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MOLECULAR AND CLINICAL MARKERS IN ISCHAEMIC CEREBROVASCULAR DISEASE

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ABSTRACT

Ischaemic stroke and TIA, together labelled as Ischaemic Cerebrovascular Disease (ICVD), is a heterogeneous, complex disorder with great impact on morbidity and mortality worldwide. Genetic predisposition and environmental factors interact and confer susceptibility to different pathologies that are associated with increased ICVD risk. The most common aetiological mechanisms include large vessel atherosclerosis, sources of cardioembolism and small vessel disease. Distinct pathologies underlying ICVD may occur alone or co-exist in various combinations where their causal relation to the actual ischaemic event is difficult to determine. The TOAST system has been the most widely accepted classification approach that assigns patients in one of the three main aetiopathogenic subtypes, if evidence for a causal relation exists, or to unknown if the investigation has been inconclusive. The ASCO system has recently suggested a different approach by which all the three pathologies are evaluated and graded for the likelihood to have a causative association with the ischaemic event. Related comorbidities, risk factor profile, treatment choices and prognosis differ in each aetiological subtype of ICVD, yet with several overlapping features. One common aspect, though with diverse pathways, involves inflammatory mechanisms that contribute to the initiation and progress of pathologies mediating ICVD, and have an ambiguous role in brain tissue damage during cerebral ischaemia. Moreover, factors of coagulation and metabolism have been suggested to correlate with aetiological subtypes of ICVD and to serve as predictors of long-term disability. The purpose of this project was to investigate the role of genetic and blood-based biomarkers in ICVD susceptibility, aetiology and prognosis.

Ischaemic stroke and TIA patients admitted over different periods in the stroke-unit of Karolinska University Hospital in Huddinge, as well as healthy individuals comprised the population of our studies. In the first, case-control study on delta32 mutation that abolishes CCR5 from cell surface, we found that the presence of the mutated allele was less common in patients with cardioembolic ICVD compared to those with other aetiological subtypes. Hence, we assumed that CCR5 might play a role in predisposition to cardiac conditions that confer increased embolic risk. In the subsequent studies we investigated the association of blood-borne markers with aetiological subtypes and long-term survival in ICVD. Our results imply that increased levels of glucose, white blood cell count and fibrinogen, as well as low levels of cholesterol on admission, may be associated with poorer long-term survival after stroke and TIA. Investigation of aetiological phenotypes according to TOAST and ASCO showed very good agreement between the two classification systems in all subgroups except for large vessel stroke where the matching was good. Long-term survival was higher in patients with small vessel stroke and poorer in patients with incomplete work-up followed by cardioembolic and cryptogenic aetiology.

Overall, our findings indicate that molecules involved in inflammation and metabolism may play a role in the pathology and prognosis of ICVD. We also addressed the issue of aetiological classification in stroke, and how different approaches may improve research accomplishments. Our studies are not designed to serve as sources of conclusions on causal mechanisms associated with disease occurrence and outcome. Future studies on larger, well-described populations are needed in order to clarify the processes involved in different aetiopathogeneses of stroke.
I. Kostulas N, Markaki I, Kostulas V, Hillert J, Kostulas K. The CCR5 Δ32 polymorphism differentiates cardioembolism from other aetiologies of ischaemic cerebrovascular diseases. Scandinavian Journal of Immunology, 2009; 70: 475-480

II. Kostulas N, Markaki I, Cansu H, Masterman T, Kostulas V. Hyperglycaemia in acute ischaemic stroke is associated with an increased 5-year mortality. Age and Ageing, 2009; 38: 590-594


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<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ALOX5AP</td>
<td>Arachidonate 5-lipoxygenase-activating protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine (C-C motif) ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>C-C chemokine receptor</td>
</tr>
<tr>
<td>CDKN2B</td>
<td>Cyclin-dependent kinase inhibitor 2B</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphates</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>E-sel</td>
<td>E-selectin</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GCS</td>
<td>Glasgow Coma Scale</td>
</tr>
<tr>
<td>HDAC9</td>
<td>Histone deacetylase 9</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virous</td>
</tr>
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<td>HR</td>
<td>Hazard ratio</td>
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<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>ICA</td>
<td>Internal carotid artery</td>
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<tr>
<td>ICAM</td>
<td>Intracellular adhesion molecule</td>
</tr>
<tr>
<td>ICVD</td>
<td>Ischaemic cerebrovascular disease</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile range</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Macrophage chemoattractant protein-1</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>mRS</td>
<td>modified Rankin Scale</td>
</tr>
<tr>
<td>NIHSS</td>
<td>National Institutes of Health Stroke Scale</td>
</tr>
<tr>
<td>OCSP</td>
<td>Oxford Community Stroke Project</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDE4D</td>
<td>Phosphodiesterase 4D</td>
</tr>
<tr>
<td>PFO</td>
<td>Patent foramen ovale</td>
</tr>
<tr>
<td>PITX2</td>
<td>Paired-like homeodomain 2</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on activation, normal T cell expressed and secreted</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper type 2</td>
</tr>
<tr>
<td>TIA</td>
<td>Transient ischaemic attack</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TOAST</td>
<td>Trial of Org 10172 in Acute Stroke Treatment</td>
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<tr>
<td>ZFHX3</td>
<td>Zinc finger homeobox 3</td>
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1 INTRODUCTION

In a large volume of medical literature of antiquity, stroke symptoms – including impairment of motion, sensation and mental function – were described by the term *apoplexy*. Aristotle (d. 322 BC) suggested that the heart was the organ that controlled motor function, and the physician Diocles (320 BC) was the first to explain apoplexy and paralysis as a consequence of large vessel obstruction by cold and thick phlegm [1]. In Hippocrates’ writings (d. 370 BC), several theories on the aetiology of apoplexy are presented, however the perception that symptoms somehow relate to the brain dominates [2].

The World Health Organization has estimated that in 2004 there were nine million new cases of first-ever stroke worldwide and over 30 million stroke-survivors. Approximately 5.7 million deaths were attributed to cerebrovascular diseases, accounting for nearly 10% of all deaths. All cardiovascular deaths, including stroke, are projected to increase from 17 million in 2004 to 23 million in 2030 [3].

Over the past four decades, stroke incidence has decreased by 42% (from 163 per 100 000 person-years in 1970-79 to 94 per 100 000 person-years in 2000-08) in high-income countries. In contrast, incidence rates in low to middle income countries have increased by over 100%, and have exceeded those of high income countries, with 117 cases per 100 000 person-years reported in 2000-08 [4].

1.1 PATHOPHYSIOLOGIC CLASSIFICATION OF CEREBROVASCULAR DISEASE

Cerebrovascular disease is caused by one of several pathophysiologic processes affecting the blood vessels of the brain [5].

- The process may be located within the vessel, as in atherosclerosis, lipohyalinosis and arterial dissection.
- The process may arise remotely, as occurs when an embolus from the heart or an extracranial vessel obstructs an intracranial artery.
- The process may be due to inadequate cerebral blood flow caused by decreased perfusion pressure or increased blood viscosity.
- The process may accompany the rupture of a vessel in the subarachnoid space or into the brain.

The first three processes may underlie transient ischaemic attack (TIA) or ischaemic stroke, while the fourth is associated with subarachnoid and intracerebral haemorrhage. According to the latest report from American Heart Association on heart disease and stroke statistics, the proportions of ischaemic stroke, intracerebral haemorrhage and subarachnoid haemorrhage are 87%, 10% and 3%, respectively, in USA [6]. In Europe, the proportion of ischaemic stroke varied from 63% in Menorca, Spain, to 88% in Dijon, France, at the beginning of 21st century [7].
1.1.1 Brain ischaemia

The symptoms of brain ischaemia may be transient, totally resolving within seconds to minutes, or may persist for longer periods of time. Symptoms and signs are permanent if the brain becomes irreversibly damaged and infarction occurs [5]. However, neurological examination does not accurately reflect the presence or absence of infarction, and the temporal pattern of symptom progression does not indicate the cause of ischaemia [8].

1.1.1.1 TIA

TIA is now defined as “a transient episode of neurologic dysfunction caused by focal brain, spinal cord, or retinal ischaemia, without acute infarction” [9]. This tissue-based definition of TIA relies on the absence of end-organ injury, as assessed by imaging techniques.

1.1.1.2 Ischaemic stroke subtypes

The main subtypes of brain ischaemia comprise large vessel disease, small vessel disease, embolism, systemic hypoperfusion, and coagulation disorders.

In large vessel disease, the pathophysiologic process involves thrombus formation in a large artery that leads to infarction, either by reduced blood flow distally, or by an embolic fragment that breaks off and travels to a more distal vessel (artery-to-artery embolism) [10]. Large vessel disease affects both the extracranial and intracranial arterial system, and may be attributed to atherosclerosis, dissection or other less common pathologies (Table 1). Atherothrombosis is, however, the most common pathological process, accounting for approximately 15% of all ischaemic strokes in population-based studies [11]. Extracranial internal carotid artery (ICA) stenosis is the most common type of the disease, compared to extracranial ICA occlusion and intracranial atherosclerosis [12]. Extracranial atherosclerosis is associated with increasing age, male gender and hyperlipidaemia, whereas patients with intracranial atherosclerosis have more often metabolic syndrome [13].

Small vessel disease refers to several pathologies that may affect the small arteries, arterioles, venules, and capillaries of the brain. In contrast to large arteries, small vessels cannot be visualized in vivo, and deep brain tissue changes caused by these pathological processes have been used as markers of small vessel disease [14]. Several types of small vessel disease have previously been described, but the most prevalent pathologies will be discussed here, leaving aside inherited and immunemediated forms of the disease, as well as venous collagenosis and other, rare aetiologies (Table 1).

Arteriolosclerosis is the most common aetiology underlying small vessel disease; it results in a systemic, age-related process affecting the brain and other organs (e.g., kidneys and retinas) and is associated mainly with hypertension and diabetes [15]. Histopathological changes observed in arteriolosclerosis include atherosclerosis of small perforating arteries, associated with lacunar infarcts (5-20 mm), and fibrinoid
necrosis and lipohyalinosis of the very small arterioles, associated with smaller lesions (up to 7 mm). Recently, in a cross-section analysis of magnetic resonance imaging (MRI) data from individuals included in a community-based prospective cohort study, it has been suggested that very small lacunes (≤ 7mm) are associated with HbA1c and diabetes, whereas larger infarcts (8-20 mm) are associated with LDL-cholesterol levels. Increasing age, hypertension and ever-smoking were associated with both types of infarcts [16]. These findings provide further support for the theory favouring distinct vascular pathologies in small vessel disease, and explain, at least partly, previous discrepancies in studies of risk factor profiles in small vessel disease [17].

Cerebral amyloid angiopathy affects small-to-medium sized arteries and arterioles in the leptomeningeal space and cortex, where progressive accumulation of amyloid protein takes place [14]. This pathology is present in Alzheimer’s disease [18], and in the general elderly population, with a frequency of near 50% in individuals in the 9th decade of life [19], and is associated with recurrent lobular haemorrhages of the brain, and with white matter lesions and microinfarcts [20].

**Embolism** refers to particles of debris originating elsewhere that impede arterial supply to a specific brain region [10]. Emboli may originate from the heart and the aortic arch (Table 1) or the large arteries (discussed above, in large vessel disease). Rare sources of embolism include air, fat and tumour tissue that may compromise cerebral blood flow. Cardiac emboli result from one of the following mechanisms: a. blood stasis and thrombus formation in an affected large chamber of the heart; b. release of potentially harmful material from an abnormal valvular surface; c. paradoxical passage of material from venous to arterial circulation [21]. Here, atrial fibrillation that is the most common cause of cardioembolic stroke, and aortic arch atheroma, which is poorly recognised and often overlooked, are briefly discussed.

Atrial fibrillation is the most common source of cardioembolism and also the most common arrhythmia in the elderly, with a prevalence that increases from 0.1% in individuals younger than 55 years to 9% in 80-year old adults [22]. Atrial fibrillation is more frequent in men than women but due to their longer life expectancy, women are overrepresented (60%) among people with atrial fibrillation after the age of 75 years [23]. Sustained atrial fibrillation is easily identified by electrocardiogram (ECG), however paroxysmal atrial fibrillation may become apparent after several days of observation in the stroke-unit. Continuous ECG monitoring during hospital-stay is more sensitive than 24-hour Holter ECG, as studies have shown that median time to paroxysmal atrial fibrillation detection is 36-43 hours [24, 25].

Aortic arch atheroma with presence of plaques ≥ 4 mm thick is an important risk factor for new [26] and recurrent ischaemic stroke [27]. It has also been shown that aortic arch atheroma is a result of a dynamic process that may advance and thereby contribute to further increase of the risk for ischaemic stroke recurrence, myocardial infarction and vascular death [28]. There are no randomized trials on the treatment of aortic arch atheroma in ischaemic stroke, however it has been suggested that
secondary prevention with antiplatelet and statin treatment is reasonable in patients with symptomatic plaques ≥ 4mm, and oral anticoagulants should be administered in those with mobile thrombi superimposed on the plaques [29].

**Systemic hypoperfusion** is a general circulatory problem that may affect the brain and perhaps also other organs. Cerebral perfusion may be compromised due to cardiac pump failure caused by cardiac arrest or arrhythmia, or due to reduced cardiac output secondary to myocardial infarction, pulmonary embolism, pericardial effusion, or bleeding. Hypoxaemia may further diminish the amount of oxygen delivered to end-organs including brain tissue. Symptoms are usually non-focal and signs of circulatory instability may co-exist [10].

**Coagulation disorders** (Table 1) account for 1-5% of all ischaemic strokes and this proportion is higher in patients younger than 45 years and in patients with cryptogenic stroke [30]. Specific coagulation tests should be performed in young adults with ischaemic stroke or TIA, and in patients with no cause identified in the routine work-up.

1.1.1.3  **Ischaemic stroke pathophysiology**

Cerebral blood flow in healthy individuals is maintained rather constant through endogenous mechanisms that involve vasoactive molecules responding in changes of perfusion pressure [31]. This phenomenon is known as cerebral autoregulation [32] and it can compensate arterial pressure changes between 60 and 150 mmHg [31]. In ischaemic stroke, autoregulation deteriorates as perfusion pressure drops, and flow rates lower than 15 mL/100 g per minute result in failure of membrane ion homeostasis in neurons and infarction development [33]. Electrical failure during brain ischaemia causes glutamate receptor activation and ion channel opening with subsequent sodium and calcium influx and potassium efflux in neurons [34]. Sodium influx results in oedema, and glutamate accumulation causes over-excitation of neurons and subsequent rise of calcium ions followed by activation of detrimental cellular signalling pathways [35]. Brain hypoxia stimulates also the nitric oxide synthase activity followed by production of large amounts of nitric oxide that are directly toxic to the brain tissue [36]. Accumulation of reactive oxygen species causes increased mitochondrial membrane permeability and metabolic failure, release of initiators of apoptosis, and DNA damage [37]. Subsequently, metabolic failure causes further depletion of ATP levels, thereby resulting in cell death by necrosis rather than ATP-requiring apoptosis [38]. Finally, byproducts of cellular death caused by necrosis activate inflammatory mechanisms that result in increased blood flow and leukocyte migration, with an ambiguous effect on the ischaemic brain tissue [39]. Tissue damage and neurovascular disruption, mediated by proteases including MMPs, are inevitable consequences of cerebral ischaemia, with further adverse effects as cerebral oedema and haemorrhagic transformation of the infarction due to BBB breakdown [40].
Table 1. Pathophysiologic classification of ischaemic stroke

**Large vessel disease**
- Atherosclerosis
- Dissection
- Giant cell arteritis
- Fibromuscular dysplasia
- Takayasu’s arteritis
- Moyamoya syndrome
- Vasoconstriction

**Small vessel disease**
- Arteriolosclerosis
- Sporadic and hereditary cerebral amyloid angiopathy
- Inherited small vessel diseases distinct from amyloid angiopathy (e.g., CADASIL, Fabry disease)
- Inflammatory and immunologically mediated small vessel diseases
- Venous collagenosis
- Other small vessel diseases (i.e. post-radiation angiopathy)

**Cardioaortic embolic disease**

**High risk sources of embolism**
- Atrial: atrial fibrillation, sustained atrial flutter, sick sinus syndrome, left atrial thrombus, left atrial appendage thrombus, left atrial myxoma
- Ventricular: left ventricular thrombus, left ventricular myxoma, acute myocardial infarction, dilated cardiomyopathy
- Valvular: mitral stenosis, prosthetic valve, infective endocarditis, non-infective endocarditis

**Low risk/uncertain sources of embolism**
- Atrial: patent foramen ovale, atrial septal aneurysm, atrial auto-contrast
- Ventricular: akinetic/dyskinetic ventricular wall segment, subaortic hypertrophic cardiomyopathy, congestive heart failure
- Valvular: mitral annular calcification, mitral valve prolapse, calcified aortic stenosis, fibroelastoma

**Ascending aortic atheromatous disease (>4 mm)**

**True unknown source of embolism**

**Systemic hypoperfusion**

**Coagulation disorders**
- Antiphospholipid syndrome, protein C and S deficiency, antithrombin III deficiency, polycythaemia vera, sickle cell anaemia, essential thrombocythaemia, hyperhomocysteinaemia.

