infarctions ($p < 0.02$).

Discussion

This was a follow-up case-control study of IS patients, with serial measurements of markers of coagulation, fibrinolysis, and vasoactivity and repeated neurological examinations. The purpose was to investigate serial changes in these markers and to determine whether the markers could predict short-term (3 months) and long-term (3 years) outcome, including recurrent IS. We selected patients with mild or moderate IS to maximize the likelihood of survival over the study period and to allow repeated neurological evaluations and a long-term follow-up. We had a special interest in serial measurements of several coagulation-associated markers, as previous studies have typically included one or a few sets of markers.

Spesific objectives were to elucidate (1) whether repeated measurements of markers assessing coagulation, fibrinolysis, and vasoactivity disclose any patterns that might help in evaluating IS patients, including early diagnosis of stroke subtypes, in estimating prognosis and risk of recurrence, and in selecting a treatment for secondary prevention of stroke, and (2) whether patients with FVLm and IS share common characteristics that may arouse suspicion of FVLm on clinical grounds and indicate the test to verify or exclude the presence of FVLm.

Repeated measurements of ET-1 levels did not disclose information that could foster the diagnostic evaluation of IS patients (I). Plasma Hcy levels were significantly lower in IS patients than in controls in the acute stage, increasing and remaining stable during the convalescent period (II). Our 3-year follow-up examination showed that F1+2 level at 3 months after stroke had a positive correlation with recurrence of IS and may be used as a predictive marker of subsequent cerebral events (III). D-dimer and AT levels on admission and D-dimer level at 1 week were positively correlated with stroke outcome and disability (III, IV). IS patients with heterozygous FVLm more often had a positive family history of thrombosis, a higher prevalence of peripheral vascular disease, and multiple infarctions in brain images, most of which were 'silent infarcts' (V).

This study has certain limitations. The first blood samples were mostly taken between 24 and 48 h after stroke, and consequently, a hyperacute peak in fibrinolytic or coagulation activity could have been missed. The study population was male-dominant. Our patients were in better condition than the average stroke patient to maximize the likelihood of their survival until the end of follow-up. Severely ill stroke patients are frequently bedridden and incur such confounding factors as fever, infections, and DVT, which affect

the markers of coagulation, fibrinolysis, and vasoactivity. The most serious limitation in our study was that 27–36% of patients used anticoagulant therapy. This treatment has an effect on markers of coagulation and fibrinolysis, thus influencing study results. The decision for FVLm testing was reached by physicians responsible for the patients; possibly, severely affected stroke patients and patients who died early were not tested for FVLm. For this reason, our study cannot rule out whether FVLm-positive patients had a poor prognosis. In addition, only some of the patients in Study V were evaluated to exclude patent foramen ovale (PFO).

Changes in markers measuring coagulation, fibrinolysis, and vasoactivity over time

ET-1 (Study I)

ET-1 could be involved in the pathogenesis of IS because it has a strong and long-lasting vasoconstrictor effect. Vasoconstriction due to ET-1 in atherosclerotic arteries is significantly more pronounced than in normal vessels (41). Recent studies have demonstrated a rapid increase in circulating plasma ET-1 levels in the acute phase (20, 23, 86) as well as in the chronic phase (23) of IS. Normal levels of ET-1 have also been reported (111). Endothelial cells release ET-1 in response to various stimuli such as hypoxia (216), transforming growth factor (151), and thrombin (286). Experimentally, the depth of injury to the vessel wall was found to be an important determinant of the amount of thrombus formation, and ET-1 levels may be higher in extensive brain tissue necrosis (86).

