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ASPECTS OF MIGRAINE AND PATENT FORAMEN OVALE IN ISCHEMIC STROKE

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Aspects of migraine and patent foramen ovale in ischemic stroke
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ABSTRACT

Stroke is one of the main causes of death and disability worldwide, and only in Sweden approximately 25 000 individuals suffer from stroke each year. This thesis focuses on two common conditions; migraine and patent foramen ovale (PFO) and their role in stroke.

Migraine is a common primary headache disorder, affecting approximately 11-13 % of the population with a 3:1 female preponderance. One third of the patients have an initial aura of neurological symptoms before the headache, and clinically migraine can be divided into migraine with and without aura. The pathophysiological mechanisms are highly complex, and involve cortical spreading depression (CSD) as the substrate for migraine aura, and activation of the trigeminovascular system causing the headache.

PFO is an inborn anomaly, a remnant from the fetal circulation, that is prevalent in approximately 25 % of the population. PFO enables shunting of venous blood to the arterial circulation, bypassing the pulmonary system and enables paradoxial embolization. PFO is associated with ischemic stroke, as well as migraine with aura. The reason for the latter is unknown, but may relate to micro-embolisms through a PFO triggering migraine attacks.

For decades, migraine has been suggested as a risk factor for stroke and cardiovascular disease. The risk seems to be mostly related to migraine with aura, female gender and young age. The reasons for this are still unknown and probably multifactorial. Different theories involve increased prevalence of cardiovascular risk factors among migraineurs, co-existence of other co-morbid conditions increasing the risk for stroke (i.e. PFO and cervical artery dissection), and association to endothelial dysfunction with subsequent hypercoagulability and decreased vascular reactivity. Migraine with aura may also be associated with a phenotype, leading to an increased susceptibility for CSD, and an increased sensitivity to cerebral ischemia. This thesis involves four different projects concerning PFO and migraine in relation to stroke. The projects are performed in stroke populations (Study I-III) and in a population-based twin sample (Study IV).

In Study I, patients with ischemic stroke investigated with transesophageal echocardiography were included (N=117), and dichotomized depending on the co-existence of a PFO. The prevalence of PFO was 11.9 %. Patients were analyzed regarding cardiovascular risk factors and allele frequency of 100 different genetic markers, previously associated with cerebrovascular disease. Four genetic markers, located in the Prothrombin-, Selectin E- and Apolipoprotein C III- genes, were significantly associated with PFO. The strongest association was for Prothrombin 20210 G/A (p= 0.0049), which is a marker associated with increased risk of venous thromboembolism. There were no differences regarding risk factors in the two groups.

In Study II, patients with a diagnosis of TIA, ischemic or hemorrhagic stroke, admitted to the stroke ward during a six-month period, were included (N=175). Prevalence of migraine was investigated using a structured questionnaire, and patients were analyzed regarding cardiovascular risk factors and clinical characteristics of their stroke, depending on co-existing migraine or not. The prevalence of migraine was 20 %, which

is comparable to prevalence estimates in the general population. However, migraine with aura was more prevalent than expected (61 % of those with migraine). Migraine was associated with PFO. Most stroke patients had a favorable outcome after stroke and there was no difference in stroke severity depending on migraine status.

In Study III, patients with cryptogenic stroke and PFO, planned for closure of their PFO (N=20) or on medical treatment only (N=7), were included. Patients were followed prospectively from baseline to six month after closure. Fifty percent of the patients had co-existing migraine, whereof the majority had migraine with aura (84.6 %). Endothelial dysfunction was assessed at baseline and after one day, one month and six months. A majority of patients had an impaired endothelial function at baseline, but there was no change after PFO closure. In a few patients, migraine frequency was impacted after closure with a distinct increase in migraine attacks after the procedure. At a second, long-time follow-up, the majority of patients were improved regarding frequency of migraine attacks.

In Study IV, the risk for stroke related to migraine was investigated in a population from the Swedish Twin Registry (N=53 404). A diagnose of migraine with or without aura was identified through a symptom based algorithm. The twins were followed longitudinally for more than 10 years for the outcome of stroke, using data from national patient registries. There was no general increased risk for stroke associated with migraine, but twins with migraine with aura had a border-significant 27 % increased risk for stroke. Further analysis suggested that this could be impacted by familial factors. However, in comparison with previous studies, our results showed a considerably weaker association to stroke related to migraine.

LIST OF SCIENTIFIC PAPERS

I. Patent foramen ovale and ischemic stroke – risk factors and genetic profile. **Maria Lantz**, Christina Sjöstrand, Konstantinos Kostulas.
Published in J Stroke Cerebrovascular Diseases. 2013 Aug; 22(6):841-5

II. Prevalence of migraine in an inpatient stroke population. **Maria Lantz**, Elisabet Waldenlind, Christina Sjöstrand.
Published in Acta Neurologica Scandinavia. 2015 May;131(5):290-7

III. Impairment of endothelial function in patients with cryptogenic stroke and patent foramen ovale is not affected by closure. **Maria Lantz**, Konstantinos Kostulas, Magnus Settergren, Christina Sjöstrand.
Accepted for publication in J of Interventional Cardiology.

IV. Migraine and the risk of stroke – a national population-based twin study. **Maria Lantz**, Johanna Sieurin, Arvid Sjölander, Elisabet Waldenlind, Christina Sjöstrand, Karin Wirdefeldt.
Manuscript.

These articles will be referred to in the text by their roman numbers (I-IV).