1.1.2 Intracerebral haemorrhage

Spontaneous intracerebral haemorrhage usually occurs in the deep brain parenchyma due to small artery disease, associated with hypertension [41]. Underlying pathology of the small vessels is similar to that related with lacunar infarcts, and is characterised by lipohyalinosis, microaneurysms and fibrinoid necrosis [42]. Hypertensive cerebral
bleedings are typically localized in the basal ganglia, thalamus, cerebellum and pons [43]. Cerebral amyloid angiopathy is another cause of intracerebral haemorrhage in the elderly, among whom the risk for recurrence is three times higher in carriers of epsilon2 or epsilon4 alleles of apolipoprotein E gene [44]. Other causes of primary intracerebral haemorrhage include coagulopathies and illicit drug use (mainly sympathomimetics). The course of intracerebral haemorrhage is characterized by “acute neurologic deficit with rapid progression”, probably due to haematoma expansion [43], and it is often accompanied by headache secondary to intracranial pressure increase or likely to direct involvement of pain-sensitive structures [45].

1.1.3 Subarachnoid haemorrhage

This type of stroke occurs most commonly due to rupture of aneurysms at the bifurcations of large arteries at the inferior surface of the brain (75% of the cases), and less often due to trauma, arterio-venous malformations, dural sinus thrombosis, drugs and coagulopathies [46]. Typically, subarachnoid haemorrhage due to aneurysmal rupture results in a sudden, very severe headache that occurs during activity, and may be accompanied by transient loss of consciousness or vomiting [47]. Clinical examination usually reveals neck stiffness and other signs of meningismus, whereas motor deficits, speech disturbance, seizures and visual symptoms are less common [46]. Underlying pathology involves blood release directly into the CSF under arterial pressure, with subsequent quick spreading and rapid increase of the intracranial pressure, the extent of which reflects the severity of stroke [48]. Subarachnoid haemorrhage has been suggested to exhibit a temporal pattern with seasonal (winter and spring), diurnal (late morning) and daily (Sunday) peaks [49].

1.2 AETIOLOGICAL CLASSIFICATION OF ISCHAEMIC CEREBROVASCULAR DISEASE (ICVD)

Identification of the underlying cause of ischaemic events is an important element of daily clinical practice that guides treatment decisions and prognosis for individual patients.

1.2.1 TOAST classification

In 1993, the investigators of the Trial of Org 10172 in Acute Stroke Treatment (TOAST) suggested a classification system comprising five aetiological subtypes of ischaemic stroke (Table 2), based on clinical features and results from ancillary diagnostic studies [50]. Each subtype was further categorized as “probable” if other aetiologies had been excluded, or “possible” when clinical findings and results of performed tests were consistent with one subtype but other aetiologies had not been investigated.
Table 2. TOAST Classification of subtypes of acute ischaemic stroke

Large artery atherosclerosis
Cardioembolism
Small-vessel occlusion

Stroke of undetermined aetiology
   a. Two or more causes identified
   b. Negative evaluation
   c. Incomplete evaluation

Stroke of other determined aetiology


Since the original TOAST classification scheme was developed, advances in diagnostic imaging and clinical evaluation in stroke have resulted in increasing number of ischaemic events being classified as “undetermined”. Subsequently, an evidence-based modification of the TOAST criteria has been developed under the name SSS-TOAST [51]. That system divides each of the original TOAST subtypes into three subcategories – “evident”, “probable”, or “possible” – according to the level of diagnostic evidence, based on predefined clinical and imaging criteria (Table 3). A 3-step algorithm (Fig. 1) is applied to interpret evidence for the identification of the level of confidence in assigning a cause. Further improvement has been achieved by introducing a computerised version of the SSS-TOAST called the Causative Classification System (CCS), an automated algorithm consisting of a questionnaire-style classification scheme [52]. The CCS is available online at https://ccs.mgh.harvard.edu.

Figure 1. The decision algorithm to assign a mechanism.

<table>
<thead>
<tr>
<th>Stroke Mechanism</th>
<th>Level of Confidence</th>
<th>Criteria</th>
</tr>
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</table>
| Large artery atherosclerosis     | Evident             | 1. Either occlusive or stenotic (≥50% diameter reduction) vascular disease judged to be due to atherosclerosis in the clinically relevant extracranial or intracranial arteries, and  
2. The absence of acute infarction in vascular territories other than the stenotic or occluded artery |
|                                  | Probable            | 1. Prior history of one or more episodes of TMB, TIA, or stroke from the territory of index artery affected by atherosclerosis within the last month, or  
2. Evidence of near-occlusive stenosis or nonchronic complete occlusion judged to be due to atherosclerosis in the clinically relevant extracranial or intracranial arteries (except for the vertebral arteries), or  
3. The presence of ipsilateral and unilateral internal watershed infarctions or multiple, temporally separate, infarctions exclusively within the territory of the affected artery |
|                                  | Possible            | 1. The presence of an atherosclerotic plaque protruding into the lumen and causing mild stenosis (<50%) in a clinically relevant extracranial or intracranial artery and prior history of two or more TMBs, TIA, or strokes from the territory of index artery affected by atherosclerosis, at least one event within the last month, or  
2. Evidence for evident large artery atherosclerosis in the absence of complete diagnostic investigation for other mechanisms |
| Cardioaortic embolism            | Evident             | The presence of a high-risk cardiac source of cerebral embolism                                                                                                                                 |
|                                  | Probable            | 1. Evidence of systemic embolism, or  
2. Presence of multiple acute infarctions that have occurred closely related in time within both right and left anterior or both anterior and posterior circulations in the absence of occlusion or near-occlusive stenosis of all relevant vessels; other diseases that can cause multifocal ischaemic brain injury such as vasculitides, vasculopathies, and hemostatic or hemodynamic disturbances must not be present |
|                                  | Possible            | 1. The presence of a cardiac condition with low or uncertain primary risk of cerebral embolism, or  
2. Evidence for evident cardioaortic embolism in the absence of complete diagnostic investigation for other mechanisms |
| Small-artery occlusion           | Evident             | Imaging evidence of a single clinically relevant acute infarction less than 20 mm in greatest diameter within the territory of basal or brainstem penetrating arteries in the |
absence of any other pathology in the parent artery at the site of the origin of the penetrating artery.

**Probable**
The presence of stereotypic lacunar TIAs within the past week.

**Possible**
1. Presenting with a classical lacunar syndrome in the absence of imaging that is sensitive enough to detect small infarctions, or
2. Evidence for evident small artery occlusion in the absence of complete diagnostic investigation for other mechanisms.

<table>
<thead>
<tr>
<th>Other causes</th>
<th>Evident</th>
<th>Presence of a specific disease process that involves clinically appropriate brain arteries</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Probable</td>
<td>A specific disease process that has occurred in clear and close temporal relation to the onset of brain infarction such as arterial dissection, cardiac or arterial surgery, and cardiovascular interventions</td>
</tr>
<tr>
<td></td>
<td>Possible</td>
<td>Evidence for an evident other cause in the absence of complete diagnostic investigation for mechanisms listed above</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Undetermined causes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evident: Presence of a specific disease process that involves clinically appropriate brain arteries</td>
</tr>
<tr>
<td>Probable: A specific disease process that has occurred in clear and close temporal relation to the onset of brain infarction such as arterial dissection, cardiac or arterial surgery, and cardiovascular interventions</td>
</tr>
<tr>
<td>Possible: Evidence for an evident other cause in the absence of complete diagnostic investigation for mechanisms listed above</td>
</tr>
<tr>
<td>Unknown: Cryptogenic embolism:</td>
</tr>
<tr>
<td>1. Angiographic evidence of abrupt cutoff consistent with a blood clot within otherwise angiographically normal looking intracranial arteries, or</td>
</tr>
<tr>
<td>2. Imaging evidence of complete recanalization of previously occluded artery, or</td>
</tr>
<tr>
<td>3. Presence of multiple acute infarctions that have occurred closely related in time without detectable abnormality in the relevant vessels</td>
</tr>
<tr>
<td>Other cryptogenic: those not fulfilling the criteria for cryptogenic embolism</td>
</tr>
<tr>
<td>Incomplete evaluation: absence of diagnostic tests that, up to the examiner's judgment, would have been essential to uncover the underlying cause</td>
</tr>
<tr>
<td>The presence of more than one evident mechanism where there is either probable evidence for each or no probable evidence to be able to establish a single cause</td>
</tr>
</tbody>
</table>

TMB: Transient monocular blindness; TIA: Transient ischaemic attack
Table 4. Grades of pathology by ASCO

Grades for atherothrombosis (A)

1. Definitely a potential cause of the index stroke
   Atherothrombotic stroke defined as:
   (a) Patients with any atherosclerotic stenosis 70–99% in an intra-/or extracranial artery supplying the ischaemic field diagnosed by level A or B evidence; or,
   (b) Any atherosclerotic stenosis <70% in an intra-/or extracranial artery supplying the ischaemic field with attached luminal thrombus diagnosed by level A or B evidence; or
   (c) A mobile thrombus in the aortic arch; or
   (d) Occlusion with imaging evidence of atherosclerosis in an intra-/or extracranial artery supplying the ischaemic field.

2. Causality uncertain
   (a) Patients with any atherosclerotic stenosis 70–99% in an intra-/or extracranial artery supplying the ischaemic field diagnosed by level C evidence; or
   (b) Any atherosclerotic stenosis <70% in an intra-/or extracranial artery supplying the ischaemic field with attached luminal thrombus diagnosed by level C evidence; or
   (c) Aortic arch plaques >4 mm in thickness without a mobile component.

3. Unlikely a direct cause of index stroke (but disease is present)
   (a) Presence of carotid or vertebral artery plaque without stenosis; or
   (b) Aortic arch plaque <4 mm; or
   (c) Stenosis (any degree) in a brain artery, contralateral to the brain infarction or in the opposite circulation.
   (d) History of myocardial infarction or coronary revascularization or peripheral arterial disease.

Grades for small vessel disease (S)

1. Definitely a potential cause of the index stroke
   Association of:
   (a) Deep branch artery stroke: small, deep infarct with diameter <15 mm on MRI (or CT) in the territory corresponding to symptoms; and either
   (b) One or several old or silent lacunar infarcts in territories different from the index stroke; or
   (c) Leukoaraiosis on MRI (or CT), microbleeds on MRI (gradient echo imaging), dilatation of the perivascular spaces on MRI (or CT); or
   (d) Recent repeated similar TIAs – when they preceded the brain infarct by 1 month or less and attributable to the same territory as the subsequent brain infarction (which increase the prediction for lacunar stroke from 57 to 80%, and are therefore supportive).

2. Causality uncertain

3. Unlikely a direct cause of index stroke (but disease is present)
   (a) Single, deep branch artery stroke; or
   (b) Clinical syndrome suggestive of deep branch artery stroke with no MRI/CT evidence of stroke (clinical syndrome suggestive of a deep branch artery stroke – classic lacunar
syndromes: pure motor hemiparesis, pure sensory syndrome, ataxic hemiparesis, dysarthria clumsy-hand syndrome, and sensorimotor syndrome; or other “nonlacunar” clinical syndromes. e.g. hemichorea, hemiballism, isolated dysarthria, etc.).
Leukoaraiosis on MRI (or CT), and/or microbleeds on MRI (gradient echo imaging), and/or dilatation of perivascular spaces on MRI (or CT), and/or one or several lacunar infarcts (silent or old) in territories different from the index stroke.

<table>
<thead>
<tr>
<th>Grades for cardioembolism (C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. Definitely a potential cause of the index stroke</strong></td>
</tr>
<tr>
<td>(a) Mitral stenosis;</td>
</tr>
<tr>
<td>(b) Prosthetic heart valve;</td>
</tr>
<tr>
<td>(c) Myocardial infarction within the past 4 weeks;</td>
</tr>
<tr>
<td>(d) Mural thrombus in left cavities;</td>
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<tr>
<td>(e) Left ventricular aneurysm;</td>
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<tr>
<td>(f) Any documented history of permanent or transient atrial fibrillation or flutter with or without spontaneous echo contrast or left atrial thrombus;</td>
</tr>
<tr>
<td>(g) Sick sinus syndrome</td>
</tr>
<tr>
<td>(h) Dilated cardiomyopathy;</td>
</tr>
<tr>
<td>(i) Ejection fraction &lt;35%;</td>
</tr>
<tr>
<td>(j) Endocarditis;</td>
</tr>
<tr>
<td>(k) Intracardiac mass;</td>
</tr>
<tr>
<td>(l) PFO plus in situ thrombosis;</td>
</tr>
<tr>
<td>(m) PFO plus concomitant PE or DVT preceding the brain infarction.</td>
</tr>
<tr>
<td><strong>2. Causality uncertain</strong></td>
</tr>
<tr>
<td>(b) PFO and concomitant DVT or PE (but not preceding the index stroke);</td>
</tr>
<tr>
<td>(c) Spontaneous echo contrast;</td>
</tr>
<tr>
<td>(d) Apical akinesia of the left ventricle and impaired ejection fraction (but &gt;35%);</td>
</tr>
<tr>
<td>(e) Only suggested by history of myocardial infarction or palpitation and multiple repeated brain infarcts on both sides or in both the anterior and posterior circulation;</td>
</tr>
<tr>
<td>(f) Only suggested by abdominal CT/MRI or autopsy demonstration of the presence of systemic infarction (e.g. kidney, splenic, mesenteric) or lower limb embolism (in addition to the index stroke).</td>
</tr>
<tr>
<td><strong>3. Unlikely a direct cause of index stroke</strong></td>
</tr>
</tbody>
</table>
Grades for other causes (O)

1. Definitely a potential cause of the index stroke (examples)
   (a) Arterial dissection by A or B evidence;
   (b) Dolichoectasia with complicated aneurysm;
   (c) Polycythemia vera, thrombocythemia >800,000/mm3;
   (d) Lupus erythematosus;
   (e) Disseminated intravascular coagulation;
   (f) Criteria for antiphospholipid antibody syndrome;
   (g) Fabry’s disease;
   (h) Concomitant meningitis;
   (i) Sickle cell disease;
   (j) Ruptured cerebral aneurysm with or without demonstration of spasm in the territory of the brain infarct;
   (k) Homozygosity for hyperhomocystinuria

2. Causality uncertain
   (a) Arterial dissection diagnosed by level C evidence (only suggestive history or clinical syndrome, e.g. isolated acute painful Horner’s syndrome, or only history of previous dissection);
   (b) Fibromuscular dysplasia.

3. Unlikely a direct cause of index stroke (but disease is present)
   (a) Kinking or dolichoectasia without complicated aneurysm or plication;
   (b) Arteriovenous malformation or saccular aneurysm;
   (c) Thrombocytosis >450,000 and <800,000/mm3;
   (d) Antiphospholipid antibodies <100 GPL units;
   (e) Mild hyperhomocysteinaemia heterozygosity

MRI: Magnetic resonance imaging; CT: Computed tomography; TIA: Transient ischaemic attack; PFO: Patent foramen ovale; DVT: Deep-vein thrombosis; PE: Pulmonary embolism; ASA: Atrial septal aneurysm


1.2.2 ASCO Classification System

In 2009, the ASCO classification system was introduced in an attempt to better define clinically and pathogenically meaningful groups, while losing as little information as possible [53]. The strength of this system is that it is factual rather than interpretative, and recognises that many patients belong to several aetiological categories, some of which may be causally related to the index ischaemic event, and others may simply co-exist. In order to estimate the level of diagnostic evidence of each category, this classification system evaluates the completeness, the quality and the timing of performed investigation.