We expected plasma ET-1 levels in IS patients to be higher than in controls, although this was not the case. This may be due to the following factors. Only patients with mild to moderate IS were included in our study because of their anticipated longer survival. Thus, most of our patients had small- to medium-sized infarctions and were in better condition than the average stroke patient. In addition, increased local production of ET-1 may not be correctly detected in peripheral blood because ET-1 is a local regulating factor and is higher at the interface of the endothelium than in plasma (161). Lampl et al. (155) reported an elevated ET-1 in CSF among IS patients, whereas plasma values did not show any significant changes. Measurement from the CSF during the hyperacute phase may yield more accurate results. Plasma ET-1 levels increase within 60–150 min of thrombotic occlusion (95). In our patients, the first blood samples were usually taken between 24 and 48 h after stroke; thus, we may have missed the elevation of ET-1 at the hyperacute phase. Regardless of these limitations, our study does not provide evidence of a major role for ET-1 in IS.

Hcy (Study II)

Although a rise of plasma Hcy levels exceeding 15 mmol/L is at present considered an independent risk factor for vascular disease, including IS (16), it remains uncertain whether Hcy is a causal risk factor for stroke or is a marker of other stroke-associated factors (4, 39).

Plasma Hcy levels were significantly lower in our IS patients than in controls on admission, but increased in the subacute stage and remained stable during the convalescent period. Hcy levels were similar to those of control subjects after the acute stroke stage. Lindgren et al. (163) found that in 17 patients re-examined in the convalescent phase of IS, plasma Hcy values were significantly higher than in the acute phase. By contrast, plasma Hcy levels did not change significantly over time in the 20 re-examined control subjects. Meiklejohn et al. (188) showed that plasma Hcy levels after recent atherothrombotic stroke did not differ from control subjects. However, plasma Hcy levels increased significantly in the convalescent period and were then significantly higher than in control subjects (188). In a recent study, Hcy levels were shown to increase during the acute period after stroke (122). We do not know whether this acute decrease in Hcy levels upon onset of IS is a protective reaction of the human body aimed at limiting brain injury or is solely a nonspecific acute-phase reaction (81). Plasma Hcy levels on admission could reflect a premorbid state, with environmental factors, such as low dietary folate or several widely used drugs (e.g. statins, metformin, insulin), altering Hcy levels (77). Genetic factors may also play a role in explaining the study results. Racial and geographic differences are known to exist in the atherosclerotic process in cerebral arteries (238). Studies from African white and black populations have shown a significant difference in favor of the black population in Hcy levels, highlighting the importance of genetic factors (269, 277). The lack of association between Hcy and IS in our study and similar results in previous studies of the Finnish population (19, 138) suggest a role for genetic factors and a low overall prevalence of hyperhomocysteinemia in Finland.

Fibrinolysis and coagulation markers (F1+2, TAT, t-PA:Ag, PAI-1, D-dimer, Study III)

F1+2, TAT, and t-PA:Ag levels were not significantly different from those of control subjects at any time point. These findings are at odds with several earlier studies (21, 103, 164, 249), probably due to patient selection; our patients had milder than average strokes, suffering small- to medium-sized infarcts. The results may be different in severely ill stroke patients who have large infarcts, probably a larger mass of clot material, and more frequently suffer from other confounding factors such as fever, infections, and a depressed level of consciousness, and thus, are more prone to other thromboembolic complications. PAI-1 activity was increased on admission and at 3 months, but normal at 1 week and at 1 month after IS. The results

confirm previous findings of increased PAI-1 activity both in the acute phase and in the convalescent phase after stroke (21, 164). Increased PAI-1 activity in stroke patients may be associated with an acute-phase reaction (131) or it might reflect an endogenous predisposition to thrombogenesis (130). Normalized post-stroke PAI-1 levels at 1 week and 1 month may suggest enhanced in fibrinolytic activity in these patients, possible also being due to use of antithrombotic agents, vigorous physical rehabilitation, cessation of smoking, hospital diet, better care for diabetes and hypertension in hospital, or other yet unknown factors. High PAI-1 activity at 3 months post-stroke may be associated with a rebound hypercoagulable state or may reflect a high rate of synthesis and release from atherosclerotic vessels (227).