CONTENTS

LIST OF ABBREVIATIONS

In alphabetical order;

ANOVA	Analysis of Variance
ASA	Atrial Septal Aneurysm
CAD	Cervical Artery Dissection
CADASIL	Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy
CDR	Cause of Death Registry
CE	Cardioembolism
CGRP	Calcitonin Gene Related Peptide
CI	Confidence Interval
CLOSURE I	Evaluation of the STAR Flex Septal Closure System in Patients With a Stroke and/or Transient Ischemic Attack Due to Presumed Paradoxical Embolism Through a Patent Foramen Ovale
CSD	Cortical Spreading Depression
CS	Cryptogenic stroke
ED	Endothelial Dysfunction
ELISA	Enzyme-Linked Immuno-Sorbent Assay
EPC	Endothelial Progenitor Cell
ESUS	Embolic Stroke of Unknown Source
ET-1	Endothelin-1
FHM	Familial Hemiplegic Migraine
FMD	Flow Mediated Dilatation
GWAS	Genome Wide Association Study
Hcy	Homocysteine
hs-CRP	High sensitive, C-Reactive Protein
ICD-10	International Classification of Disease, 10:th version
ICHD-III	International Classification of headache Disorders, 3:rd version
HIS	International Headache Society
IQR	Inter Quartile Range
LAA	Large Artery Atherosclerosis
MIST	Migraine Intervention With STAR Flex Technology trial
MRI	Magnetic Resonance Imaging
mRS	modified Rankin Scale
NIHSS	National Institute of Health Stroke Scale
NO	Nitric oxide
NOMAS	Northern Manhattan Study
OSAS	Obstructive Sleep Apnea Syndrome
PFO	Patent Foramen Ovale
PC-trial	Patent Foramen Ovale and Cryptogenic Embolism trial
PRIMA	The Percutaneous Closure of PFO in Migraine with Aura trial
RCT	Randomized Controlled Trial
RESPECT	Randomized Evaluation of Recurrent Stroke Comparing PFO Closure to Established Current Standard of Care Treatment
RHI	Reactive Hyperemia Index

RoPE	Risk of Paradoxial Embolism
SALT	Screening Across The Lifetime Study
SAO	Small Artery Occlusion
SD	Standard Deviation
SNP	Single Nucleotide Polymorphism
SPARC	Stroke Prevention: Assessment of Risk in a Community Study
SSISS	South Stockholm Ischemic Stroke Study
STAGE	The Study of Twin Adults: Genes and Environment
STR	Swedish Twin Registry
TIA	Transitory Ischemic Attack
TOAST	Trial of Org 10172 in Acute Stroke Treatment
TTE	Transthoracic Echocardiography
TEE	Transesophageal Echocardiography
vWf	Von Willebrand factor
WHO	World Health Organization
WML	White Matter Lesion

1 BACKGROUND

Stroke has a huge impact on disease burden worldwide, affecting millions of people each year. Even if there has been a decline in cardiovascular mortality in recent years, stroke is still one of the leading causes of death and disability (1, 2). In Sweden, each year approximately 25 000 individuals suffer from a stroke and 8 000 patients have a TIA. Of all stroke cases, approximately 25 % are recurrent events (3).

This thesis focuses on two common conditions and their role in relation to stroke; migraine, which is a common primary headache disorder, and patent foramen ovale (PFO), an inborn cardiac anomaly.

1.1 WHAT IS STROKE?

Stroke is the collective name for ischemic and hemorrhagic stroke. The major part of all strokes (85 %) are ischemic, caused by the obstruction of a cerebral blood vessel, which leads to brain ischemia unless the blood flow is restored. Different pathophysiological mechanisms can lead to an ischemic stroke. It can arise due to a thrombus formed locally in the blood vessel, or from an embolus of a remote origin traveling with the blood stream to the sight of infarction. It can also be due to insufficient cerebral blood flow caused by hypoperfusion. The remaining 15 % of stroke cases are caused by rupture of a blood vessel in the brain or in the subarachnoid space (i.e. hemorrhagic stroke) (4). A TIA (transient ischemic attack) is an episode of focal neurological symptoms of presumed vascular origin; regressing completely within 24 hours, without signs of ischemic injury (5).

The mean age for having a stroke is approximately 75 years of age. Stroke is equally affecting men and women, however the mean age for stroke is lower in men than in women; 73.1 years for males compared to 77.9 years for females (3). Approximately 25-30 % of the patients are under 65 years of age at stroke onset (6), and 10 % of admitted stroke patients are under 55 years of age (7).

1.1.1 Etiology of Stroke

Stroke is a heterogeneous disease that can be caused by a wide range of disorders; however, the most common causes for stroke are large artery atherosclerosis, small vessel occlusion and cardio-embolic stroke, the latter mainly due to atrial fibrillation. Other causes range from non-atherosclerotic disorders of the cerebral arteries, genetic disorders, abnormal coagulation, and infections and systemic disorders involving the cerebrovascular system (8). Ischemic stroke can be divided into different categories based on the probable underlying pathophysiological mechanism and stroke etiology. The TOAST system is the most widely used classification system; dividing patients into Large artery atherosclerosis (LAA), Small artery occlusion (SAO), Cardio-embolism (CE), stroke of other determined etiology and stroke of undetermined etiology (9).

1.1.1.1 Risk factors of stroke

Both genetic and environmental factors affect the risk for stroke. The incidence for stroke increases exponentially with age (6), and the risk doubles for every decade after 55 years of age (10). Other important risk factors are conditions that predispose for atherosclerosis like smoking, hypertension, hyperlipidemia and diabetes mellitus. Cardiac disease like atrial fibrillation, recent myocardial infarction and valvular disease are important risk factors for cardio-embolic stroke.