Patients are evaluated for four phenotypes: atherosclerosis (A), small vessel disease (S), cardiac disease (C), and other causes (O), and the grade of likelihood (1-3) that each subtype has a causative relation to the index ischaemic event, is decided (Table 4). When no disease is present, patients are graded 0, and when the likelihood of causality cannot be determined due to failure to perform appropriate investigation,
patients are graded 9. The level of diagnostic evidence for the presence of each phenotype is A if it is directly demonstrated by gold-standard diagnostic tests or criteria; B if there is indirect evidence, or less sensitive or specific tests or criteria are applied; and C in cases of weak evidence in the absence of specific tests or criteria.

1.2.3 ASCO vs. TOAST - how do they differ?

The original purpose of the TOAST classification system was to better characterize a multicentre cohort of patients in order to investigate the efficacy of danaparoid (an anticoagulant with factor X inhibitory activity) in different stroke subtypes [50]. Since then, the TOAST classification system has been widely used in stroke studies with various purposes including genetic association studies, epidemiologic surveys and clinical studies on novel risk factors and biomarkers.

The presence of one “probable” or “possible” TOAST category is sufficient to allocate the patient to the corresponding TOAST group, even if the work-up on remaining categories has not been completed. This can potentially mask the cessation of clinical investigations, and also lead to erroneous allocations. More precisely, a small vessel stroke as defined by the clinical syndrome and the size of infarct, may overlap with the clinical picture of a distal M1 middle cerebral artery atherosclerotic occlusion. Also, the presence of medium risk sources of cardioembolism is sufficient to assess an ischaemic event as of cardiac origin, which may be a weak assumption in cases of incomplete work-up on remaining categories. Finally, the “undetermined aetiology” group is highly heterogeneous, comprising patients with several potential causes, and patients with no identified cause and either entirely normal, or mildly abnormal work-up [54].

The strengths of the TOAST classification system are its reliability, which has been further increased in the computerized version (CCS), and the improved SSS-TOAST algorithm, which resulted in lower number of “undetermined” cases. The latter fact remains, however, a matter of controversy, as it is a result of “forcing” patients into categories by accepting weak levels of evidence.

The application of the ASCO classification system [53] is consistent with everyday clinical practice, where physicians individually evaluate the presence of atherosclerosis, small vessel disease, cardiac sources of embolism and other causes of stroke, to decide which is the most likely cause of the index ischaemic event. Also, it recognizes the overlap between the four categories and retains all available information concerning these. That makes the system highly flexible for different research purposes, by facilitating the selection of appropriate phenotypes to study in each context. Finally, it provides data for the level of diagnostic evidence.

In our project, the TOAST classification system has been used for the description of patients in Studies I, III and IV, and the ASCO system was applied in Studies III and IV.
1.3 INFLAMMATION AND ICVD

Atherosclerosis is an inflammatory condition that has been associated with increased risk for ischaemic stroke of all aetiological subtypes [55].

1.3.1 Role of inflammation in Large Artery Stroke

Endothelial activation through lipid accumulation, followed by chemokine production and sub-intimal mononuclear cell infiltration, is the first sign of inflammation in the arterial wall, leading to the formation of atherosclerotic plaques [56]. Chemokines and their receptors play a crucial role in platelet activation and adhesion, and in mononuclear cell recruitment in the atherosclerosis-prone vessels [57]. Experimental studies on knockout mice have shown that CCR2 and CCR5 deficiency is associated with less atherosclerosis and more stable plaque phenotypes [58]. Homozygous delta32 deletion of CCR5 gene in humans was first reported to offer complete protection against HIV-1 infection [59], and was later associated with lower CRP levels, and decreased carotid intima-media thickness and cardiovascular disease risk, in a homogenous population in Italy [60]. Heat shock proteins (HSPs) are a group of evolutionary conserved proteins with chaperone activity and cell-protective function in response to stress stimuli [61], which have been suggested to act both as protectors [62] and activators [63] of inflammatory mechanisms underlying atherosclerosis. HSP70 may link macrophage lipid accumulation with initiation of inflammatory processes in the atherosclerotic plaques, but also drive anti-inflammatory response mediated by Th2 cell induction and IL-10 production [64]. Studies on patients with carotid atherosclerosis have shown lower plasma HSP70 levels and increased elastase levels and activity, indicating that HSP70 may have an atheroprotective role and its levels in peripheral blood may reflect the balance between its production from healthy arteries and degradation by atherosclerotic proteases [65].

1.3.2 Inflammation in Cardioembolic Stroke

Cardioembolic stroke is attributed to conditions that predispose to intracardiac thrombus formation, including ischaemic heart disease and atrial fibrillation. Subclinical atherosclerosis, inflammation and oxidative stress have been suggested to participate in pathophysiological mechanisms underlying atrial fibrillation [66]. Increased levels of CRP and IL-6 have been correlated with initiation and perpetuation of atrial fibrillation by inducing local inflammation and complement activation, which in turn leads to atrial remodelling [67]. HSPs have been reported to prevent atrial remodelling and progression from paroxysmal to chronic, persistent atrial fibrillation [68].

1.3.3 Inflammation and small vessel stroke

White matter lesions (leukoaraiosis) and silent brain infarcts have been attributed to small vessel disease, particularly arteriolosclerosis [69], and have been associated
with increased risk for cognitive deficits [70], future stroke [71] and motor function impairment [72]. A population-based study on elderly Europeans without dementia has previously indicated a strong association of increased CRP levels with progression of leukoaraiosis and lacunar infarcts, after adjustment for vascular risk factors, carotid plaques and intima-media thickness [73]. In another population-based, longitudinal cohort in the US, higher CRP and IL-6 levels were associated with white matter lesions and silent brain infarcts in both blacks and whites, and a common haplotype of IL-6 gene was associated with the presence of the disease only in whites [74].

1.4 EPIDEMIOLOGY OF ICVD

1.4.1 Risk factors

1.4.1.1 Non-modifiable risk factors

Age, gender, ethnicity and heredity serve as markers for stroke risk and contribute to the identification of high-risk populations that may require preventive treatment [75]. Stroke incidence increases with age, and stroke risk doubles for every decade above 55 years [76]. Men have 1.25 times greater stroke incidence rates compared to women, but more women than men die each year of stroke because they live longer [77]. Incidence and mortality rates of stroke vary among different ethnicities; in a large community-based cohort in Manhattan, blacks and Hispanics had a two-fold increased stroke incidence compared to whites [78]. Family history was associated with increased risk of stroke in an offspring analysis of the Framingham Study [79].

1.4.1.2 Potentially modifiable risk factors

Hypertension is the single most important modifiable risk factor for ischaemic stroke, with an estimated relative risk of stroke of 4 for systolic blood pressure over 160 mmHg and/or diastolic blood pressure over 95 mmHg [77].

Cardiac disease is associated with increased stroke risk, and atrial fibrillation is the most powerful predisposing heart condition [21]. Non-valvular atrial fibrillation is independently associated to a three to fivefold increased risk for stroke, according to the Framingham Study [80]. Valvular diseases, especially mitral stenosis, and left atrial enlargement, PFO and atrial septal aneurysm, coronary heart disease, left ventricular hypertrophy, interventional cardiology treatments and cardiac surgery are also associated to cardioembolic stroke.

The presence of diabetes increases the susceptibility to atherosclerosis and is associated with increased prevalence of other atherogenic factors, including hypertension, obesity and dyslipidaemia. Epidemiological studies report a twofold increase of ischaemic stroke risk in patients with diabetes compared to non-diabetics [77].
Dyslipidaemia, and specifically hypercholesterolaemia with increased total and LDL cholesterol levels, is associated to increased risk for extracranial carotid atherosclerosis [81], and its presence motivates the initiation of statin treatment in order to reduce atherosclerotic stroke risk [82, 83]. Treatment recommendations for triglycerides and HDL cholesterol are not equally well established. A recent study on a Korean population reports that triglyceride and HDL cholesterol levels are associated to atherosclerotic stroke only in patients with low LDL cholesterol levels [84]. Further studies are warranted to validate the effect of non-LDL cholesterol lowering on atherosclerotic stroke risk reduction.

Cigarette smoking is associated with a twofold increased risk for ischaemic stroke [85], and smoking cessation leads to risk reduction within two to four years, across whole age range and in both heavy and moderate smokers [86].

Lifestyle factors, including obesity, physical inactivity, dietary habits and emotional stress are associated with the presence of atherogenic factors and with increased stroke risk [87].

Homocysteine is a product of methionine metabolism, and its levels in peripheral circulation are influenced by genetic factors and dietary intake of vitamins B6 and B12 and folic acid. Elevated plasma levels of homocysteine are atherogenic and prothrombotic, and are associated to increased stroke risk in middle-aged men [88]. However, folic acid supplementation appears inefficient in reducing the cardiovascular disease risk or all-cause mortality in patients with pre-existing vascular disease [89].

Silent brain infarcts identified by neuroimaging, in the absence of associated symptoms of stroke or TIA in patient’s history, are common in elderly populations, and are associated with an increased risk for ischaemic stroke [90], and cognitive impairment [91].

TIAs are associated with increased stroke risk within 90 days from symptom onset, ranging from 4% to 15% in population-based studies [92, 93]. Recently, the ABCD2 scoring system, based on five factors (age, blood pressure, clinical features, duration of symptoms and diabetes), was suggested for the evaluation of short-term stroke risk after TIA [94]. Subsequent studies have validated the predictive value of ABCD2 score in identifying TIA patients at high risk for stroke [95], and have also confirmed an association between high ABCD2 score and increased long-term vascular morbidity and mortality [96].

Asymptomatic carotid stenosis has long been treated surgically with carotid endarterectomy, and more recently of interventional treatment with carotid artery stenting, based on evidence provided by large clinical trials [97-100]. However, increasing evidence shows that best medical treatment with beta-blockers, statins, antiplatelet drugs and good diabetes and blood pressure control, is effective against carotid plaque progression and is associated with reduced risk for cardiovascular
events [101]. Focus is now put on identification of high-risk patients who may benefit from endovascular treatment. Clinical features and imaging findings including mild cognitive impairment and rapid progressive dementia; presence of embolic signals on transcranial Doppler ultrasound, and thin fibrous cap, neovascularization and intraplaque haemorrhage, are high-risk factors for future vascular events [102].

**Systemic inflammation** is associated with increased stroke susceptibility. Atherosclerosis is considered a chronic, inflammatory disease of the vascular wall, where endothelial dysfunction and activation trigger the expression of adhesion molecules that promote the recruitment of monocyte-derived macrophages and T lymphocytes. Continuous turns of immune cell recruitment and activation contribute to the acceleration of atherosclerotic disease and in plaque instability [103]. Acute infections of the respiratory and urinary tract are suggested to be associated with a transient increase of the risk for myocardial infarction and stroke [104]. This may be due to a pro-atherogenic effect of acute infections that are suggested to accelerate the maturation of plaques and to promote plaque instability, or to a shift towards a pro-thrombotic state facilitated by impairment of coagulation and fibrinolytic mechanisms [105]. Chronic infectious pathogens, including *Chlamydia pneumoniae* [106], and gram-negative induced periodontitis [107], have been associated with chronic, low-grade inflammatory activity and increased cardiovascular risk. Systemic inflammatory markers, as CRP, IL-6 and soluble CD40 ligand have been studied in relation to stroke risk prediction [108], but whether they are causally involved and mediate a common underlying mechanism for infection-related vascular events, remains unclear.

1.4.2 Prognostic factors

1.4.2.1 Non-modifiable prognostic factors

**Age** is a strong risk factor of in-hospital mortality in ischaemic stroke, accounting for increased hazard ratio (HR) of 1.8 [95% confidence interval (CI) 1.3-2.4] in ages 65 to 74 years; HR 3.5 (95% CI 2.7-4.7) in patients 75-84 years old; and HR 5.7 (95% CI 4.3-7.7) in the group aged over 85 years, compared to patients younger than 65 years [109].

**Male gender** is associated with increased mortality rates, after adjustment for age, in large epidemiological studies [110].

**Genetic factors** are not extensively studied in correlation to stroke outcome, however apolipoprotein E epsilon3/epsilon4 genotype in men, and epsilon2/epsilon3 genotype in women have been associated with increased 30-day mortality after ischaemic stroke [111].

1.4.2.2 Clinical prognostic factors

Aetiological and clinical subtypes of stroke have various risk profiles; **cardioembolic** aetiology [112] and **total anterior circulation infarcts** [113] are associated with
highest mortality rates, compared to the remaining sub-groups of the TOAST and OCSP classification system, respectively.

**Stroke severity**, in terms of neurological deficits (paresis, aphasia, dysarthria, disturbed level of consciousness), is a strong predictor of outcome; for patients with two deficits, the HR for in-hospital death is 2.5 (95% CI 1.8-3.3); three deficits are associated with HR 6.7 (95% CI 4.9-9.3), and in patients with four deficits the HR is 11.1 (95% CI 7.6-16.2), compared to the reference group with zero or one deficit [109].

**Clinical score scales** are useful instruments for the evaluation of the presence and severity of symptoms after ischaemic stroke. The National Institutes of Health Stroke Scale (**NIHSS**) is a widely used score, with high reliability when used by neurologists, and also by non-neurologists and nurses, after appropriate, rapid training [114]. The NIHSS score measured within six hours from stroke onset, together with age, constitutes an accurate prognostic model for three-months functional outcome and mortality [115]. Glasgow Coma Scale (**GCS**) is a simple and widely appreciated score with good predictive value of short-term mortality after ischaemic stroke [116]. The **six simple variables model**, including age at stroke onset, living alone, independence in activities of daily living before stroke, verbal component of GCS, arm power and ability to walk, has also been validated and suggested as a valuable tool of long-term prognosis [117].

1.4.2.3 Laborotary tests as prognostic factors

**White blood cell count** and **CRP** correlate to stroke volume and severity of symptoms, and successful thrombolysis leads to reduction of their levels [118]. White blood cell count over 9.7x10³ cells/µl is associated with odds ratio (OR) for in-hospital death 8.3 (95% CI 3.4-17.3), compared with the remaining stroke patients [119]. High sensitivity CRP levels on admission predict mortality after thrombolysis treatment, even in patients in whom recanalization was successful [120]. However, in elderly people, CRP levels have been associated with mortality of both vascular and non-vascular aetiology, suggesting that it is a non-specific prognostic marker of fatal events [121]. As discussed above, temporary infections and markers of systemic inflammatory response are associated with increased risk for ischaemic stroke onset, which may confound the investigation of inflammatory markers in relation to stroke outcome. However, this risk has been considered and overcome by selecting patients without concurrent acute infections and inflammatory diseases, and by adjusting for other factors that may affect the outcome in the above-mentioned studies.

**IL-6** has also been studied in stroke patients and has been shown to correlate with infarct volume, severity of symptoms and long-term functional outcome [122]. In a large, prospective cohort in Edinburgh, IL-6 was independently associated with poor outcome and death at six months, and its inclusion improved the predictive value of six simple variables model, albeit slightly [123].
TNF-alpha, a pro-inflammatory cytokine, has been studied in ischaemic stroke patients and has been suggested to correlate with cardioembolic aetiological subtype, with stroke severity and poor outcome [124].

Fibrinogen gamma is an isoform of fibrinogen with additional binding sites for thrombin, that is increased in the acute phase of ischaemic stroke and has been independently associated with poor modified Rankin Scale (mRS) score at hospital discharge [125].

Adiponectin is a protein derived from adipose tissue that contributes to glucose metabolism and has been suggested to reduce atherosclerosis in experimental models [126]. Low plasma levels in humans have been associated with increased five-year mortality rates within five years of ischaemic stroke onset [127], but also with lower all-cause mortality in patients with known carotid atherosclerosis, without stroke [128].

Hyperglycaemia is observed within eight hours from ischaemic stroke onset and a second peak takes place at 48-88 hours, in both diabetic and non-diabetic patients [129]. Admission hyperglycaemia in acutely ill patients has been attributed to a stress response mediated by a complex interaction between cytokines and catecholamines that leads to high hepatic glucose output and insulin resistance, and promotes a catabolic state [130]. Persistent post-stroke hyperglycaemia has been associated with increased infarct volume [131], and higher short-term (30-days) mortality and worse functional outcome [132]. Triggering of vasoconstriction and pro-inflammatory and pro-thrombotic mechanisms have been suggested to mediate the deleterious effect of hyperglycaemia in stroke patients [133]. Administration of thrombolysis as treatment in patients with admission hyperglycaemia has been associated with less effective recanalization [134] and increased risk for symptomatic intracranial haemorrhage [135].

Insulin-like growth factor 1 (IGF-1) is a protein secreted by the liver in response to growth hormone stimulation, and has a role in neurogenesis and neuroprotection [136]. Increased plasma levels of IGF-1 in ischaemic stroke patients are associated with increased three-month survival and improved functional outcome [137].