We found D-dimer levels to be significantly higher in the subacute phase of IS (1 week and 1 month), but not in the chronic phase, which is in accordance with previous reports (21, 123). Elevated D-dimer levels may reflect an acute-phase response (28), an activation of coagulation secondary to tissue damage, or a complication in the course of stroke (infection or venous thromboembolism) (32). Elevated D-dimer levels could also be caused by continuous formation and subsequent lysis of fibrin (93). Anticoagulant treatment can normalize D-dimer levels in the convalescent phase of stroke (198).

Natural anticoagulants (AT, PC, and PS, Study IV)

A distinct pattern of natural anticoagulant activity is present in IS; while AT level remains significantly decreased at all time points after stroke, PC level is increased on admission, becoming normalized later. PS level in turn is normal in the acute phase of stroke and decreases later.

In patients with IS, AT level was previously reported as either decreased (21) or normal (285). Takano et al. (249) showed that AT activity was significantly lower at the time of admission in cardioembolic IS patients compared with controls. The low AT levels probably reflect an increased consumption of active AT in the acute phase. Because unfractionated heparin (UFH) indirectly inhibits thrombin through an antithrombin-dependent mechanism, frequent use of UFH in our patients in the acute stage of IS could also explain this finding (192). Our patients had lower AT levels up to 3 months after stroke, suggesting that the coagulation system had not returned to normal even then, whereas in two previous studies (249, 259), the AT level increased with time and normalized in a few weeks following stroke. This is likely because of a chronic activation of the fibrinolysis system.

PC level has been found to be low (248) or normal (285) during the acute phase and normal in the convalescent phase of IS (285). In our study, we observed a significantly high PC level on admission and normal PC levels thereafter. The variation in results between other studies and ours most likely reflects differences in patient selection since in the previous studies, the lowest PC levels were observed in the most severely ill patients, and such patients were excluded from our study. The normalized PC levels at later

time points may also reflect the effect of oral anticoagulant therapy (153).

We found PS levels to be significantly low in the subacute and chronic phases of IS but not in the acute phase. This is in agreement with results of some studies (21, 136), while another study (24) reported low free PS levels in 8 of 43 patients with IS in an acute setting. The varying and partly conflicting results of different studies may be due to different patient population characteristics, laboratory methods, time points of examination, stroke classifications used, or whether warfarin treatment was administered.

Markers measuring coagulation, fibrinolysis, and vasoactivity and stroke risk factors (Studies I–V)

The relationship between markers of coagulation, fibrinolysis, and vasoactivity and several IS risk factors has previously been investigated, with conflicting results (16, 166, 204, 218, 226, 287). We found a significant correlation between these markers and such major stroke risk factors as age (Hcy, AT, PAI-1, and D-dimer), sex (PS), BMI (ET-1, PAI-1), and LDL-cholesterol (AT, PC), whereas AH, DM, dyslipidemia, smoking, and sleep apnea did not affect hemostatic markers. In general, patient selection, type and duration of concomitant diseases, regimen and efficiency of treatment, existence of complications, and some other confounding factors may have had an influence on our results. The results of previous studies have been highly variable in the literature, and therefore, allow no firm conclusions to be drawn.

Our results, in agreement with those of Lalouschek et al. (154), showed that no significant correlation exists between the presence of FVLm and the major stroke risk factors. Moreover, we found no association between classical migraine and FVLm positivity in IS patients, which is again consistent with a previous report (124). We did observe a higher prevalence of peripheral vascular disease among FVLm-positive patients, comparable to the findings of Sykes et al. (245). In accordance with previous studies (108, 265), carrier status for FVLm was associated with family history of thrombosis. Since FVLm has been established as an autosomal dominant trait, this finding is quite plausible. However, a positive family history of thrombosis was found in only a minority of our patients, thus, its predictive value was only modest. Positive family histories of venous thrombosis and cardiovascular events were not present among the same patients. Interestingly, FVLm carriers did not suffer from early venous thrombosis more often than those without FVLm, contrary to earlier reports (194, 225). FVLm positivity in IS patients was not associated with deficiencies in natural anticoagulants. Dahlbäck et al. (65) found no significant link between the presence of FVLm and cardiolipin IgG antibodies or lupus anticoagulant; our results are consistent with this.