1.1.1.2 Endothelial dysfunction as a risk factor for stroke

The innermost layer of the vessel wall consists of endothelial cells, which have vital biological functions. Endothelial cells produce a large number of substances that play a role in maintaining vascular tone, in thrombosis and homeostasis, as well as in inflammation and angiogenesis (11, 12). Important mediators in maintaining vascular tone are nitric oxide (NO) and prostacyclin, which have vasodilator capacity, and endothelin-1 (ET-1) which is a potent vasoconstrictor. In the healthy endothelium, ET-1 and NO works inter-dependently in regulating vascular tone (13).

Endothelial dysfunction (ED) is considered one of the first steps in the development of atherosclerosis. ED is characterized by a reduction in the bioavailability of vasodilators and an increase in endothelial-derived contracting factors. This leads to an impairment of endothelium-dependent vasodilatation. Another characteristic of ED is endothelial activation causing a pro-inflammatory and pro-thrombotic state. ED develops with increasing age and in the presence of risk factors for cardiovascular disease; such as smoking and hypertension. It is associated with the development of atherosclerosis and future cardiovascular disease (11, 14, 15). There are several biomarkers that may be used evaluating ED; ET-1, von Willebrand factor (vWf), homocysteine (Hcy), hs-CRP, endothelial progenitor cells (EPC) and endothelial microparticles are examples of biomarkers for ED (16-19). In the section below, biomarkers used in this thesis (Study III) are described.

- ET-1 is mainly produced by endothelial cells, but also by smooth muscle cells and macrophages. ET-1 works as a potent vasoconstrictor by binding to receptors in vascular smooth muscle cells, but it also has pro-inflammatory effects. The plasma levels of ET-1 are, for example, affected by shear stress and pro-inflammatory cytokines (20). Higher concentrations of ET-1 have been shown to predict future cardiovascular events and also to predict mortality in patients with acute heart failure (21, 22) .

- vWF is produced by endothelial cells and mega-karyocytes, but under normal conditions vWf is only produced by endothelial cells. vWf works pro-coagulatory by increasing platelet aggregation, and it’s also a carrier protein for Factor VIII. It is released as a response to vessel injury, but elevated plasma levels of vWf have also been seen in patients with ischemic heart disease, diabetes mellitus and stroke (20, 23, 24).

- Hcy is a metabolite in cysteine and methionine metabolism, and high levels of Hcy have been associated with cardiovascular disease. High levels of Hcy leads to increased oxidative stress and reduced bioavailability of NO, and has also been associated with higher concentrations of ET-1 (25, 26).

- CRP is a plasma-protein and a well-known marker of inflammation. It is an acute-phase protein, produced by the liver where the production is regulated by different cytokines. CRP affects the endothelium by downregulating the production of NO, and also by inducing the production of different adhesion molecules. Several studies have shown that hsCRP (high sensitive CRP) is an independent predictor of future cardiovascular disease (27, 28).

1.1.1.3 Aspects of age in stroke etiology

Age is an important aspect to consider in the work-up of a stroke patient. Younger and older patients differ; both regarding gender representation and etiological causes for stroke. Among younger stroke patients (<60 years); female gender is more common in the youngest patients (< 35 years of age), while there is a male dominance in middle-aged individuals (29-31). Regarding etiology of stroke, major causes like atrial fibrillation, atherosclerosis or small vessel occlusion plays a crucial role in older patients, while these are less common in younger stroke patients (29, 32). Instead, in younger stroke patients, other determined etiologies for stroke, like cervical artery dissection (CAD), PFO or migraine, are more common. A large portion of younger stroke patients have an undetermined cause of stroke (29, 30). However, traditional risk factors, especially smoking, have been shown to be highly prevalent also in younger stroke cohorts (31), and even in young patients with stroke of unknown source, atherosclerosis is more prevalent compared to controls (33).

1.1.1.4 Genetic aspects of stroke

There are many different genetic traits that may increase the risk of stroke. Though rare, there are around 50 different monogenetic diseases increasing the risk of stroke. Of these, Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) is one with particular interest in regards to migraine and stroke, since the phenotype of CADASIL often starts with migraine with aura, followed by early onset of stroke and dementia. CADASIL is caused by a mutation in the NOTCH-3, leading to sub-cortical infarctions (32, 34).

Foremost, stroke is a polygenic disease, where different gene variants, each with a small individual effect, may increase the risk of stroke; either by affecting the likelihood of cardiovascular risk factors, or interacting with risk factors and environmental factors, increasing the risk of stroke. Through candidate-gene studies, and genome-wide association studies (GWAS), a large number of different single nucleotide polymorphisms (SNPs) have been found, affecting the risk for stroke. Many of these markers have been located in genes involved in inflammation, lipid metabolism, coagulation and hemostasis and the renin-angiotensin-aldosteron system (35, 36). Most genetic markers have been associated to a specific subtype of stroke etiology (37, 38).

1.1.2 Stroke of unknown origin

After a standard diagnostic work-up, the cause of stroke cannot be found in approximately 20-25 % of all stroke. These are often referred to as cryptogenic stroke (CS). However, the term CS complies both of patients with an unknown source of stroke, but also of patients with multiple causes of stroke or un-complete diagnostic work-up (39), and in stroke centers using more extensive investigations the rate of CS is consequently lower. In comparison with stroke of other determined causes, CS are often less severe and have lower mortality rate (40). CS is more common in younger age groups (30, 39), and has often more similarities with cardio-embolic stroke than stroke of other etiologies. In studies of patients with CS, prolonged cardiac monitoring has shown transient atrial fibrillation on follow-up, in an additional 10-15 % of the patients (41, 42).