Glial fibrillary acidic protein, a brain specific molecule rapidly released out of injured brain, has been studied in peripheral blood of ischaemic stroke patients and has been suggested to correlate with infarct volume, severity of symptoms, recanalization after thrombolysis and three-month functional outcome [138].

Glutamate is an excitotoxic aminoacid that has a key role in ischaemic brain injury and neuronal cell death. High plasma levels of glutamate are independently and strongly associated with early neurological deterioration after ischaemic stroke [139].

Matrix metalloproteinase protein-9 is an enzyme with important role in BBB disruption that increases at stroke onset and correlates with infarct volume, stroke
severity and functional outcome; it has also been suggested as a potential predictor of haemorrhagic transformation after thrombolysis treatment [140].

**Brain natriuretic peptide** is produced by cardiac tissue in response to myocyte distension, and has been suggested as a surrogate marker of left atrial appendage dysfunction and cardioembolic stroke [141], and as a predictor of neurological worsening and in-hospital mortality after ischaemic and haemorrhagic stroke [142].

**Troponin** levels have been found elevated in every seventh patient of an acute ischaemic stroke cohort, and was associated with the presence of coronary artery disease, ischaemic damage of the insular cortex, renal insufficiency, absence of hypercholesterolaemia and stroke severity [143]. In the same study, troponin levels were independently associated with in-hospital mortality; however, in another study with a newly developed assay of highly sensitive troponin T, there was no predictive association with long-term mortality confirmed, although elevated troponin levels were more frequently detected in stroke patients whose previous levels, measured by less sensitive tests, were found to be normal [144]. Troponin I has been suggested to predict new onset atrial fibrillation in ischaemic stroke and TIA patients and indicate poorer three-month prognosis when found elevated [145].

**Cholesterol** levels have been studied in correlation with ischaemic stroke origin, severity and outcome. An inverse association, between low cholesterol and increased severity of symptoms at ischaemic stroke onset, has been reported by a few investigators [146, 147]. These studies showed that patients with atrial fibrillation and severe cardioembolic strokes had lower cholesterol levels compared to patients with less severe injury due to small vessel disease. Also, increased short- [148] and long-term [146] mortality rates have been reported in ischaemic stroke patients with low admission cholesterol levels, independent of pre-stroke statin use. A large, multicentre study on thrombolysis treated patients recruited in five European countries, has recently showed that lower HDL and triglyceride levels were associated with increased all-cause 3-month mortality [149].

Admission glucose and cholesterol levels were investigated as prognostic markers of long-term survival in Studies II and IV respectively. A panel of biochemical markers of inflammation, coagulation and metabolism were investigated in Study III for their association with stroke aetiology and long-term survival.

### 1.5 GENETICS OF STROKE

Several monogenic disorders, including non-atherosclerotic vasculopathies, and connective and metabolic diseases, are associated with increased risk for ischaemic stroke in young patients with positive family history and absence of conventional vascular risk factors [150]. Apart from these well-established causes of ischaemic stroke, there is growing evidence supporting genetic predisposition to ischaemic stroke risk and prognosis, based on family [151] and twin [152] research studies.
Genetic factors may differ among aetiological subtypes of stroke, and may also contribute to different pathological processes by predisposing to conventional risk factors, by modifying the effect of these risk factors on target organs or by a direct effect on stroke risk and extent of ischaemic brain injury [153].

1.5.1 Candidate gene association and genome-wide linkage approaches

Candidate gene association studies have been very popular in the investigation of disease risk, however the results of studies on ischaemic stroke have not been consistently replicated, possibly due to lack of power and methodological differences among studies [154]. Genome-wide linkage analysis for stroke susceptibility has been performed in the genetically homogeneous, isolated population of Iceland, and evidence for linkage with stroke was found in a region of human chromosome 5q12 that included the PDE4D gene [155]. Since then, several replication studies have been performed, with variable results. A subsequent meta-analysis showed that no single nucleotide polymorphism (SNP) examined in PDE4D had a robust association to ischaemic stroke, thus suggesting that possible existing associations may be weak and restricted to specific sub-groups [156]. The majority of subjects in the studies included in that meta-analysis were white. A multi-centre study involving over 2500 patients from the southern and western regions of Sweden did not confirm an association of PDE4D SNP 45 with stroke risk, and meta-analysis of 17 previously published reports showed no significant overall estimate, with significant heterogeneity for random effects [157]. However, a recent meta-analysis on known genetic polymorphisms associated with ischaemic stroke in South Asians, showed that PDE4D SNP 83 and 32 conveyed increased stroke risk [158]. Regulation of inflammatory processes underlying atherosclerosis has been suggested as a possible mechanism in which PDE4D influences susceptibility to ischaemic stroke [155].

Another study on the population of Iceland revealed a susceptibility locus predisposing to stroke and myocardial infarction in chromosome 13q12-13, and ALOX5AP gene in this region was proposed as stroke susceptibility locus [159]. The investigators hypothesized that ALOX5AP genetic variants contributed to the risk for ischaemic vascular events by increasing leukotriene production and inflammation in the arterial wall. Subsequent replication studies in Swedish [160] and Sardinian [161] populations provided no evidence of association of ALOX5AP gene variants with ischaemic stroke risk. In a meta-analysis of over 5000 stroke patients (including haemorrhages), where the majority were white individuals, no significant associations of HapA and HapB haplotypes, and SG polymorphisms of ALOX5AP with stroke risk were confirmed [162].

Expansion of molecular genetic research during the last decade, and increasing appreciation of the role of inflammatory genes in ICVD risk has stimulated scientific interest. Genetic variants of polymorphisms encoding prototypical mediators of inflammation, including IL-6, ICAM-1, MMP-3, MCP-1 and E-sel, have been reported to occur more often in individuals with history of ischaemic stroke compared to controls [163]. CC chemokines have been suggested to play crucial role
in pathogenesis of atherosclerosis and ischaemic brain injury [164], and CC chemokine receptor polymorphisms have been reported to confer increased risk for ischaemic stroke in hypertensive individuals [165].

In Study I we have investigated the role of a common mutation of CCR5 gene in ICVD.

1.5.2 Genome-wide association (GWA) approach

Genome-wide genotyping has enabled simultaneous investigation of thousands of genes and the identification of novel genetic loci associated with disease risk without requiring a pre-specified hypothesis. In a first phase GWA scan in ischaemic stroke patients, where over 400,000 SNPs were assayed, no single locus was associated with stroke risk [166]. Genotyping results were publicly released for 88% of patients who consented, offering a valuable resource for further investigation. Several GWA studies on stroke have been published since then, and a number of genetic loci have been suggested to contribute to stroke risk. However, effect sizes are very small and not all results have been consistently reproducible. Loci consistently replicated were shown to be associated with aetiological subtypes of ischaemic stroke; PITX2 [167] and ZFHX3 [168] were associated with atrial fibrillation and cardioembolic stroke, and genetic variants in 7p21 [169], 9p21 [170] and 6p21.1 [171] loci were shown to confer risk for large vessel stroke. The protein encoded by PITX2 is important in cardiac development by directing the asymmetric morphogenesis of the heart [172]. ZFHX3 encodes for a transcription factor, and is associated with muscle-cell and neuronal growth-regulation and differentiation [173]. HDAC9 on chromosome 7p21, is associated to myogenesis and heart development [174].

The METASTROKE collaboration was established in order to combine all previous GWA datasets on ischaemic stroke, and to validate previously suggested associations as well as to identify novel genetic loci through meta-analysis of the available data. In their first publication, METASTROKE investigators confirmed previous associations for cardioembolic and large-vessel stroke in PITX2 and ZFHX3, and at the 9p21 locus, respectively [175]. As a result, the investigators suggested that thorough aetiological classification is necessary for successful genetic studies in ischaemic stroke, and that different genetic pathophysiological mechanisms may mediate the different aetiological subtypes.
2 AIMS OF THE THESIS

The overall purpose of this thesis was to investigate the role of genetic and biochemical markers in ICVD, and explore possible associations with underlying aetiological factors, and with clinical outcome. The aims of our studies were the following:

• Investigate the role of inflammatory genes in different aetiological subtypes of ICVD, and further describe the genetic profile of each aetiological mechanism.

• Describe the temporal profile of biochemical markers of inflammation, haemostasis and metabolism in peripheral blood of ICVD patients.

• Measure the levels of biochemical markers in peripheral blood during the acute phase of ischaemic events, and illustrate possible correlations with long-term clinical outcome, in terms of all-cause mortality.

• Distinguish the biochemical profile of different aetiological subtypes of ICVD.
3 PATIENTS AND METHODS

3.1 STUDY DESIGN AND INDIVIDUAL POPULATIONS

Patients with ischaemic stroke or TIA diagnosis, admitted to the stroke-unit of the Department of Neurology of Karolinska University Hospital, Huddinge, at different time periods between 1997 and 2010, comprised the populations of our studies. No pre-selection was performed since all patients who had experienced a stroke in the catchment area of the hospital were admitted to our department regardless of age, severity of stroke and comorbidities. Diagnoses were coded according to ICD-10 and were verified by stroke neurologists in the stroke-unit. Ethnically matched, healthy blood-donors recruited from the same geographic area, without previous cerebrovascular diseases comprised the control population enrolled in the first study.

3.1.1 Case-control study design

In the first, observational, case-control study (Study I), ischaemic stroke (n=562) and TIA (n=97) patients admitted from January 1998 to December 1999 and from January 2003 to December 2006, and 803 healthy controls were genotyped. Medical records were reviewed retrospectively for data extraction.

3.1.2 Retrospective cohort studies

In Study II, ischaemic stroke (n=395) and TIA (n=114) patients admitted from January 1997 to December 2002 were enrolled. Glycaemic status (hyperglycaemia vs. normoglycaemia), was the exposure factor, and all-cause death was the outcome variable, examined with survival analysis.

In Study IV, 190 ischaemic stroke patients admitted in 2006 and 2010 were included in the analysis. Cholesterol status (high vs. low) was the exposure factor, and all-cause death was investigated with survival analysis.

3.1.3 Acute Inflammation in Stroke Study (AISS), a prospective cohort study

The AISS was designed as a prospective, hospital-based, follow-up cohort study aiming to investigate the impact of inflammation on ICVD aetiology and clinical outcome. For that purpose, consecutive patients over 18 years of age, evaluated within 48 hours from sudden onset of hemisymptoms, and without acute infections or chronic inflammatory and/or malignant diseases, were included in the study. Clinical examination, including measurement of body temperature, blood pressure, and NIHSS, was performed upon inclusion, at day 2 and day 7 or upon discharge if earlier, and at 1, 3 and 12 months. Modified Rankin Scale before stroke was registered upon inclusion and re-assessed at each time-point of the follow-up period. Barthel Index was measured at 1, 3 and 12 months. At all time-points, blood samples were drawn and sent for direct analyses of a number of biochemical markers. Also, plasma and serum were separated from whole blood after centrifugation of EDTA
and serum tubes in 3000 rpm for 15 minutes, and additional samples of whole blood drawn in specially designed tubes for RNA stabilisation, were stored first in −20°C and later in −70°C. Comorbidities at baseline and investigation test results during the follow-up period were registered for all patients.

In Study III, ischaemic stroke (n=84) and TIA (n=17) participants of AISS, admitted from May 2006 to May 2010 were enrolled. Aetiological subtypes of ICVD defined the levels of exposure, and all-cause death was the outcome variable, examined with survival analysis. All patients included in this study were evaluated by the study investigators, and clinical examination and biochemical analysis of blood samples were performed at admission and at follow-up time-points. However, in this first work based on AISS dataset, only admission data were presented, as the purpose was to introduce the cohort and describe patients’ profile at symptom onset.

The studies were approved by the Local Ethics Committee.

3.2 AETIOLOGICAL CLASSIFICATION

Patients included in Studies I and III were classified according to the TOAST classification system, after data extraction based on a standard form suggested by Goldstein et al. in 2001 [176]. In Study IV, the computerized algorithm (CCS) of SSS-TOAST was applied for aetiological classification of patients [52]. Also, the ASCO classification system was applied in Studies III and IV. One investigator performed all assignments. Frequencies of aetiological subgroups by TOAST and ASCO are presented in Table 5.

The proportion of Large Artery Atherosclerosis, Cardioembolism and Small Artery Occlusion in ischaemic stroke is similar to previously reported numbers of population-based studies in Europe and in the US [177]. Yet, frequency of cardioembolism in study III was somewhat higher and small artery occlusion was slightly lower than expected. Patients with unknown aetiology due to incomplete work-up were rather few in Study III, perhaps owing to the study-design and purpose, which entailed more intensive investigation. Aetiological subtypes by ASCO showed similar proportions as previously stated in a population-based study in Dublin [178].

In Study I delta32 allele frequencies were compared among four aetiological subgroups: large artery atherosclerosis, cardioembolism, small artery occlusion and cryptogenic (patients with unknown aetiology despite complete work-up). Cryptogenic group was thereby a subgroup of the originally described “Group of undetermined aetiology”, which comprises undetermined aetiology with and without complete work-up, and multiple aetiologies.
### Table 5. Frequency of TOAST and ASCO subtypes in patients of Studies I, III, IV

<table>
<thead>
<tr>
<th></th>
<th>Study I (IS=562)</th>
<th>Study III (IS=84)</th>
<th>Study IV (IS=190)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>by TOAST</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAA</td>
<td>9.4 (53)</td>
<td>15.4 (15)</td>
<td>58.8 (10)</td>
</tr>
<tr>
<td>CE</td>
<td>29 (163)</td>
<td>23.7 (23)</td>
<td>58.8 (10)</td>
</tr>
<tr>
<td>SAO</td>
<td>19.8 (111)</td>
<td>6.2 (6)</td>
<td>10.7 (9)</td>
</tr>
<tr>
<td>CRYPT</td>
<td>13.2 (74)</td>
<td>34 (33)</td>
<td>19 (16)</td>
</tr>
<tr>
<td>Unknown</td>
<td>23.1 (130)</td>
<td>19.6 (19)</td>
<td>9.5 (8)</td>
</tr>
<tr>
<td>Other</td>
<td>5.5 (31)</td>
<td>1.1 (1)</td>
<td>-</td>
</tr>
<tr>
<td><strong>by ASCO</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAA pure</td>
<td>-</td>
<td>13.1 (11)</td>
<td>11.6 (22)</td>
</tr>
<tr>
<td>CE pure</td>
<td>-</td>
<td>36.9 (31)</td>
<td>34.7 (66)</td>
</tr>
<tr>
<td>SAO pure</td>
<td>-</td>
<td>9.5 (8)</td>
<td>22.1 (42)</td>
</tr>
<tr>
<td>Mixed</td>
<td>-</td>
<td>15.5 (13)</td>
<td>8.4 (16)</td>
</tr>
<tr>
<td>CRYPT</td>
<td>-</td>
<td>14.3 (12)</td>
<td>4.7 (9)</td>
</tr>
<tr>
<td>Unknown</td>
<td>-</td>
<td>10.7 (9)</td>
<td>18.4 (35)</td>
</tr>
</tbody>
</table>

Numbers are percentages with absolute numbers in parentheses. IS: Ischaemic Stroke; TIA: Transient Ischaemic Attack; LAA: Large Artery Atherosclerosis; CE: Cardioembolism; SAO: Small Artery Occlusion; CRYPT: Cryptogenic.

Similar approach was applied in Study III, but one patient with multiple aetiologies was integrated in the CRYPT group according to TOAST, and Unknown due to incomplete work-up consisted a separate group. Patients were also classified by ASCO, and seven groups were produced: pure large artery atherosclerosis, pure cardioembolism, pure small artery occlusion, large artery atherosclerosis plus cardioembolism, small artery occlusion plus cardioembolism, unknown despite complete work-up (Neg Wup), and Unknown (due to incomplete work-up). There were no patients with all the three pathologies present, and with the combination large artery atherosclerosis plus small artery occlusion. Pure phenotypes included patients with grade 1 or 2 of one pathology and grade 0, 3 or 9 for the remaining ones. Mixed phenotypes included patients with grade 1 or 2 of two pathologies. Neg Wup by ASCO corresponded to cryptogenic by TOAST.

In Study IV, the three main phenotypes by TOAST and ASCO (large artery atherosclerosis, cardioembolism and small artery occlusion) are presented in the manuscript. Pure phenotypes by ASCO were determined as described above for Study III.