Markers measuring coagulation, fibrinolysis and vasoactivity, and stroke etiology, severity, outcome, and recurrence rate (Studies I-IV)

Pathogenetic mechanisms may differ in various subtypes of IS. Consequently, some investigators have suggested that coagulation and fibrinolytic parameters may distinguish various stroke subtypes. Our results did not indicate any relationship between plasma ET-1 levels (Study I) and cause of stroke, which is in agreement with data reported by Estrada et al. (86). Previous studies (20, 23, 155) have described significantly higher plasma ET-1 levels in large cortical and cardiogenic infarctions that could be explained by anticoagulant use, as also the case in our study.

High Hcy levels have been associated with subtypes of IS caused by large-artery and small-artery disease (82, 288) as well as cardiogenic disease (233). In Study II, Hcy levels did not vary with the etiology of IS, supporting the findings of Bushnell et al. (50).

Concerning natural anticoagulants (Study III), we found that PC and PS levels were significantly lower in patients with cardioembolic infarction. PC and PS levels are lowered by warfarin treatment (248), which is the most likely explanation for this finding. In Study IV, we found no significant relationship between the etiology of brain infarction and AT, F1+2, TAT, t-PA:Ag, PAI-1, or D-dimer levels. The discrepancy between this finding and previous results (21, 87, 89, 96, 142, 248) can be explained in several ways. The small number of patients in each stroke subgroup hampers the reliability of results. In addition, IS has been classified according to different criteria in earlier studies. Furthermore, treatment regimen (antithrombotic vs. anticoagulant treatment) will have an effect on the levels of the markers of coagulation and fibrinolysis.

We were unable to reveal an association between the subtypes of IS and the presence of FVLm (Study V), similar to previous studies (115, 290).

We hypothesized that activation of coagulation and fibrinolysis could be an important cause in severely affected stroke patients because of disturbed endothelial function, large masses of thrombi, and large tissue necrosis in the brain. In Study II, elevated Hcy levels showed a significant inverse correlation with MMSE, in agreement with previous reports (230). This finding is likely an epiphenomenon, reflecting the parallel influence of aging on both cognitive performance and Hcy levels, in opposite directions, rather than an independent causal effect of Hcy levels on cognition.

AT levels (Study III) were significantly correlated with stroke severity and outcome. These results probably reflect an increased consumption of active AT due to the the extent of thrombosis (203). The elevated D-dimer levels on admission and 1 week after IS (Study IV) were strongly associated with stroke outcome and disability, supporting the results of two earlier reports (32, 88). In fact, elevated levels of D-dimer may reflect the level of activation

of both the fibrinolytic and the coagulation systems, and thus, the ongoing thrombotic process. The other markers measuring coagulation, fibrinolysis, and vasoactivity (Studies I–IV) did not disclose information that could aid in identifying patients with poor prognosis. This finding indicates that there is considerably less activation of the fibrinolytic and coagulation systems in mild or moderately ill IS patients, corroborating previous reports (24, 47, 189, 248). Interestingly, our follow-up examination of patients after stroke showed that F1+2 levels in the chronic phase (Study IV) had a positive correlation with recurrence of thromboembolic events and thus these marker may be used as a predictor of subsequent cerebral events, as also observed previously (61, 240). These data support the idea that stroke patients with sustained activation of the blood coagulation system and increased thrombin generation of F1+2 levels may have an unfavorable prognosis.

No reports are available concerning a correlation between the presence of FVLm and stroke severity and prognosis. We found that stroke severity, outcome, and recurrence rates were not different among FVLm-positive and -negative patients (Study V). However, a firm conclusion on this matter necessitates recruitment of a large number of consecutive patients, including those who die early or remain bedridden. Apparently, such patients are not considered prime targets for FVLm testing; those included herein were mostly young, vigorous patients without obvious etiologic factors for stroke, which explains the rather mild to moderate strokes observed.