In recent years, another concept has been proposed; embolic stroke of unknown origin (ESUS) (43). In comparison to the term cryptogenic stroke, ESUS is defined as non-lacunar stroke of unknown origin and requires a complete work-up, ruling out other potential sources of stroke. Possible underlying mechanisms might be atrial fibrillation, PFO or aortic arch atherosclerosis (44).

1.2 MIGRAINE

Migraine is a common neurological disorder and consists of recurrent headache episodes with certain diagnostic criteria. Like stroke, migraine has a high impact on disease burden and is according to the WHO one of the major causes of disability worldwide (45, 46).

1.2.1 Definition of migraine

A diagnose of migraine relies on the clinical description of the recurrent headache attacks and is assigned according to criteria set by the International Headache Society (IHS). Many patients experience prodromal symptoms before the development of headache; such as fatigue, frequent yawning, feeling of sadness or cognitive dysfunction (47, 48), and 1/3 of the patients have an initial aura of neurological symptoms (49). Currently diagnostic criteria are defined in ICHD-III (International Criteria of Headache Disorders, third version) (50). Depending on the clinical description, migraine can be defined as different subtypes.

1.2.1.1 Migraine without aura

The typical migraine headache follows certain characteristics regarding duration of the headache, type of headache and associated symptoms. For a definite diagnosis, there must have been at least 5 recurrent episodes (Table 1). There are large differences in the clinical expression of migraine, regarding frequency and severity of the attacks, both within the individual patient as well as between patients. It has been reported that approximately 50 % of the patients have an impact on their daily activities, or are at bed rest during a migraine attack (51).

1.2.1.2 Migraine with aura

A diagnose of migraine with aura is based on the description of the aura only (Table 1). The aura can consist of single or multiple neurological symptoms and the most common aura presentation is visual symptoms with flickering of light, zigzag lines or scotomas in the visual field. A migraine aura evolves gradually, and in the case of multiple symptoms these come after one another, where each symptom last between 5-60 minutes. Headache usually evolves at the same time, or within one hour after the aura, but is not essential for diagnosis and approximately 5 % have recurrent migraine auras without subsequent headache (47, 50). One criteria for diagnosis, is the exclusion of TIA as a cause to the symptoms. In comparison, TIAs usually present with sudden onset of symptoms, compared with the gradual onset in a migraine aura.

1.2.1.3 Probable migraine

Patients fulfilling all but one criterion for a migraine diagnosis according to ICHD are categorized as probable migraine. Probable migraine follows the same epidemiological patterns as definite migraine and responds to migraine specific treatments (52, 53).

Table 1. Diagnostic criteria of migraine headache according to ICHD-III

Migraine without aura	
Criterion A:	At least five attacks fulfilling criteria B-D
Criterion B:	Headache attacks lasting 4-72 h (untreated or unsuccessfully treated)
Criterion C:	Headache has at least two of the following four characteristics: [1] Unilateral location, [2] Pulsating quality, [3] Moderate to severe intensity, [4] Aggravation with physical activity.
Criterion D:	During headache, at least one of the following: [1] Nausea/vomiting or [2] Photophobia/phonophobia
Criterion E:	Not better accounted for by another ICHD-3 diagnosis

Migraine with aura	
Criterion A:	At least two attacks fulfilling criteria B and C
Criterion B:	One or more of the following fully reversible aura symptoms; [1] Visual, [2] Sensory, [3] Speech, [4]Motor, [5]Brainstem, [6] Retinal.
Criterion C:	At least two of the following four characteristics; [1]At least one aura symptom spreads gradually over ≥5 min, and if two or more symptoms, they occur in succession. [2]Each aura symptom last 5-60 minutes. [3]At least one aura symptom is unilateral. [4]The aura is accompanied/followed by headache within 60 minutes.
Criterion D:	Not better accounted for by another diagnosis, and TIA has been excluded.

1.2.2 Migraine epidemiology

Migraine affects approximately 11-13 % of the adult population (54, 55). The median age of onset is 20-25 years of age (54), and the prevalence of migraine increases with age, reaching a peak between 30-40 years of age, while onset after the age of 40 is rare (56, 57). Thus, migraine prevalence is lower in older age groups. The overall migraine prevalence is about the same in the western world, and large population-based trials have shown a stable prevalence over the last decades (49, 51, 56).

The incidence and prevalence of migraine also varies with gender. In childhood, boys have an earlier peak incidence rate compared to girls, resulting in equal prevalence of migraine in boys and girls at young age. After puberty, the incidence increase more rapidly in females, and during the reproductive years' prevalence of migraine is 2-3 times more common in females. In older age groups, the differences between the sexes again diminishes (51, 57). Data from Danish twin studies suggest that the female preponderance may be less apparent in migraine with aura (58).

1.2.3 Migraine pathophysiology

Migraine is a neurovascular disorder and the pathophysiological mechanisms are very complex and still not completely understood. The migraine aura and the migraine headache have different underlying mechanisms. The pain derives from activation of the trigeminovascular system, with release of neurotransmitters, mainly Calcitonin Gene Related Peptide (CGRP). The trigeminovascular systems have afferent nociceptors innervating meninges and cerebral blood vessels. Upon activation, the release of CGRP, leads to a vasodilatory response and release of cytokines and inflammatory substances causing neurogenic inflammation. Also. Neurons in the brainstem and the thalamus are activated (47, 59, 60).