#### 3.3 Risk Factors

Frequencies of conventional vascular risk factors of patients are presented in all studies. Data on vascular comorbidities were not available for the control population of Study I.
• **Hypertension** was considered present when the patient was on antihypertensive treatment on admission, or when diagnosed during the hospital-stay by repeated detection of blood pressure measurements greater than 140/90mmHg.

• **Diabetes Mellitus** was considered present in patients with known diagnosis on admission in all studies, and also in patients with admission HbA1c ≥ 6.5% in Studies III and IV.

• **Hyperlipidaemia** was considered present in patients that were on statin-treatment or had increased fasting cholesterol levels during hospital-stay. Different cut-off values were used in each study.

• **Atrial fibrillation** was considered present when mentioned in patients’ past medical history in all studies, and additionally when present on admission ECG or on any ECG during hospital-stay, in Studies II, III and IV.

• Presence of **Heart failure** was evaluated in Studies I and III, and **Angina** in Studies I, III and IV, and considered positive when mentioned in patients’ past medical history.

• **Smoking** was considered present in all patients smoking any kind of tobacco on daily basis, and former smokers who have abstained from smoking for at least three months were coded as non-smokers. Data on smoking habits were missing in 11% of all patients in Studies I and II (n=71 and n=55, respectively) and in 8% (n=15) in Study IV. In Study III, there were no missing data on smoking habits.

• **Admission Hyperglycaemia** was the exposure variable investigated in Study II, and was defined as glucose > 8 mmol/L within 3 days from ischaemic stroke or TIA onset.

• **Admission Hypercholesterolaemia** was the exposure variable investigated in Study IV, and was defined as fasting total cholesterol ≥ 4.6 mmol/L within three days from symptom onset.

### 3.4 CLINICAL INVESTIGATIONS AND BIOCHEMICAL STUDIES

All patients underwent relevant clinical investigations, including brain imaging with computed tomography (CT) or MRI, and ancillary diagnostic tests including duplex ultrasonography of extracranial carotid and vertebral arteries, transthoracic and/or transoesophageal echocardiography, CT- and/or MR-angiography and standardized blood tests. Investigation profiles for patient populations of Study III and IV are presented in Table 6.

Severity of stroke was measured with NIHSS in Studies III and IV, whereas in the remaining studies no data were available about the severity of symptoms at disease onset. Several biochemical markers were evaluated in Studies III and IV, as presented in the published papers. Data on antithrombotic and statin treatment on admission and at discharge were available for patients of Study IV.
Table 6. Investigations performed in patients of Studies III and IV

<table>
<thead>
<tr>
<th></th>
<th>Study III (n=101)</th>
<th>Study IV (n=190)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral Imaging (CT or MRI)</td>
<td>99 (100)</td>
<td>100 (190)</td>
</tr>
<tr>
<td>Extracranial vascular imaging</td>
<td>84 (85)</td>
<td>63 (119)</td>
</tr>
<tr>
<td>Echocardiography</td>
<td>67 (58/10)</td>
<td>53 (67/33)</td>
</tr>
<tr>
<td>ECG or Holter</td>
<td>99 (100)</td>
<td>99 (188)</td>
</tr>
<tr>
<td>CT or MR angiography</td>
<td>39 (33/6)</td>
<td>30 (51/15)</td>
</tr>
</tbody>
</table>

Numbers are percentages with absolute numbers in parentheses. Echocardiography is presented as number of TTE/TEE, and angiography as number of CT/MR. CT: computed tomography; MRI: magnetic resonance imaging

3.5 MOLECULAR GENETIC ANALYSIS

3.5.1 DNA extraction

In Study I DNA was isolated by salting out procedure applied on mononuclear cells from 10 ml EDTA anticoagulated venous blood of the patients recruited in 1998-99. A multitude of other DNA extraction procedures were used during the following years, and some of the blood samples of patients recruited in 2003-2006 were purified at KI Biobank Core Facility by Autopure LS Robot from Gentra Systems. DNA concentration was then measured by UV absorption at 260 nm with 260/280 ratio as quality control. DNA samples were subsequently diluted in even concentrations 20ug/ml and stored in 96-well blocks.

3.5.2 Genotyping

CCR5 delta32 genotypes were determined using the TaqMan discrimination assay. The wild type allele was coded as wt and the allele that carried the deletion was coded as Δ32. PCRs were carried out in a final volume of 25 µl, containing 40 ng genomic DNA, 22.5 pmol of each primer (forward 5’-GTCTTCAATTACACCTGCAGCTCTC-3’ and reverse 5’-GGACCGCCCCAAGATGAC-3’), 5 pmol of each probe (wt-allele probe 5’-(6-carboxyfluorescein, FAM)-TCTGGAAGAATTTC-3’ and Δ32-allele probe 5’-(VIC)-ATTITCCATACATTAAGATA-3’) and TaqMan Universal PCR Mastermix. The PCRs were performed with the following amplification protocol: denaturation at 92°C for 15 seconds and annealing and extension at 60°C for 1 minute for 40 cycles. Post-PCR, the genotype of each sample was automatically attributed by measuring the allele-specific fluorescence in the ABI Prism 7500 Sequence Detection System, using SDS 1.2.3 software for allele discrimination (Applied Biosystems, Stockholm, Sweden).
3.5.3 RNA extraction and cDNA synthesis

In the AISS study, whole blood (2.5 ml) of patients was stored in PAXgene tubes filled with a stabilizing reagent for immediate intracellular RNA-stabilisation and subsequent RNA purification. Samples were incubated at room temperature for at least 2 hours before being transferred to freezer (-20°C).

Purification was performed manually in 100 samples according to the manufacturer’s protocol. The procedure begins with a centrifugation step and the pellet is then washed and resuspended, and thereafter incubated in optimized buffers together with proteinase K for protein digestion. An additional centrifugation step is being performed to homogenize the cell lysate and to remove residual cell debris. Ethanol is added to adjust binding conditions, and the lysate is applied to a spin column. During a brief centrifugation, RNA is selectively bound to a silica membrane and several efficient washing-steps are performed prior to treatment with DNAsase I to remove trace amounts of bound DNA. Total RNA is finally eluted in elution buffer and heat-denatured. RNA quality was determined by calculating the A260/A280 ratio of absorbance and values over 2 were accepted as indicators of pure RNA. Only three samples had low ratio under 2. Subsequently, RNA was converted to cDNA in a separate reverse transcriptase PCR step, where 30 µl RNA were added to 70 µl reaction mixture containing reverse transcriptase, an optimized transcription buffer, dNTPs and random primers. Thermal cycling conditions were as following: 10 minutes in 25°C and 30 minutes in 37°C.

3.5.4 Gene expression analysis

Real-time, quantitative PCR was performed to investigate CCR5 gene expression, and the ∆∆CT method was applied for relative quantification. A pool of cDNA from five healthy controls was used as calibrator, and GAPDH was used as internal control (housekeeping gene). All samples were run in duplicates, and mean CT values were used for the calculation of ∆∆CT according to the formula:

\[
\Delta\Delta C_T = (C_{T,CCR5} - C_{TGAPDH})_{\text{patient}} - (C_{T,CCR5} - C_{TGAPDH})_{\text{controls}}
\]

Relative expression was then calculated as fold change, defined as \(2^{-\Delta\Delta C_T}\).

3.6 ELISA STUDIES

Ligands of CCR5 including CCL3, CCL4 and CCL5 were studied in plasma samples of ischaemic stroke patients with ELISA technique. The same method was used to investigate serum levels of HSP70 and anti-HSP70. Instant kits of human immunoassays were used for all studies. All samples were run in duplicates, and the mean absorbance was calculated. Duplicates that were within 20% of the mean were accepted. The limit of detection of CCL3, CCL4 and CCL5 was < 6, 3 and 4 pg/ml respectively, and for HSP70 and anti-HSP70 detection, the sensitivity of the assays was 60 pg/ml and 7 ng/ml respectively.
3.7 STATISTICAL TESTS

Demographic and baseline characteristics of patients were presented as means +/- SD for continuous variables, and as absolute numbers and percentages for dichotomous, categorical variables. Non-parametric tests were used for comparisons of non-normally distributed, continuous variables; Wilcoxon-Mann-Whitney test was applied in two-group comparisons, and Kruskal-Wallis test was performed when several groups were examined. The student t-test was applied in two-group comparisons of normally distributed, continuous variables. Chi-square test was used to describe differences among categorical variables, and the Fisher’s exact test was applied in cases with low frequencies (≤5) in one more cells of the contingency table.

OR calculation was performed in Study I to measure the strength of association between the presence of delta32 allele (exposure) and ICVD (outcome).

Logistic regression analysis was applied in Study I to investigate the influence of delta32 polymorphism on cardioembolic vs. non-cardioembolic ICVD, and in Study IV to assess predictors of one-month mortality after ischaemic stroke.

Survival analysis was performed in Studies II, III and IV, and time to death was examined with Kaplan-Meier method. The log rank test was used to compare survival curves between two groups. Cox proportional hazards regression analysis was performed to calculate HRs with 95% CIs for the effect of several covariates on survival. The assumption of proportional hazards was tested for all variables. In Studies II and IV, one main factor of interest was examined for its association with survival (hyperglycaemia and hypercholesterolaemia, respectively), and other factors of known relevance were added in the model (i.e. age, gender and vascular risk factors). In study III, a collection of factors was under investigation, for their potential association with survival, without a pre-specified hypothesis. In all these three studies, univariate analysis was performed for all variables of interest, and variables with significant p-values were included in the multivariate model.
4 RESULTS

4.1 STUDIES ON INFLAMMATION

4.1.1 CCR5 delta32 polymorphism and aetiological subtypes of ICVD

A total of 1462 individuals (659 ICVD patients and 803 healthy controls) were genotyped for CCR5 delta32 polymorphism in Study 1. The genotypic frequency distributions did not deviate from Hardy-Weinberg equilibrium (chi square: 0.21 and 0.03 for patients and controls respectively). Genotype and allele frequencies, and ORs for comparisons between groups are presented in Table 7. Data on age at sampling and gender, but not on common risk factors for cerebrovascular diseases were available for healthy controls. Control individuals were younger than ICVD population (53±12 vs. 69±11, p < 0.0001 with t-test), and had higher proportion of women (44% vs. 36%, p=0.002 with the chi-square test). Delta32 genotype frequencies did not differ between patients and controls (13% vs. 12% respectively, p=0.2 with the chi-square test). Stratification for age and gender was performed and no differences in genotype frequencies were reported within strata.

Table 7. Genotype and allele frequencies of CCR5 delta32 polymorphisms in patients and controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>ICVD (n=659)</th>
<th>HC (n=803)</th>
<th>OR (95% CI)</th>
<th>p</th>
<th>CE (n=186)</th>
<th>non-CE (n=292)</th>
<th>OR (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt/wt</td>
<td>78 (511)</td>
<td>75 (601)</td>
<td>reference</td>
<td></td>
<td>85 (158)</td>
<td>71 (209)</td>
<td>reference</td>
<td></td>
</tr>
<tr>
<td>wt/delta32</td>
<td>21 (140)</td>
<td>23 (188)</td>
<td>1.14</td>
<td>0.3</td>
<td>14 (27)</td>
<td>27 (78)</td>
<td>2.2</td>
<td>0.001</td>
</tr>
<tr>
<td>delta32/delta32</td>
<td>1 (8)</td>
<td>2 (14)</td>
<td>1.49</td>
<td>0.4</td>
<td>1 (1)</td>
<td>2 (5)</td>
<td>3.78</td>
<td>0.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Allele frequency</th>
<th>ICVD (n=659)</th>
<th>HC (n=803)</th>
<th>OR (95% CI)</th>
<th>p</th>
<th>CE (n=186)</th>
<th>non-CE (n=292)</th>
<th>OR (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>88 (1162)</td>
<td>87 (1390)</td>
<td>reference</td>
<td></td>
<td>92 (343)</td>
<td>85 (496)</td>
<td>reference</td>
<td></td>
</tr>
<tr>
<td>delta32</td>
<td>12 (156)</td>
<td>13 (216)</td>
<td>1.16</td>
<td>0.2</td>
<td>8 (29)</td>
<td>15 (88)</td>
<td>2.1</td>
<td>0.0008</td>
</tr>
</tbody>
</table>

Numbers are percentages with absolute count in parentheses, and odds ratios (OR) with confidence intervals (CI) in parentheses. ICVD: Ischaemic cerebrovascular disease; HC: Healthy Controls; CE: Cardioembolic stroke; wt: wild type.

Of 659 ICVD patients included in the study, 73% (n=478) were assigned in one of four TOAST aetiological subtypes under investigation. Baseline characteristics are presented in Table 8; patients with cardioembolic stroke were older and had more often heart disease, but smoked less, compared to the remaining aetiological
subgroups. The proportion of men was higher in small artery occlusion and cryptogenic groups. Delta32 allele frequency was lower in cardioembolic group compared to other aetiological ICVD subtypes and to controls. Multivariate logistic regression analysis was performed and cardioembolic ICVD vs. healthy status was the dependent variable. After adjustment for age and gender, we found that presence of at least one delta32 allele was associated with decreased risk for cardioembolism (OR 0.41; 95% CI 0.18-0.92; p=0.03).

Logistic regression analysis was also performed with cardioembolic vs. non-cardioembolic ICVD set as outcome variable. After adjustment for age, gender, hypertension, diabetes, and hyperlipidaemia, presence of at least one delta32 allele in patients' genotype was associated with reduced probability for cardioembolism (OR 0.5; 95% CI 0.28-0.75; p=0.007). Exploration of interactions between gender and genotype showed that women with wild type genotype had increased risk for cardioembolism compared to men with at least one delta32 allele (OR 2.5; 95% CI 1.37-4.63; p=0.003).

**Table 8. Baseline characteristics of patients included in Study I, by TOAST subtype**

<table>
<thead>
<tr>
<th></th>
<th>LAA (n=68)</th>
<th>CE (n=186)</th>
<th>SAO (n=117)</th>
<th>CRYPT (n=107)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>68±9</td>
<td>71±10</td>
<td>68±10</td>
<td>65±10</td>
<td><strong>0.0001</strong></td>
</tr>
<tr>
<td><strong>Male gender</strong></td>
<td>69 (47)</td>
<td>63 (118)</td>
<td>72 (84)</td>
<td>79 (84)</td>
<td><strong>0.05</strong></td>
</tr>
<tr>
<td><strong>Hypertension</strong></td>
<td>57 (39)</td>
<td>56 (105)</td>
<td>68 (80)</td>
<td>48 (51)</td>
<td><strong>0.02</strong></td>
</tr>
<tr>
<td><strong>Diabetes Mellitus</strong></td>
<td>26 (18)</td>
<td>16 (29)</td>
<td>27 (31)</td>
<td>19 (20)</td>
<td>0.07</td>
</tr>
<tr>
<td><strong>Hyperlipidaemia</strong></td>
<td>66 (45)</td>
<td>59 (109)</td>
<td>74 (86)</td>
<td>68 (73)</td>
<td>0.06</td>
</tr>
<tr>
<td><strong>Heart Disease</strong></td>
<td>28 (19)</td>
<td>58 (107)</td>
<td>20 (23)</td>
<td>11 (12)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Current Smoking</strong></td>
<td>31 (21)</td>
<td>14 (26)</td>
<td>26 (30)</td>
<td>21 (23)</td>
<td><strong>0.01</strong></td>
</tr>
</tbody>
</table>

Age is presented in mean years ± standard deviation and risk factors in percentages with absolute numbers in parentheses. LAA: Large Artery Atherosclerosis; CE: Cardioembolism; SAO: Small Artery Occlusion; CRYPT: Cryptogenic

### 4.1.2 CCR5 mRNA expression

Gene expression of CCR5 was measured in peripheral blood of 16 patients on day 1, 2 and 30 from stroke onset. The mean fold change of CCR5 gene, normalized to GAPDH and relative to that of control pool, was approximately 0.85 at all time-points. There were no differences among TOAST subtypes, but the sample was very small for group-comparisons. An inverse correlation was observed between fold change of CCR5 gene expression and NIHSS on admission (rho = -0.46; p=0.1 with Spearman correlation). Also, CCR5 expression was lower at all time-points in patients with unfavourable outcome at 3 months (mRS > 1), and a more pronounced difference was observed on day 2 (Fig. 2; p=0.03 with Wilcoxon-Mann-Whitney test).
4.1.3 CCR5 ligands in AISS patients

The presence of a common mutation of CCR5 gene and the fold change of its expression levels in peripheral blood of ICVD patients have been reported above. One would expect that CCR5 gene expression would be higher in patients with CE, as the delta32 mutation that abolishes the receptor from cell surface, was less common in this group. We did not confirm this hypothesis, yet we decided to proceed by investigating the receptor’s ligands on protein level.