Influence of markers measuring coagulation, fibrinolysis, and vasoactivity on radiological findings (Studies II, V)

The hypothesis underlying Study II was that plasma Hcy levels promote brain injury after IS, thus increasing ischemic lesion size. Endres et al. (84) reported that elevated Hcy levels in folate-deficient mice were associated with increased brain lesion size. We found no correlation between Hcy levels and brain infarction volumes.

The links between FVLm and radiological findings have been examined in a few case-control studies, with contradictory results (208, 246). We recognized that the radiological findings were nonspecific with regard to FVLm positivity and could not be used to show patterns indicative of FVLm. However, FVLm carriers more often had multiple ischemic lesions in brain images than FVLm-negative IS patients; in FVLm-positive patients, most infarctions were 'silent infarcts'. This is a novel finding. Karttunen et al. (139) observed factor V Leiden or prothrombin G20210A mutation to be associated with cryptogenic stroke. Among patients with these prothrombotic states, the Valsalva maneuver was common at stroke onset. In our study, only 31 patients with FVLm were evaluated to exclude PFO. Thus, we do not know whether multiple silent infarcts in brain images are related to an underlying

paradoxical embolism via PFO (139). No previous reports exist regarding a correlation between FVLm and leukoaraiosis. We found no significant differences between FVLm-positive and -negative IS patients in the presence or degree of leukoaraiosis.

Summary and conclusions

IS is a common and devastating disease frequently leading to death, disability, and suffering. Evidence indicates increased thrombin generation and fibrin turnover, altered fibrinolytic activity, and disturbed endothelial function in IS. We hypothesized that changes in coagulation and fibrinolytic factors and in endothelial function would disclose patterns that might help in evaluating IS patients, including the early diagnosis of stroke subtypes, in estimating prognosis and risk of recurrence, and in selecting a treatment for secondary prevention of stroke. In Studies I–IV, we performed repeated measurements of markers assessing coagulation, fibrinolysis, and vasoactivity in a defined population of IS patients to shed light on these issues. In Study V, we examined IS patients with FVLm to determine whether the presence of FVLm was associated with specific clinical, laboratory, radiological, or prognostic characteristics.

All five studies were carried out at the Department of Neurology, Helsinki University Central Hospital, after being approved by the Ethics Committee. One hundred and two IS patients were included in Studies I and II, and 55 consecutive patients were chosen from this group for Studies III and IV. Another 42 patients were selected for Study V from 740 patients with IS without an obvious etiology. Patients underwent blood sampling four times, at each time point undergoing a full neurological evaluation, including several neurological scores, and assessment of multiple markers of endothelial and hemostatic function. Vasoconstrictor agent ET-1, Hcy, indicators of thrombin formation (F1+2), thrombin inactivation (TAT) and fibrinolysis (t-PA, PAI-1, and D-dimer), and natural anticoagulants (AT, PC, and PS) were measured. In addition, DNA analysis to determine whether FVLm was present was included. Short-term (3 months) and long-term (3 years) follow-ups were performed to investigate stroke outcome and recurrence. During this study, 27–36% of patients were receiving anticoagulant therapy.

Repeated measurements of ET-1 or Hcy levels did not disclose any useful information. D-dimer and AT levels on admission and D-dimer levels at 1 week were positively correlated with stroke outcome and disability. Our 3-year follow-up examination showed that F1+2 level at 3 months after stroke had a positive correlation with recurrence of thrombotic events, and thus, this marker can used to predict recurrent IS. IS patients with heterozygous FVLm more often had a positive family history of thrombosis, a higher prevalence of peripheral vascular disease, and multiple infarctions in brain images, most of which were 'silent infarcts'.

In summary, changes in markers measuring coagulation, fibrinolysis, and

vasoactivity can be useful in estimating prognosis of IS patients. A clear need exists for a randomized prospective study to determine whether a subgroup of IS patients with markers indicating activation of fibrinolytic and coagulation systems could benefit from more aggressive secondary prevention of stroke.

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