1.2.3.1 Migraine aura

The migraine aura is believed to be caused by cortical spreading depression (CSD) (61, 62). CSD, first described by Lao in 1944 (63), is a wave of slow depolarizing activity spreading across the brain cortex and followed by an inhibition in neuronal activity. During CSD changes in the cerebral blood flow has been seen, with an initial increase followed by a longer lasting decrease in blood flow, moving along the cortex, however staying above the threshold for ischemic injury (64). Neuroimaging studies have shown that the decrease in regional blood flow normally starts in the occipital cortex (62), corresponding to the most common aura presentation of a visual scotoma. Data from animal studies suggests that CSD can be triggered by many factors, for example vasoactive substances like ET-1, but also by cerebral ischemia and micro emboli's (65). An elevation in extra-cellular potassium has been describes as a critical event in inducing CSD (61). The initiation of CSD is influenced by gender, where female sex hormones increase the susceptibility to evoke CSD. On the other hand several migraine prophylactic treatments have been shown to inhibit development of CSD (61). Data from animal experiments show that CSD can activate nociceptors within the trigeminovascular system, where activation seems to be initiated with a delay

corresponding to the time between migraine aura and the development of headache (62, 66).

1.2.3.2 Genetic aspects

Migraine is a complex disease with both genetic and environmental factors being important for the clinical expression (62, 67). The genetic background of migraine has been investigated in several candidate gene- and GWAS, and a meta-analysis of 28 GWAS studies found 12 different gene variants associated with migraine (68).There is also a strong familial aggregation in at least 50 % of migraine patients (67, 69), and studies of the heritability of migraine ranges between 34-57 % (70, 71).

Also, there are monogenetic variants of migraine. Familial hemiplegic migraine (FHM) is a rare dominant autosomal inherited disorder causing migraine attacks with an initial aura including motor weakness (72). In FHM, there are four different forms caused by mutations in different ion channels. The mutations lead to an enhanced susceptibility to induce CSD, and transgenic mouse models of FHM has been valuable in the studies of different pathophysiological mechanisms in migraine (62).

1.2.3.3 ED and hypercoagulability in migraine pathophysiology

ED has been implied in migraine pathophysiology, both as a cause and a consequence of migraine headaches. Numerous studies have investigated vascular reactivity and biomarkers of endothelial activation in migraine, both ictally and in between of attacks. Plasma concentrations of vWf and ET-1 have been shown to be higher ictally in migraine attacks (73). Another study showed that migraineurs had a decreased number of circulating EPC, serving as a marker for endothelial repair capacity (74). Migraineurs have also been shown to have an altered arterial function with reduced dilatory response to shear stress, which was also seen in patients with migraine of recent onset (75). Whether ED is involved in migraine pathophysiology is still a matter of debate, and different studies have shown conflicting results, where some even showed an improved endothelial function in migraineurs (76, 77). There have also been conflicting results whether ED is limited to the cerebral circulation, as opposed to also involve the systemic circulation (78). However, some of the prophylactic treatments used in migraine have an effect on improving endothelial function, and recently also statin treatment, which is known to improve endothelial function, was shown to reduce the number of migraine days in a randomized double-blind study (79).

As discussed previously, ED leads to a pro-coagulatory milieu, with increased levels of for example vWf, which induces platelet aggregation. The role of platelet activation in migraine has previously been extensively investigated, with conflicting results, showing both increased and decreased platelet activation during, and in between of migraine attacks. A state of hypercoagulability in female patients with migraine, may also be associated with elevated levels of estrogen, which is pro-coagulatory. The fact that micro embolisms may trigger CSD and thus cause migraine attacks, have also been lifted in support of the association between migraine and hypercoagulability (80, 81).

1.3 PATENT FORAMEN OVALE (PFO)

Patent foramen ovale (PFO) is a common cardiac anomaly, and a normal part of the fetal circulation. The PFO is a tunnel or flap, caused by the unfused structures that builds the atrial septum. It allows right-to-left shunting of the blood, thus bypassing the pulmonary system. After birth the pulmonary blood flow increases, leading to a raised pressure of the left atria and the flap is then pressed toward the left septum wall. Over the first few months after birth this leads to a permanent closure of the PFO (82-84). However, in a large part of the population the PFO stays open. A PFO can be diagnosed by using cardiac ultrasound or transcranial ultrasound to detect a right-to left shunt at rest or valsalva. The golden standard is the use of contrast enhanced transesophageal echocardiography (TEE) (85, 86). PFO is sometimes associated with an atrial septal aneurysm (ASA), which consist of a bulging movement of the septum membrane of more than 10 mm (87).

1.3.1 Epidemiology

The prevalence of PFO has been investigated in both autopsy studies and clinical populations with estimates ranging from 15-40 % (77, 84, 88), and an equal prevalence in men and women. Autopsy studies suggest that the prevalence of PFO decreases with increasing age (89). The reason for the unsuccessful closure of PFO is unknown, however there are data suggesting that PFO might be a familial trait (90).

1.3.2 PFO and possible pathophysiological mechanisms

Usually, a PFO does not impact the normal physiological function of the heart. However, the finding of a PFO have been associated with a range of conditions; ischemic stroke and systemic embolism, obstructive sleep apnea syndrome (OSAS), migraine and decompression sickness (91-94). A PFO allows for shunting of deoxygenated blood from the right atrium to the left. This enables paradoxical embolism; i.e. clots formed on the venous side allows bypassing the lungs and instead reaching the arterial circulation. This mechanism has been implicated both in migraine and stroke pathophysiology, where the size of the clot could lead to either a migraine attack, or an ischemic stroke depending on the size (65). It’s also suggested that the presence of a PFO can induce transient arrhythmias due to atrial vulnerability (95) or in the combination of a PFO and ASA, promote formation of thrombi within the ASA (96).