MIP-1-alpha (CCL3), MIP-1-beta (CCL4) and RANTES (CCL5) were defined on admission and at day-90. CCL3 plasma levels on day-1 were measurable in 51 of 68 tested patients, and on day-90 in 43 of 59 tested patients. A wide range of values was observed (median 16.5 pg/ml; range 2.7-1663 pg/ml on day 1), and logarithmic transformation was performed to enable statistical calculations. CCL3 levels on admission were slightly higher than three months later, and analysis by aetiological phenotypes did not show any significant differences among TOAST or ASCO groups.

CCL4 levels were measurable in 44 of 48 patients investigated on day-1 (median 13.5 pg/ml; range 4.8-406 pg/ml), and in 45 of 46 patients tested on day-90. Logarithmic values on admission did not differ from day-90, and investigation of the main aetiological phenotypes showed similar progress over time among groups.

CCL5 levels were measured in 22 patients on day-1 (median 8618 pg/ml; range 4636-11 515 pg/ml), and 21 patients on day-90 (median 8580 pg/ml; 3546-12 989 pg/ml). The distribution of values was normal and no logarithmic transformation was performed. CCL5 plasma levels were similar on admission compared to day-90, and also among TOAST and ASCO groups.

No association was observed between plasma levels of CCL3, CCL4 and CCL5, and day-90 disability (mRS score > 1).
4.1.4 Serum levels of HSP70 and anti-HSP70 in AISS patients

Serum levels of HSP70 were measured in 61 patients on day-1, and in 57 patients on day-90, but no significant difference was observed over time [median 1.9 ng/ml; interquartile range (IQR) 2.2 ng/ml vs. median 1.8 ng/ml; IQR 2.3 ng/ml respectively; p=0.7]. In ASCO subgroups, HSP70 on admission was highest in patients with small artery occlusion (median 2.8 ng/ml; IQR 2.6; n=6), compared to large artery atherosclerosis (median 2.1 ng/ml; IQR 2.7; n=11), cardioembolism (median 2.1 ng/ml; IQR 1.9; n=22) and cryptogenic disease that was lowest (median 0.8 ng/ml; IQR 0.7; n=10; p=0.006 with the Kruskal-Wallis test).

Anti-HSP70 serum levels were investigated in 47 patients on day-1 (median 86 ng/ml; IQR 63 ng/ml) and 42 patients on day-90 (median 86 ng/ml; IQR 61 ng/ml; p=0.6). No differences were observed among aetiological phenotypes. No significant associations were observed between serum levels of HSP70 and anti-HSP70 and 3-month functional outcome.

4.2 SURVIVAL STUDIES

4.2.1 Admission glucose and long-term survival after ischaemic stroke and TIA

In Study II we investigated plasma glucose levels within 72 hours from symptom onset in 395 ischaemic stroke and 114 TIA patients. Hyperglycaemia was defined as plasma glucose > 8 mmol/L, and was present in 28% of all ischaemic stroke patients (24% of non-diabetic and 42% of diabetic patients; p=0.002). Median observation time was 69 months for all patients; 67 and 76 months for ischaemic stroke and TIA respectively. Mean admission glucose levels were higher in patients with ischaemic stroke than in patients with TIA, (7.6±3.2 mmol/L vs. 6.7±2.3 mmol/L respectively; p=0.002), and groups were investigated separately. Baseline characteristics of ischaemic stroke patients by glycaemic status are presented in Table 9.

Kaplan-Meier survival curves of hyperglycaemic vs. normoglycaemic patients with ischaemic stroke are presented in Fig. 3. One-month mortality rates were 4% in non-hyperglycaemic and 6% in hyperglycaemic stroke patients. In non-hyperglycaemic patients 1-, 5- and 10-year mortality rates were 9%, 29% and 52% respectively, compared to 16%, 42% and 54% in hyperglycaemic stroke (p=0.04 with the log rank test).

Univariate Cox proportional hazards regression was performed and only age and glycaemic status were significant predictors of survival. In multivariate model, after adjustment for age and gender, hyperglycaemia on admission was associated with increased probability of death (HR 1.4; 95% CI 1.02-1.97). After further adjustment for hypertension, diabetes, heart disease, known hyperlipidemia and smoking, the effect of hyperglycaemia was still apparent, yet with marginal statistical significance (HR 1.4; 95% 0.99-1.92). The effect sizes are slightly different than those reported in the original paper, where age was managed as dichotomous variable.
Table 9. Baseline characteristics of ischaemic stroke patients included in Study II by glycaemic status

<table>
<thead>
<tr>
<th></th>
<th>Normoglycaemia (n=286)</th>
<th>Hyperglycaemia (n=109)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>70±11</td>
<td>70±12</td>
<td>0.8</td>
</tr>
<tr>
<td>Male gender</td>
<td>59 (170)</td>
<td>52 (57)</td>
<td>0.2</td>
</tr>
<tr>
<td>Deceased</td>
<td>39 (112)</td>
<td>49 (53)</td>
<td>0.09</td>
</tr>
<tr>
<td>Hypertension</td>
<td>50 (142)</td>
<td>59 (64)</td>
<td>0.1</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>15 (44)</td>
<td>29 (32)</td>
<td>0.002</td>
</tr>
<tr>
<td>Hyperlipidaemia</td>
<td>17 (50)</td>
<td>27 (29)</td>
<td>0.05</td>
</tr>
<tr>
<td>Heart Disease</td>
<td>42 (120)</td>
<td>40 (44)</td>
<td>0.8</td>
</tr>
<tr>
<td>Atrial Fibrillation</td>
<td>21 (61)</td>
<td>23 (25)</td>
<td>0.7</td>
</tr>
<tr>
<td>Current Smoking</td>
<td>26 (73)</td>
<td>14 (15)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Age is presented in mean years of age ± standard deviation and risk factors in percentages with absolute numbers in parentheses.

Figure 3. Kaplan-Meier curves illustrating mortality in ischaemic stroke by glycaemic status on admission. (HG: Hyperglycaemia)

4.2.2 Long-term survival in aetiological phenotypes of ICVD

In Study III, we analysed clinical and biochemical parameters of patients included in AISS, and their association to long-term survival. A total of 101 individuals were enrolled in the final analysis, and phenotypic classification was performed by TOAST and ASCO classification systems, as presented in the original paper. Kappa
statistic indicated very good agreement between the two systems for all subtypes except for large artery atherosclerosis were the matching was good.

Demographic characteristics, and risk-factor and biochemical profile of the main aetiological subtypes (Large artery atherosclerosis, Cardioembolism and Small artery occlusion by ASCO) are presented in Table 10. Patients with cardioembolism were older, and had higher fibrinogen levels and white blood cell count, and lower iron and cholesterol levels on admission. Creatinine and urea levels were lower in small artery occlusion.

Table 10. Risk factor and biochemical profile of patients by ASCO subtype

<table>
<thead>
<tr>
<th></th>
<th>LAA pure (n=18)</th>
<th>CE pure (n=36)</th>
<th>SAO pure (n=8)</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>68±9</td>
<td>77±10</td>
<td>63±9</td>
<td>0.0002</td>
</tr>
<tr>
<td>Male gender</td>
<td>78 (14)</td>
<td>58 (21)</td>
<td>63 (5)</td>
<td>0.4</td>
</tr>
<tr>
<td>Hypertension</td>
<td>50 (9)</td>
<td>53 (19)</td>
<td>25 (2)</td>
<td>0.4</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>17 (3)</td>
<td>17 (6)</td>
<td>25 (2)</td>
<td>0.8</td>
</tr>
<tr>
<td>Atrial fibrillation</td>
<td>6 (1)</td>
<td>53 (19)</td>
<td>0</td>
<td>(&lt;0.0001)</td>
</tr>
<tr>
<td>Heart failure</td>
<td>6 (1)</td>
<td>28 (10)</td>
<td>0</td>
<td>0.05</td>
</tr>
<tr>
<td>Angina</td>
<td>6 (1)</td>
<td>22 (8)</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>78 (14)</td>
<td>64 (23)</td>
<td>75 (6)</td>
<td>0.5</td>
</tr>
<tr>
<td>Current smoking</td>
<td>28 (5)</td>
<td>11 (4)</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>hs-CRP</td>
<td>2.5±2.1</td>
<td>2.7±2.3</td>
<td>2.5±2.3</td>
<td>0.9</td>
</tr>
<tr>
<td>White blood cell count</td>
<td>9.2±3.3</td>
<td>9.1±4.4</td>
<td>5.9±1.1</td>
<td>0.05</td>
</tr>
<tr>
<td>Platelets</td>
<td>234±45</td>
<td>245±72</td>
<td>288±115</td>
<td>0.5</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>3.7±0.6</td>
<td>4.1±1.4</td>
<td>2.9±0.5</td>
<td>0.04</td>
</tr>
<tr>
<td>Glucose</td>
<td>6.2±0.9</td>
<td>6.1±1.2</td>
<td>6.6±2</td>
<td>0.7</td>
</tr>
<tr>
<td>HbA1c</td>
<td>4.9±0.6</td>
<td>4.8±0.6</td>
<td>5.2±1.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Iron</td>
<td>16±6</td>
<td>13±6</td>
<td>22±5</td>
<td>( 0.006 )</td>
</tr>
<tr>
<td>Urea</td>
<td>6.6±3</td>
<td>7.5±3.1</td>
<td>4.6±1.3</td>
<td>0.01</td>
</tr>
<tr>
<td>Creatinin</td>
<td>85±25</td>
<td>89±23</td>
<td>72±16</td>
<td>0.1</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>15.9±6.4</td>
<td>15.4±5.2</td>
<td>15.6±8.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>5.5±1.4</td>
<td>4.5±1.1</td>
<td>5±0.7</td>
<td>( 0.06 )</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.2±0.5</td>
<td>1.3±1</td>
<td>1.2±0.7</td>
<td>0.9</td>
</tr>
<tr>
<td>LDL</td>
<td>3.6±1.4</td>
<td>2.5±0.9</td>
<td>2.9±0.7</td>
<td>( 0.03 )</td>
</tr>
<tr>
<td>HDL</td>
<td>1.4±0.4</td>
<td>1.4±0.5</td>
<td>1.6±0.5</td>
<td>0.6</td>
</tr>
<tr>
<td>Albumin</td>
<td>39±3</td>
<td>36±4</td>
<td>39±3</td>
<td>0.1</td>
</tr>
<tr>
<td>Kobalamine</td>
<td>34±121</td>
<td>393±207</td>
<td>476±230</td>
<td>0.5</td>
</tr>
<tr>
<td>Folate acid</td>
<td>15±3</td>
<td>20±10</td>
<td>20±9</td>
<td>0.1</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>15.2±6.8</td>
<td>15.2±5.6</td>
<td>10.8±4.3</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Age and biochemical parameters are presented in means ± standard deviations, and risk factors in percentages with absolute numbers in parentheses. LDL: low-density lipoprotein; HDL: high-density lipoprotein.
A total of 12 patients died within three months from symptom onset, and 1-, 2- and 4-year cumulative risk was 15%, 22% and 31% respectively. Kaplan-Meier survival curves of aetiological subtypes by ASCO are presented in Fig. 4 (p<0.0001).

Figure 4. Kaplan-Meier curves indicating mortality in ASCO subtypes.

Univariate Cox proportional hazards analysis showed that increased age, presence of pure cardioembolic subtype and angina, as well as elevated levels of white blood cells, fibrinogen, bilirubin, and urea were associated with increased probability of death. Also, lower total cholesterol, LDL cholesterol, iron and albumin were predictors of unfavourable outcome. After adjustment for age, and stroke severity, only white blood cell count (HR 1.2; 95% CI 1.1-1.4), fibrinogen (HR 2.4 95% CI 1.2-4.8), and bilirubin (HR 1.14; 95% CI 1-1.3 were independently associated with mortality.

4.2.3 Long-term survival in ischaemic stroke patients with increased cholesterol

In Study IV, we investigated the effect of admission cholesterol levels and statin treatment on long-term survival after ischaemic stroke. Of 190 patients included in the analysis, 21 (11%) died within one month from symptom onset and 61 (32%) patients died over a 7-year observation period. Baseline characteristics of patients by survival status are presented in Table 11. Cholesterol values were missing in 67% of patients that died early, and they were investigated separately as presented in the manuscript. Of 169 one-month survivors, cholesterol values were available in 94% (n=159; mean±SD 4.6±1.1 mmol/L).
During a median follow-up period of 52 months, 52 of 159 (33%) patients with available cholesterol levels at admission died. Patients with admission cholesterol ≥4.6 mmol/L had 1-year survival rate of 98% compared to 87% in patients with lower cholesterol levels (Fig. 5; p=0.0001 with the log rank test). The annual mortality rate in patients with higher cholesterol remained close to 3.5% for the following five years, and reached 30% at the end of follow-up period. In contrast, patients with lower cholesterol had an average annual mortality rate of 8.5%, approaching 60% by the end of observation period. Low admission cholesterol was also associated with older age, increased presence of angina and atrial fibrillation, lower admission blood pressure, and higher rate of antiplatelet and statin treatment on admission. Cox proportional hazards regression analysis was performed and admission cholesterol < 4.6 mmol/L was independently associated with mortality, after adjustment for age and admission NIHSS. After further adjustment for angina and blood pressure, the effect was still obvious, albeit no longer statistically significant (HR 1.87; 95% CI 0.94-3.32).

**Figure 5.** Kaplan-Meier survival curves by admission cholesterol levels of one-month survivors.
Table 11. Baseline characteristics of patients by survival status

<table>
<thead>
<tr>
<th></th>
<th>Alive (n=169)</th>
<th>Deceased (n=21)</th>
<th>p</th>
<th>Alive (n=108)</th>
<th>Deceased (n=61)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>72 ± 13</td>
<td>86 ± 8</td>
<td>&lt;0.0001</td>
<td>68 ± 12</td>
<td>80 ± 9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Male gender</td>
<td>59 (100)</td>
<td>19 (4)</td>
<td>0.005</td>
<td>63 (68)</td>
<td>52 (32)</td>
<td>0.2</td>
</tr>
<tr>
<td>Hypertension</td>
<td>62 (105)</td>
<td>67 (14)</td>
<td>0.7</td>
<td>64 (69)</td>
<td>59 (36)</td>
<td>0.5</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>24 (41)</td>
<td>19 (4)</td>
<td>0.8</td>
<td>23 (25)</td>
<td>26 (16)</td>
<td>0.7</td>
</tr>
<tr>
<td>Angina</td>
<td>22 (38)</td>
<td>14 (3)</td>
<td>0.6</td>
<td>15 (16)</td>
<td>36 (22)</td>
<td>0.001</td>
</tr>
<tr>
<td>Smoking</td>
<td>18 (29)</td>
<td>0</td>
<td>0.08</td>
<td>19 (20)</td>
<td>17 (9)</td>
<td>0.8</td>
</tr>
<tr>
<td>Atrial Fibrillation</td>
<td>27 (45)</td>
<td>48 (10)</td>
<td>0.05</td>
<td>20 (22)</td>
<td>38 (23)</td>
<td>0.01</td>
</tr>
<tr>
<td>NIHSS admission</td>
<td>3 (4)</td>
<td>19 (10)</td>
<td>&lt;0.0001</td>
<td>2 (3)</td>
<td>4 (7)</td>
<td>0.0001</td>
</tr>
<tr>
<td>SBP</td>
<td>164 ± 28</td>
<td>159 ± 27</td>
<td>0.4</td>
<td>166 ± 29</td>
<td>161 ± 27</td>
<td>0.3</td>
</tr>
<tr>
<td>DBP</td>
<td>89 ± 16</td>
<td>90 ± 17</td>
<td>0.8</td>
<td>92 ± 15</td>
<td>85 ± 15</td>
<td>0.003</td>
</tr>
<tr>
<td>BMI</td>
<td>26.3±4.7</td>
<td>24.6±4.5</td>
<td>0.2</td>
<td>26.7±4.5</td>
<td>25.7±5.1</td>
<td>0.2</td>
</tr>
<tr>
<td>LAA by TOAST</td>
<td>17 (28)</td>
<td>5 (1)</td>
<td>0.2</td>
<td>20 (22)</td>
<td>10 (6)</td>
<td>0.08</td>
</tr>
<tr>
<td>LAA pure by ASCO</td>
<td>12 (21)</td>
<td>5 (1)</td>
<td>0.5</td>
<td>17 (18)</td>
<td>5 (3)</td>
<td>0.03</td>
</tr>
<tr>
<td>CE by TOAST</td>
<td>30 (51)</td>
<td>57 (12)</td>
<td>0.01</td>
<td>27 (29)</td>
<td>36 (22)</td>
<td>0.2</td>
</tr>
<tr>
<td>CE pure by ASCO</td>
<td>30 (51)</td>
<td>71 (15)</td>
<td>&lt;0.0001</td>
<td>27 (29)</td>
<td>36 (22)</td>
<td>0.2</td>
</tr>
<tr>
<td>SAO by TOAST</td>
<td>22 (33)</td>
<td>0</td>
<td>0.03</td>
<td>20 (23)</td>
<td>16 (10)</td>
<td>0.4</td>
</tr>
<tr>
<td>SAO pure by ASCO</td>
<td>25 (42)</td>
<td>0</td>
<td>0.005</td>
<td>25 (27)</td>
<td>25 (15)</td>
<td>0.9</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>4.6 ± 1.1</td>
<td>4.4 ± 1.6</td>
<td>0.6</td>
<td>4.8 ± 1.1</td>
<td>4.2 ± 1.2</td>
<td>0.001</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>2.7 ± 0.9</td>
<td>2.4 ± 1.3</td>
<td>0.4</td>
<td>2.9 ± 0.9</td>
<td>2.4 ± 0.9</td>
<td>0.007</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>1.4 ± 0.7</td>
<td>1 ± 0.4</td>
<td>0.2</td>
<td>1.3 ± 0.4</td>
<td>1.4 ± 0.4</td>
<td>0.8</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.4 ± 0.4</td>
<td>1.6 ± 0.5</td>
<td>0.1</td>
<td>1.4 ± 0.7</td>
<td>1.4 ± 0.7</td>
<td>0.9</td>
</tr>
<tr>
<td>White blood cell count</td>
<td>8.2 ± 2.3</td>
<td>9.3 ± 2.7</td>
<td>0.06</td>
<td>7.9 ± 2.4</td>
<td>8.6 ± 2.1</td>
<td>0.07</td>
</tr>
<tr>
<td>Glucose</td>
<td>6.2 (1.9)</td>
<td>6.7 (2.3)</td>
<td>0.4</td>
<td>6 (1.8)</td>
<td>6.3 (2)</td>
<td>0.4</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>138 ± 14</td>
<td>132 ± 15</td>
<td>0.07</td>
<td>142 ± 13</td>
<td>132 ± 14</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>14 (6)</td>
<td>11 (12)</td>
<td>0.6</td>
<td>13 (6)</td>
<td>14 (5)</td>
<td>0.3</td>
</tr>
<tr>
<td>Antiplatelets on admission</td>
<td>47 (80)</td>
<td>48 (10)</td>
<td>0.9</td>
<td>35 (38)</td>
<td>69 (42)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Anticoagulants on admission</td>
<td>7 (12)</td>
<td>14 (3)</td>
<td>0.2</td>
<td>7 (8)</td>
<td>7 (4)</td>
<td>1</td>
</tr>
<tr>
<td>Statins on admission</td>
<td>28 (48)</td>
<td>10 (2)</td>
<td>0.1</td>
<td>27 (29)</td>
<td>30 (18)</td>
<td>0.7</td>
</tr>
<tr>
<td>Antiplatelets at discharge</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>74 (80)</td>
<td>75 (46)</td>
<td>0.8</td>
</tr>
<tr>
<td>Anticoagulants at discharge</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>32 (35)</td>
<td>30 (18)</td>
<td>0.7</td>
</tr>
<tr>
<td>Statins at discharge</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>69 (75)</td>
<td>49 (30)</td>
<td>0.009</td>
</tr>
</tbody>
</table>

Age and normally distributed biochemical parameters are presented in means ± standard deviation, and non normally distributed continuous variables are presented as medians with interquartile range in parenthesis. Categorical variables are presented in percentages with absolute numbers in parentheses. SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure; BMI: Body Mass Index; LAA: Large Artery Atherosclerosis; CE: Cardioembolism; SAO: Small Artery Occlusion; CRYPT: Cryptogenic; LDL: low-density lipoprotein; HDL: high-density lipoprotein.
5 DISCUSSION

In this thesis I have summarized the results of our studies on patients with ischaemic stroke and TIA, recruited over various periods at Karolinska University Hospital in Huddinge. In these studies we focused on the clinical and molecular profile, as well as long-term survival, of the most common aetiological phenotypes of ICVD. Here I will attempt to place our findings in a broader context and correlate them to interesting reports of related studies.

• ROLE OF CCR5 AND ITS LIGANDS IN ATHEROSCLEROTIC DISEASE

In a prospective, population-based survey on epidemiology and pathogenesis of atherosclerosis in Bruneck, Italy, presence of delta32 polymorphism was associated with low levels of CRP, fibrinogen and procalcitonin, decreased carotid intima-media thickness, reduced incidence of cerebrovascular disease and lower risk of death [60]. In HIV patients, atherosclerosis accelerates faster than in non-infected individuals [179], and CCR5 that serves as a co-receptor of the virus, has been suggested to influence the development of both diseases. CCR5 mRNA expression in peripheral leukocytes has been reported to be higher in HIV-infected patients vulnerable to atherosclerosis progression, as measured by the increase of carotid intima-media thickness within 2-years of follow-up, and also in patients with detectable viral load [180]. In a mouse model of experimental dyslipidaemia, the use of maraviroc, a CCR5 antagonist antiretroviral drug, was associated with reduced progression of atherosclerosis [181].

In our results, large artery atherosclerosis group comprised patients with established, symptomatic atherosclerotic disease, but no difference in delta32 allele frequency was observed compared to control population. However, possible associations may be obscured by inadequate age- and gender-matching between groups. Furthermore, there were no available data on carotid intima-media thickness of control patients, and underlying asymptomatic atherosclerosis could not be assessed. Studies on centenarians in Italy have suggested that pro-inflammatory alleles – in, among other genes, those encoding pyrin and CCR5 – are associated with increased cardiovascular disease risk and are detrimental to longevity [182, 183]. Another case-control study on a Cretan population with coronary heart disease did not confirm any association between delta32 allele and cardiovascular disease risk, however the control population comprised “individuals with no significant findings in coronary angiography (defined as less than 50% stenosis in all epicardial arteries)” [184], which does not preclude presence of coronary atherosclerosis in this group.

Gene expression signature studies have demonstrated differences in molecular profiles of large artery atherosclerosis and cardioembolic groups, and genes involved in CCR5 signalling in macrophages have been associated with large vessel stroke [185]. In our study CCR5 delta32 allele frequency was lower in cardioembolic
compared to non-cardioembolic patients and to healthy controls. There are no previous or subsequent studies on CCR5 and cardioembolic stroke, or atrial fibrillation. Yet, there is accumulating evidence that inflammation may play a role in the occurrence and perpetuation of atrial fibrillation, which accounts for the majority of cardioembolic events, and it may also contribute to prothrombotic state and increased thromboembolic risk [186].

CC chemokines have been suggested to contribute to pro-inflammatory response triggered by acute cerebral ischaemia and to regulate tissue repair [164]. CCR5 ligands have been studied in correlation to stroke severity and outcome in experimental models and also in humans. CCL3 (MIP-1-alpha) serum levels have been reported to be elevated in ischaemic stroke patients compared to healthy individuals, and to have an inverse correlation to Bartel Index score at one month after ischaemic stroke, thereby suggesting that CCL3 response may predispose to poor short-term outcome [187]. CCL4 (MIP-1-beta) has been associated with monocyte recruitment in atherosclerotic plaques, and with increased plaque vulnerability [188]. Also, in a cohort of hypertensive individuals, higher plasma levels of CCL4 were independently associated with occurrence of stroke and cardiovascular events within 3 years of follow-up [189]. CCL5 (RANTES) – mediated macrophage recruitment has been shown to contribute to progress of atherosclerosis [190], and also to play an important role in reperfusion injury of ischaemic brain tissue by increasing BBB permeability [191]. In our studies, we did not find any association between plasma chemokine levels and any of stroke aetiological subtypes, nor with functional outcome at three months after ischaemic stroke onset. However, biologically relevant associations may have been obscured by the limited sample size.

• **ON ADMISSION HYPERGLYCAEMIA AND LONG-TERM SURVIVAL AFTER ICVD**

Admission hyperglycaemia in acute ischaemic stroke has been intensively investigated as prognostic marker of functional outcome and mortality. Scientific interest has been focused on “stress hyperglycaemia”, a term that describes patients without previous or newly diagnosed diabetes, with elevated glucose levels during illness that resolve to normal after discharge [130]. Several studies have suggested that elevated glucose on ischaemic stroke onset reflects a stress response to severe disease [192, 193], whereas subsequent, prospective studies have provided evidence that elevated admission glucose is an independent determinant of infarct expansion and poor outcome [131, 194]. Further investigation of diabetic vs. non-diabetic patients has illustrated an increased relative risk of short-term mortality (in-hospital and 30-day) in hyperglycaemic, non-diabetic stroke patients, but not in diabetic individuals [195]. Another study on persistent hyperglycaemia has reported glucose levels > 8.6 mmol/L at any time during the first 48 hours from ischaemic stroke onset to be associated with poor 3-month functional outcome (mRS > 2), after adjustment for age, diabetes, admission blood glucose, infarct volume and stroke severity [196]. In our study, a cut-off value of 8 mmol/L was used to define hyperglycaemia, based
on a previous long-term follow-up study [194]. Also in a recent, prospective observational study of ischaemic stroke patients recruited within 12 hours from symptom onset, blood glucose levels over 8 mmol/L were associated with increased 72-hour mortality in non-diabetics [197]. In a retrospective, observational study of 1446 ischaemic stroke patients, a J-shaped curve was described, with values between 3.7 and 7.3 mmol/L associated with favourable clinical outcome (mRS ≤ 2) at one year [198]. In our study, only one patient had admission glucose < 3.7 mmol/L (2.9 mmol/L, time to death: 9 days from symptom onset). Despite the large number of publications on admission hyperglycaemia and stroke outcome, which I only briefly discussed above, there is no established cut-off glucose value that warrants initiation of treatment. Current guidelines of American Heart Association/American Stroke Association recommend active treatment of hyperglycaemia in the acute phase of stroke, in order to maintain blood glucose levels in a range of 7.8 to 10 mmol/L (level of evidence C), yet with close monitoring to prevent hypoglycaemia [199].

In our study, admission glucose levels > 8 mmol/L were associated with increased long-term mortality in diabetic and non-diabetic ischaemic stroke patients, during 10 years of observation, after adjustment for age and gender. Data on admission NIHSS or other measurement of stroke severity, and on HbA1c values were not available. Our results may reflect the effect of uncontrolled and/or undiagnosed diabetes on long-term overall survival. We reported relatively low 1- and 3-month mortality rates, which complicated closer investigation of short-term outcome. Several mechanisms have been proposed to explain the occurrence of hyperglycaemia on the acute phase of ischaemic stroke. Impaired glucose tolerance and newly diagnosed diabetes have been reported to be present in two thirds of ischaemic stroke patients with admission hyperglycaemia who were evaluated 3 months after discharge [200]. Also, stress reaction to severe illness activates the hypothalamic-pituitary-adrenal axis with subsequent increase of serum glucocorticoid levels, sympathetic autonomic activation and increased catecholamine levels, which induce glycogenolysis and gluconeogenesis, and also insulin resistance [201]. Further support to the stress response theory is provided by studies that show a correlation between stroke severity and levels of stress hormones and blood glucose [202]. Moreover, right-hemisphere infarcts of insular cortex have been associated with more intense sympathetic activity and increased catecholamine levels [203]. Studies on imaging data of ischaemic stroke patients and experimental studies on animal models have suggested that impaired recanalization, decreased reperfusion, increased reperfusion injury and intracellular acidosis are possible mechanisms underlying the deleterious effect of glucose on ischaemic brain tissue [201].

Despite existing evidence that hyperglycaemia is associated with worse clinical outcome and survival after ischaemic stroke, clinical trials completed to date have failed to provide support for the use of intensive insulin treatment during the acute phase post ischaemic stroke [204]. However, only one trial was designed to assess treatment effect on clinical outcome, and it was complicated by low number of patients who achieved good glycaemic control, short treatment time (median 14 hours) and heterogeneous group of patients that comprised both ischaemic and
haemorrhagic strokes [205]. Recently, a randomized, blinded, multicentre, phase III trial has been designed to assess the effect of continuous intravenous insulin vs. standard sliding scale subcutaneous insulin on clinical outcome measured by baseline-severity-adjusted 90-day mRS score [206].

- **BIOMARKERS OF AETIOLOGY AND LONG-TERM SURVIVAL OF ICVD**

Ischaemic stroke is a complex disease, comprising diverse pathophysiological and clinical phenotypes [207]. Aetiological classification of stroke is essential in clinical praxis to direct appropriate secondary prevention, and to assess prognosis and risk of recurrence and mortality. It has also become evident in stroke research that well-described, homogenous phenotypes of ischaemic stroke are necessary for accurate investigation of risk factors, markers of prognosis, and treatment strategies. During the last two decades, the TOAST aetiological classification system [50] has been widely used in clinical trials, genetic studies and epidemiologic reports. However, criticism has been expressed concerning whether TOAST system is equally appropriate for different study designs [54]. The ASCO system has recently been introduced as a new approach to stroke classification by providing tools for the description of all underlying pathologies that may mediate ischaemic stroke, and the probability of causal association with the index stroke [53]. In our study, we reported very good agreement between TOAST and ASCO in all subgroups except of large artery atherosclerosis. Another study on TIA patients has shown similar distribution of aetiological subtypes by TOAST, CCS and ASCO, yet a more evident association between determined aetiology and increased ischaemic stroke risk in ASCO subgroups [208]. A prospective study of 103 ischaemic stroke patients has reported good concordance between ASCO and TOAST systems, and high inter-rater agreement, and concluded that the additional information provided by ASCO may facilitate individual treatment adaptation [209].

In our study, patients with unknown aetiology due to incomplete work-up had higher mortality rates, followed by patients with cardioembolism, cryptogenic stroke and large artery atherosclerosis. In a large observational, hospital-based cohort of ischaemic stroke patients, cardioembolic subtype was associated with poor 1-year survival, followed by cryptogenic stroke and large artery atherosclerosis, and closer investigation of cryptogenic group illustrated, in accordance with our findings, very poor survival in patients with incomplete work-up [210]. In our results, as well as in the above-mentioned study, patients with incomplete work-up were older and had higher admission NIHSS, which may explain the poorer prognosis in that group. Also, frequency distribution of subtypes was similar in our results, with somewhat lower occurrence of small artery occlusion and higher cardioembolic stroke. In a well-described ischaemic stroke population in Finland, 3-month survivors with small artery occlusion had increased mortality rates and higher risk of cardiac death during 12 years of follow-up, compared to patients with stroke of other aetiologies [211]. The authors suggested that despite the benign short-term prognosis of patients with
small vessel stroke, long-term outcome is unfavourable and may reflect end-organ manifestations of small vessel disease affecting the heart and kidneys. Another study on patients < 70 years of age has recently shown increased risk of recurrent vascular events and increased 2-year mortality in large artery atherosclerosis, followed by cardioembolism, cryptogenic and small artery occlusion [212]. The outcome variable in this study comprised recurrent stroke or TIA and coronary events, which may explain the discrepancy compared with our findings. Patients with generalised atherosclerosis may be at increased risk for the composite outcome assessed in that study, whereas younger patients with cardioembolic stroke and no contraindications for anticoagulation treatment may be more adequately treated. In an earlier population-based study and meta-analysis, patients with large artery atherosclerosis had high early risk for stroke recurrence, compared to patients with small artery occlusion who had the lowest risk [177]. Data on recurrent vascular events were not available in our study.