The shunting of blood also allows substances normally filtered in the pulmonary circulation, to reach the systemic circulation in higher concentrations (97, 98), which might explain the association with stroke and migraine. In OSAS, a PFO might exacerbate hypoxemia, and it is suggested that patients with OSAS and a PFO might get symptomatic at an earlier stage (93).

1.4 STROKE, MIGRAINE AND PFO – AN INTRIGUING CLINICAL TRIAD

Stroke, migraine and PFO, are three clinical conditions with large diversities regarding epidemiology and clinical expression. Stroke is a devastating disease affecting mostly older individuals and a leading cause of death and disability. In contrast, migraine is considered a mainly benign disease, affecting people in childhood through early adulthood and midlife, and PFO is an inborn anomaly that in most cases are clinically silent. However, despite the diversity, during the last decades' data from both clinical studies and animal experiments have implied that these conditions are related with one another.

In the following sections, I will present the current knowledge of the associations between migraine and stroke, PFO and stroke and migraine and PFO respectively. As for stroke and migraine; migraine is a primary headache disorder and cannot be caused by stroke. However, as implied previously, brain ischemia and other neurovascular conditions can induce migraine-like headache attacks, which further complicate the associations between the three conditions (99).

1.4.1 Migraine – association to stroke and cardiovascular disease

Over the last decade's numerous case-control studies and cohort studies have investigated the association between migraine and an increased risk of stroke, cardiovascular disease, and cardiovascular death (100-111). To this date, five meta-analyses have been published analyzing migraine and its role in stroke and cardiovascular disease (112-116). A meta-analysis by Spector et al. in 2010, including studies of the association to ischemic stroke, yielded an approximately two-folded increased risk (114). In the most recent meta-analysis by Hu et al., analyzing the risk for total stroke, including only prospective cohort studies, the relative risk (RR) for overall stroke was lower with RR 1.55 (95% CI 1.38-1.75) for total stroke and RR 1.64 (95 % CI 1.22-2.20) for ischemic stroke (115). For hemorrhagic stroke, results are more uncertain. There are studies indicating an increased risk (117, 118), but in the recent meta-analysis by Hu et al. there was no association to hemorrhagic stroke (115). Migraine has also been associated with an increased risk of white-matter lesions (WML) and silent infarcts (119-121), however recent data have shown conflicting results regarding this (122, 123).

In many studies, the risk for stroke related to migraine, has been carried by the subgroup of patients with migraine with aura. There is also a stronger association with female gender, young age < 45 years of age, as well as smoking and the use of oral contraceptives (113, 124). In comparison, a prospective cohort study in men by Kurth et al, did not show an increased risk for ischemic stroke (125). Also in a population based study of older individuals by Monteith et al, there was no increased risk of stroke related to migraine (126). Further, data suggests that the risk for stroke is related to active migraine and the frequency of migraine attacks, where at least monthly attacks seem to further increase the risk (105, 127).

To have a stroke at young age is still a rare event, and the absolute risk in migraineurs have been estimated to 1.8-4 additional strokes/10 000 women/ year (128) or 18

additional cardiovascular events/10 000 women with migraine with aura/year (109). There is also data suggesting that migraineurs often have stroke with good functional outcome (129).

1.4.2 Proposed mechanisms to the migraine-stroke association

If all migraineurs have an increased risk for stroke and the reason for this, is still a question of debate. Several hypotheses have been proposed, none being the sole probable mechanism between the migraine-stroke association. Most likely the cause is multi-factorial and may include both genetic and environmental factors, as well as relate to the pathophysiological mechanisms in migraine.

1.4.2.1 Migraineous infarction – a rare cause of stroke

Patients with migraine can suffer from a stroke in relation to a migraine attack, i.e. migraineous infarction. According to IHS criteria, a stroke can be diagnosed as a migraineous infarction if the patient has a previously known migraine with aura, and if the stroke is associated with a typical aura, with at least one symptom persisting for more than 60 minutes. Neuroimaging must show an ischemic lesion in a location corresponding to the aura, and the infarction must not be attributed to any other disorder (50). A migraineous infarction is a rare event; < 0.5-1% of all ischemic strokes. In a study of 2 000 consecutive stroke patients, only nine patients fulfilled criteria for migraineous infarction. However, in younger stroke patients migraineous infarctions are more common (130, 131). Data on the clinical description of these cases are scarce, however the most common presentation is a stroke in the posterior circulation, and it usually has a favorable outcome. Female gender is more common among patients with migraineous infarctions, corresponding to migraine being more prevalent among females (130, 132).

1.4.2.2 Migraine and prevalence of cardiovascular risk factors

Migraine has been associated with a wide range of co-morbid conditions, including many cardiovascular risk factors. Previous studies have found an increased risk of hypertension, current smoking and peripheral artery disease in patients with migraine (133-135). Further, patients with overall migraine, as well as migraine with aura only, have been associated with increased cholesterol levels (134, 136), and there is also an association between migraine and obesity (137). Active smoking has in several studies been strongly associated with an increased risk for stroke in migraineurs (126, 138). On the contrary, diabetes mellitus has in some studies shown a negative association with migraine (139). Further, several studies have investigated intima-media-thickness, a pre-clinical marker of atherosclerosis, in migraine, with conflicting results (140-142).

However, regardless of the association with cardiovascular risk factors, it seems like the prevalence of these risk factors do not affect the risk for subsequent stroke. On the contrary, the risk for stroke has been highest in those with the lowest Framingham risk scores, indicating a non-atherosclerotic mechanism behind the migraine-stroke association (124, 138).

1.4.2.3 Association to co-morbid conditions increasing stroke risk

Migraine has been associated with other conditions, that in turn are associated with ischemic stroke, especially in younger age groups. CAD, which is a common etiology for stroke in young patients, has been associated with migraine. In a meta-analysis of case-control studies migraine, irrespective of aura status, was associated with a two-folded increased risk for CAD. Further data from larger observational- and prospective cohort studies, have shown that migraine are more common in stroke patients with CAD, compared to stroke patients of other etiologies. The reason for the association is not clear, but may relate to shared genetic risk markers, or ED (143-145).

Migraine has also been associated to PFO, which enables cryptogenic embolism as a source of ischemic stroke. This will be further discussed in later sections.

1.4.2.4 Genetic factors predisposing for ischemic ischemia?

In recent years, another hypothesis has been proposed which suggests that migraine is associated with a phenotype of cerebral hyperexcitability, leading to an enhanced susceptibility to induce CSD, which in turn might increase the brain tissues sensitivity to ischemia (62, 146). Data supporting this hypothesis mainly comes from animal studies in mice expressing the gene for FHM, one of the monogenic disorders causing migraine.

Both stroke and migraines are heterogeneous and complex diseases with genetic traits. In ischemic stroke, many different SNPs have been associated with an increased risk of stroke. Some of these have also been associated with migraine, for example in genes involved in vasodilatation and homocysteine metabolism (147-149). There are also monogenic disorders, like previously described CADASIL, with both ischemic stroke and migraine in the clinical expression.

1.4.2.5 Association to ED and hyper-coagulability

As discussed earlier, ED has been suggested to play a role in migraine pathophysiology, and it has been implied that this could explain the increased risk for stroke and cardiovascular disease related to migraine. Circulating endothelial progenitor cells, which are inversely correlated with risk for cardiovascular disease (150), have been found to be reduced in patients with migraine (74, 151). Other studies have indicated that migraineurs have reduced vascular reactivity, as well as increased levels of biomarkers associated with endothelial activation (76, 77, 152). However, studies are inconsistent, showing both impaired as well as unchanged and improved endothelial function in migraineurs. Overall, conducted studies are small and have been using different techniques making comparisons between studies difficult.

1.4.2.6 Migraine specific treatment affecting the risk of stroke?

Migraine specific treatment, i.e. triptans, are used by many patients as an acute treatment to abort a migraine attack. Triptans are 5-hydroxytryptamine (5-HT) -receptor agonists, and have vasoconstrictor properties. They are thus contraindicated in patients with uncontrolled hypertension and cardiovascular disease (60), and have been suggested to mediate the risk for stroke in migraineurs. However, several studies have

failed to show an association between triptan use and risk of stroke among migraineurs (107, 153).

1.4.3 PFO and the risk of stroke

The risk of stroke related to a PFO has previously been investigated in two population based studies of individuals without prior cardiovascular disease. Both in the NOMAS and the SPARC study, there were no independent association between PFO and an increased risk for ischemic stroke (154, 155). However, previous studies have shown that PFO is overrepresented in patients with CS compared to patients with stroke of known etiology. A PFO have been found in 40-56% of patients with CS, while only in 4-18% of controls (96, 156-158). Studies examining the prevalence of PFO, ASA or the combination of the two, in stroke patients were analysed in a meta-analysis by Overell et al. in 2000. The results showed that the RR for stroke in a patient with PFO, regardless of age, was 1.83 compared to controls (95% confidence interval (CI), 1.25-2.66). In younger patients (<55yrs) the RR was 3.10 (95% CI, 2.39-4.21), indicating a significant association with ischemic stroke in younger patients (159).

The main hypothesis regarding pathophysiological mechanisms in PFO related ischemic stroke, is paradoxical embolism from the venous circulation due to a right to left shunt. Pro-thrombotic mutations, like prothrombin 20210 G/A and factor V Leyden, increasing the risk for venous thrombo-embolism, have been associated with an increased risk for stroke in patients with PFO (160), however the finding of a deep venous thrombosis preceding the stroke is not common (161), and other factors may influence the risk for stroke. Other theories suggest that the PFO may induce arrhythmias or promote thrombus formation when in combination with an ASA (95, 96). Also, due to the shunt-hypothesis described previously; potentially harmful substances may reach the systemic circulation in higher concentrations, increasing the risk for stroke or other cardiovascular disease. For example, high levels of Hcy, which have been associated with ED, have been shown to decrease after PFO closure (17, 162).

1.4.4 Treatment of PFO after cryptogenic stroke

There is a general agreement that there is no role for primary prevention of a PFO, either by closure of PFO or medical therapy. On the contrary, the best treatment for secondary prevention of PFO in relation to ischemic stroke, has been much debated for many years. As in all stroke care, anti-thrombotic treatment is administered in order to prevent recurrent strokes. A PFO can also be closed either by open surgery or by endovascular closure. The endovascular procedure is minimal invasive and though preferred over open surgery. It is generally accepted as a safe procedure, although complications occur; the most common being local hemorrhages at the puncture site. Other reported complications are atrial fibrillation and procedure related thrombosis (163). In Stockholm, the decision on best secondary prevention in ischemic stroke and PFO are decided on multi-disciplinary rounds at the Karolinska University Hospital.

1.4.4.1 Anti-platelet therapy vs anti-coagulant therapy

Both anti-platelet and anti-coagulant therapies are used in secondary prevention of PFO related ischemic stroke. Several studies have investigated the effectiveness of anti-platelets vs. anti-coagulants. In the TAcTiCS-PFO study, pooling individual patient data from 12 different studies, there were no benefit of anti-coagulant over anti-platelet therapy. In clinical practice, anti-coagulants seem to be more widely used in patients with large shunts or associated ASA (164, 165).