Molecular markers of aetiological subtypes in ischaemic stroke are of interest regarding risk stratification, diagnostic refinement and the effort to better understand pathophysiological mechanisms that may be involved in various disease manifestations. In a prospective hospital-based cohort, brain natriuretic peptide and D-dimer were associated with cardioembolic stroke and when combined with clinical variables, they improved the accuracy of cardioembolism prediction [213]. Also in a recent study on ischaemic stroke patients investigated with transoesophageal echocardiography, plasma brain natriuretic peptide levels were markedly increased in cardioembolic stroke and were significantly correlated with left atrial appendage dysfunction [141]. In our study, we found higher levels of white blood cell count and fibrinogen, and lower iron and cholesterol in cardioembolic compared to other stroke subtypes. In an earlier prospective controlled study, fibrinogen levels were reported higher in ischaemic stroke patients compared to healthy individuals, and also in cardioembolic patients compared to other aetiological subgroups [214]. White blood cell count has been shown to be an independent predictor of cardioembolic and lacunar stroke, in a large, prospective, population-based study [215]. Total cholesterol has previously been shown to have a negative association with cardioembolic stroke [216]. Regarding blood markers and long-term survival after ischaemic stroke, higher fibrinogen levels have previously been associated with unfavorable 3-month functional outcome (alive with Bartel Index score 95-100), after adjustment for age and stroke severity [217]. In our findings, white blood cell count and fibrinogen were predictors of long-term, all-cause mortality after adjustment for age and admission NIHSS. High-sensitivity CRP, IL-6, fibrinogen and white blood cell count have previously been shown to be independently associated with long-term survival after ischaemic stroke, adjusted for age and previous disability, but not after further adjustment for other markers [218].
In study IV we reported a possible association between admission cholesterol levels <4.6 mmol/L and increased long-term mortality after ischaemic stroke. In another study, plasma cholesterol < 4 mmol/L was associated with increased mortality at discharge and three years after ischaemic stroke, and low cholesterol was independent predictor of mortality risk after adjustment for age and gender in patients without previous statin treatment [147]. In an earlier, hospital-based cohort of patients with acute ischaemic stroke in Denmark, low cholesterol was inversely and linearly correlated with stroke severity (measured by Scandinavian Stroke Scale), and survival analysis showed that increased cholesterol was associated with improved 10-year survival [146]. The authors suggested that “higher total serum cholesterol favours the development of minor stroke and is thereby associated with improved outcome”. This conclusion has been disputed, as it contradicts the well-established association between cholesterol and large vessel atherosclerosis, and it disregards potential disturbance of serum cholesterol levels during the acute phase of stroke [219]. Serum cholesterol has been shown to decrease significantly during the first week after ischaemic stroke, and a delayed increase has been observed during the following three months [220]. It has also been stated that lifetime exposure to high cholesterol is more accurate predictor of carotid stenosis in older age [221], as cholesterol tends to decrease in the elderly, and single measurements may result in misleading conclusions [222].

In a very large meta-analysis of studies on cholesterol and cardiovascular risk as a function of age, gender and blood pressure, the authors reported that ischaemic stroke mortality was only weakly associated with serum cholesterol and only in younger individuals (< 60 years of age) with low systolic blood pressure < 145 mm Hg [223]. Moreover, a negative association between cholesterol and stroke mortality was reported at older age and in individuals with higher blood pressure. In contrast, baseline total cholesterol had a positive association with mortality from ischaemic heart disease in all age groups, and at all blood pressure levels. In a comment on the study results, it has been argued that survival bias may account for these observations, since individuals with advanced atherosclerosis that die in younger age due to coronary heart disease do not contribute later in atherothrombotic stroke risk [224]. Hence, other diseases like atrial fibrillation and small vessel disease that increase with age and may have different association with cholesterol are overrepresented in older age groups, and the relative proportion of large vessel atherosclerosis decreases. Also, stroke patients who survive the first year after ischaemic stroke onset have increased risk for subsequent cardiovascular death, rather than fatal cerebrovascular events [225], which may also influence the results of the above-mentioned meta-analysis. In our results, we report a possible association between cholesterol levels < 4.6 mmol/L and increased all-cause mortality after ischaemic stroke, after adjustment for age and stroke severity. However, patients with low cholesterol had more often history of angina and lower blood pressure on admission, and further adjustment for those variables attenuated the statistical
association. Thus, our observations may be influenced by previous statin treatment due to known angina in these patients, and the statistical association may be attributed to the effect of established ischaemic heart disease on mortality.

Clinical trials have shown that statin treatment is associated with decreased ischaemic stroke risk that is proportional to the reduction of LDL-cholesterol, which suggests that the protective effect of statins is mediated by LDL-reduction [226]. Also, pre-stroke statin use has been associated with improved 90-day functional outcome and decreased 90-day and 1-year mortality after ischaemic stroke [227]. In our results, statin treatment was three times more common among 1-month survivors, but no significant association with short-term mortality was confirmed. In a population-based, prospective cohort in Ireland, acute post-stroke statin treatment (started within 72 hours from symptom onset), and previous statin treatment were independently associated with improved in-hospital, 90-days and 1-year mortality, compared to poor outcome in statin-untreated patients [228]. Yet, it has been argued that 90-days mortality as high as 32% in the statin-untreated group, compared to 7% and 13% in patients with acute-initiated and pre-stroke statin use respectively, may indicate treatment selection bias in a patient-group with inherently poor prognosis, in which treatment restrictions are applied [229].

• LIMITATIONS OF STUDIES

Our studies have methodological limitations that may undermine the scientific strength of their content, yet they may still be interesting to the dedicated reader who keeps in mind the disadvantages discussed below.

In study I, healthy controls were younger than patients, and included higher proportion of women. There were also no available data on vascular risk factors in controls, which may have further contributed to possible systemic bias. Thereby, our findings concerning the role of CCR5 in ICVD risk should be interpreted with caution. Increasing scientific interest in genetic association studies during the last decade has resulted in myriad reports of predisposing and protective associations, only a fraction of which can be replicated. For that reason, specific guidelines for adequate study design have previously been published, and selection of appropriate, well matched control populations has been stressed as the key factor to successful case-control studies of complex diseases [230].

Investigation of CCR5 gene expression in peripheral blood was performed, yet no differences were found among phenotypic subtypes. Application of flow cytometry for the detection of CCR5 on cell surface of peripheral blood mononuclear cells would have been of interest, in order to more closely relate the transcription level of CCR5 mRNA to expression of the functional form of the receptor. However, this method was not applicable to our samples. In an attempt to investigate the receptor at the protein level, we applied ELISA to detect soluble levels of CCR5 in serum of ischaemic stroke patients, however the experiment did not succeed. Our gene
expression and ELISA studies are flawed by the lack of controls and thereby difficult to interpret.

Study II was designed as a retrospective, observational cohort study and it was thereby vulnerable for confounding bias due to suboptimal nature of data collection, which was dependent on the availability and accuracy of medical records. Also, there was no available information on HbA1c values of patients, which may have resulted in underestimation of the proportion of diabetic individuals. Missing data on stroke aetiology and severity of symptoms on admission limited our adjustments for confounders in calculations of mortality risk. Our difficulties to obtain information on the causes of death preclude closer investigation of possible associations between hyperglycaemia and vascular mortality risk. Overall, it is difficult to interpret our results, which indicate a possible association between exposure (high glucose levels on ICVD onset) and long-term outcome (5-year mortality), since exposure was defined by a single measurement of a molecule that may vary significantly over time, and we have no repeated measurements during the long follow-up period.

In study III, the limited number of patients was the major consideration, in that it limited statistical analyses. Investigation of stroke and TIA patients together may have resulted in lower mortality rates, however the proportion of TIA was low (17%). During the follow-up period, 26 patients died, which should have limited our variables included in the Cox regression model to 2-3, according to the rule of 10 events per variable [231]. However, in a large simulation study it has been indicated that this rule can be relaxed, and 5-9 events per variable may be used in statistical calculations without compromising the validity of the model [232]. In the ASCO classification, we suggested the term “pure” phenotypes, for patients with evidence of grade 1 or 2 of one pathology, and 0, 3 or 9 in the remaining pathologies. However, grade 9 refers to incomplete investigation and grade 3 indicates presence of the disease, yet probably not causally associated with the index stroke, which makes the subgroup less “pure” than intended.

In study IV, the retrospective design accounts for possible confounding bias, yet in restricted extend compared to study II, owing to the introduction in recent years of computerized medical records, which provide easier access to patients’ medical histories and more complete data. Also, patient samples were limited in size complicating subgroup analysis and closer investigation of possible associations between cholesterol and specific stroke phenotypes. Finally, missing cholesterol values on the vast majority (67%) of patients who died within one month from stroke onset precluded any conclusions on the effect of cholesterol on short-term mortality.
6 FUTURE PERSPECTIVES

In this thesis I have tried to integrate the findings of our studies during the last 6 years, which have ranged from genetic predisposition to ICVD and aspects of inflammation in relation to ICVD risk and prognosis to algorithms for classification of patients in aetiological phenotypes. Each one of these topics covers a wide field of stroke research that has been only briefly touched here. Our initial intention to limit our focus to the role of inflammation on ICVD aetiology and prognosis did not work out as expected, and several adjustments of my research plan along the way have contributed to the present shape and content of this book.

The purpose of my studies was to shed light on mechanisms underlying stroke aetiology and pathology. Here, I will try to summarize reflections that have stemmed from this thesis concerning current and future research ideas that may further serve its purpose.

- STROKE GENETICS

Risk loci that have been strongly associated with ischaemic stroke and have been possible to replicate in subsequent studies, have so far been scarce and emerged in subgroups of patients that shared common aetiological phenotypes [175]. PITX2 [233] and ZFHX3 [168] have previously been associated with atrial fibrillation and cardioembolic stroke, while the 9p21 locus, which has been correlated to coronary artery disease and abdominal aortic aneurysms [234], was also found to contribute to the risk for large-vessel stroke [235]. These findings indicate that phenotypic heterogeneity of ischaemic stroke subtypes should be accounted for by meticulous classification of patients included in genetic studies in order to support reliable conclusions. Such an attempt would only be feasible through international collaboration of several genetic research groups, given the large sample sizes required for separate investigation of different stroke phenotypes. META(STROKE) initiative is a successful example of large-scale association studies, the largest so far in stroke genetics [175]. CADISP is another multinational network that aims to advance existing knowledge on genetic predisposition to cervical artery dissection [236].

Our group has since 2007 established collaboration with deCODE genetics and participated in the Icelandic association studies discussed above. Our contribution has however been limited to a small proportion of patients who are prospectively included in SSIS (South Stockholm Ischaemic Stroke Study), an ongoing genetic epidemiologic study [237]. In the near future, we aim to keep up with patient recruitment and focus on phenotypic classification, which will support further contribution to large-scale projects. Despite advances in investigation techniques and ancillary diagnostic tests, a large proportion of patients remain unclassified regarding the aetiology of stroke. The TOAST system, which has been widely used in clinical trials as well as genetic association studies, may prove inadequate due to its interpretative character, which allows integration of several pathologies in one
phenotype. It is far from uncommon for elderly individuals that comprise the vast majority of stroke patients to present with several comorbidities associated with ischaemic stroke, like certain degree of large vessel atherosclerosis and concurrent small vessel disease or atrial fibrillation. In these cases application of the TOAST system results in selection of the comorbidity of greatest severity, or, if there is none or several equally severe, in the label of “unknown”. Factual algorithms like ASCO that retain useful information about the presence and severity of common pathologies, would be of interest to apply on existing datasets to evaluate their impact on emerging associations.

Furthermore, recent findings on the genetic profile of ischaemic stroke phenotypes indicate that different pathophysiologic processes may mediate different pathologies associated with stroke. Deeper understanding of these underlying mechanisms is of great clinical significance as it contributes to unveiling disease pathways and thereby to the development of better treatment strategies intended to cure the pathology at its point of origin. PITX2 gene that was associated with atrial fibrillation, has been shown to regulate cardiac development [172], and SNPs near its locus have been found to modulate clinical response to antiarrhythmic drugs [238] and predict atrial fibrillation recurrence after cardioversion [239]. Hence, stratification of therapeutic approaches by genotypes was suggested as possible clinical implication of these findings. The HDAC9 gene, which was associated with large vessel atherosclerosis, is known to regulate chromatin structure and gene transcription [240], and increased mRNA expression has been reported in carotid, aortic and femoral atherosclerotic plaques [241]. These findings provide further support for the original hypothesis that HDAC9 may exert its effect on disease risk by promoting atherosclerosis.

While identification of risk genes provides valuable insights in inherent factors predisposing to disease, it is highly likely that stroke phenotypes emerge through interactions between environmental factors and different combinations of genetic variants. The interplay of genes with environment can be more vigorously depicted on mRNA level, as gene expression changes constantly in response to stimuli like injury and disease. Peripheral blood is the most easily accessible and most commonly used tissue sample for transcriptional studies. The rationale for the use of blood in gene expression profiling of stroke patients is based on the inflammatory response that commences at stroke onset and involves migration and infiltration of white blood cells into the ischaemic brain tissue [242]. Moore et al. were pioneers in stroke transcriptional research and illustrated distinct gene expression profiles in peripheral blood mononuclear cells of patients with ischaemic stroke compared to control individuals [243]. Subsequent studies on RNA extracted from whole blood confirmed a distinct translational profile in ischaemic stroke [244, 245], indicating activation of genes associated with white cell activation, hypoxic stress and vascular repair.

A similar approach was then used to investigate the possibility to differentiate among aetiological subtypes of ischaemic stroke. The expression profile in the blood of large vessel stroke patients differed from that of cardioembolic patients, and a 23-gene panel was reported to distinguish the two groups with 95% sensitivity and specificity.
These results were reproducible in subsequent investigation, and the gene profile was applied to patients with cryptogenic stroke, whereupon 41% were predicted to have suffered cardioembolic stroke and 17% large vessel stroke [185]. Genes regulated in large artery atherosclerosis were expressed in platelets and monocytes, and regulated haemostasis, and gene expression in cardioembolism was associated with neutrophil response to infectious stimuli. In patients with white matter hyperdensities in the brain, considered representatives of small vessel disease, RNA expression analysis showed a unique profile of genes involved in oxidative stress, inflammation and axon repair [247]. Genes regulated in these patients showed only slight overlap with the expression profiles identified in stroke patients, thereby indicating possible distinct mechanisms between small vessel disease and remaining stroke subtypes. However, the small number of patients represents a limitation in these studies and larger cohorts of patients are warranted. Also, the inclusion of control groups with atrial fibrillation and large vessel atherosclerosis and without previous/current stroke would further facilitate interpretations on whether the expression profiles reflect an effect of the acute ischaemic event itself or arise from the baseline disease differences. Future studies on gene expression profiles of pure stroke phenotypes may further contribute to conquering disease complexity and understanding disease mechanisms.

We need first to disassemble the puzzle into parts and study them in the form of as homogeneous groups as possible, in search of common underlying pathophysiologic pathways. This knowledge may then be easier to apply in real life, where each patient carries different combinations and grades of the different pathologies.

**PLASMA BIOMARKERS AND STROKE PROGNOSIS**

The term “biomarker” has been proposed to indicate “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” [248]. Clinical and imaging measurements, as well as levels of molecules found in body fluids, are commonly used as biomarkers in research. Blood biomarkers have attracted scientific interest as diagnostic tools, and predictors of outcome and treatment response, owing to the convenience of obtaining and interpreting them. In stroke research, investigation of single candidate markers, relevant to disease mechanisms, is a simple and easy approach, yet with doubtful reliability, given the difficulties of defining pathological processes that are unique to ischaemic stroke and absent in conditions mimicking it. Panel of markers representing several processes associated with ischaemic stroke (e.g. inflammation, thrombosis and neuronal damage) may be more accurate, but also more difficult to apply and validate in different populations. Profiling of the whole proteome is an attractive, yet expensive and technically demanding approach, which would further advance the potential for discovery of novel biomarkers. During the last decade, a large number of blood-borne molecules have been associated with poor functional outcome, recurrent vascular events and mortality. However, previously published studies are generally small and vulnerable to bias due to lack of power to adjust for other confounding parameters.
like age and stroke severity, to calculations of a threshold level instead of continuous tests, and to lack of validation in separate cohorts [249]. None of the biomarkers so far identified has been utilized in clinical practice, as they were not found to improve the predictive value of already validated clinical predictive models [123, 250]. In order to minimize bias and attain high-quality, reproducible studies, researchers have been urged to apply REMARK guidelines in their study design and implementation [251].

Despite the difficulties described above, high throughput genomic and proteomic methods boaster the hope for future advances. Moreover, as already mentioned in the previous section, heterogeneity of ischaemic stroke phenotypes is an important parameter that differentiates it from other diseases and should be taken into account in clinical studies; not least in the evaluation of prognostic biomarkers. Plasma molecules that predict functional outcome and survival may differ among aetiological subtypes with different underlying pathophysiologic mechanisms and a variety of associated comorbidities.

We may be approaching the day when the collective term “ischaemic stroke” will be replaced by several terms indicating disparate origins, each of which will be studied, diagnosed and treated as a distinct entity.
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