1.4.4.2 Medical therapy vs closure of PFO.

Previous case-control studies reported a reduced risk for recurrent stroke in patients with CS and PFO, going through closure compared to medical treatment only (166, 167). On the contrary, a prospective study, comparing medical therapy vs. closure in patients with CS and PFO under the age of 45, did not prove a significant benefit from closure over medical therapy, unless in those with large shunts or under 37 years of age (168). Until now, three randomized controlled trials (RCT) have been conducted; PC-trial (169), RESPECT (170) and CLOSURE (171), while the REDUCE trial is still ongoing. All three published RCTs failed to show a significant risk-reduction for recurrent stroke by closure of the PFO, compared to medical therapy.

In all studies, recurrent events were rare, and the studies were most likely underpowered. Shortly after publication of the RCTs, three meta-analyses pooling the data were published, also failing to show a significant benefit from PFO closure (172-174). However, when stratified by device, one device (Amplatzer) showed significant benefit over medical therapy alone (174). A more recent analysis of pooled individual participant data, also controlling for age, gender and cardiovascular risk factors, showed a significant reduction of recurrent stroke with PFO closure compared to medical treatment only (175). Also, recently reported long term data (unpublished) from the RESPECT trial, have shown a significant risk reduction over time compared to medical therapy.

To be able to better determine who will benefit from PFO closure, Kent et al has constructed the Risk of Paradoxial Embolism score (RoPE) (176). This is a 10-point score, which aims at identifying a stroke-related versus incidental PFO, in patients with CS. Clinical characteristics for a stroke related PFO are young age at stroke onset, cortical infarcts and absence of cardiovascular risk factors. Overall patients with high RoPE-score (high risk for stroke-related PFO) have been shown to have a low risk for recurrent strokes, approximately one percent per year while on medical therapy.

1.4.5 PFO and migraine

Several studies have shown an increased prevalence of PFO in patients with migraine with aura; 40-60 % of patients have a PFO compared to 25% in the general population (177). These data could not be confirmed in the population-based NOMAS study, although PFO was there detected by transthoracic echocardiography (TTE), which may explain the overall low prevalence. In another observational study, MA was only associated with the combination of PFO and ASA (178, 179). Vice versa, migraine

with aura is also more prevalent in patients with PFO; 15-50% compared to 4-5% in the general population (180). It is still unclear; whether this is a causal relationship or a coincidence. However, micro emboli can trigger CSD (65) and one hypothesis is that emboli's through the PFO can trigger CSD and cause a migraine attack. Also, as mentioned previously, the shunting hypotheses suggests that a PFO may lead to higher levels of vasoactive substances, normally degraded in the lungs, that can function as a trigger for inducing a migraine attack.

Observational data on the effect of migraine after PFO closure have shown both a reduction, as well as new onset of migraine headache, and a temporary increase in migraine attacks after closure of a PFO. In a meta-analysis of observational studies, reporting migraine frequency after PFO closure in patients having the procedure due to stroke prophylaxis, the majority of patients had an improvement in migraine frequency after closure. This was especially noticed in those with migraine with aura, however included studies were mainly small and uncontrolled (181-183). The majority of stroke patients are also on secondary prevention treatment with both statins and anti-thrombotic treatment, which both have been shown to have a prophylactic effect on migraine headaches (79, 184), making it hard to draw any conclusions.

Previous studies in non-stroke migraine patients have investigated whether closure of a PFO can reduce migraine attacks. Non-randomized studies in patients with overall migraine and PFO, as well as those with large shunts indicating a high risk for paradoxial embolism, have shown improvement in migraine after closure of a PFO (183, 185). Two randomized trials have though failed to show a significant benefit on migraine from PFO closure. The MIST trial was a double-blind, sham-controlled study, evaluating the effect of PFO closure on patients with migraine with aura, with a primary endpoint of cessation of migraine. Patients were followed for six month after PFO closure. The study did not obtain its primary endpoint, however a post hoc analysis, excluding outliers who stood for 20 % of the headache days, showed a significant reduction in headache days compared to the sham group. Recently, the PRIMA trial, recruiting patients with migraine with aura, also failed to prove its primary endpoint of reduction in headache days (186, 187). There is currently no place for closure of a PFO in the treatment of migraine headaches.

1.4.6 Clinical aspects of migraine in relation to stroke

In clinical practice, we often come across patients with migraine and migraine-like features in relation to acute stroke, and it was these patients that initially sparked my interest to work within the field of migraine and stroke. In some of these patients a PFO has been a possible cause for the stroke, in others no cause has been found and the migraine-like features of the stroke cases may have been related to ischemia-induced CSD. We have previously described the clinical course of some of these cases, published in Acta Neurologica Scandinavia (188). In our clinical practice, we have also seen what might happen with frequency and clinical expression of migraine attacks after stroke onset, as well as after closure of PFO; both with temporary worsening and improvement of migraine attacks.

2 AIMS

The overall purpose of this thesis project was to explore different aspects of migraine and PFO in relation to stroke, especially ischemic stroke.

The specific aims of the thesis and its different studies were the following:

- To investigate the frequency of migraine and PFO in a stroke population, in order to explore if cardiovascular risk factors were increasing the risk of stroke related to migraine or PFO (Study I and II).

- To explore if conventional, or new risk factors could be used in assessing the risk for recurrent stroke, in stroke patients with co-existing migraine or PFO (Study I and III).

- To investigate endothelial function in cryptogenic stroke patients with PFO, and to explore whether endothelial function was altered by closure of the PFO, and if any change would be related to a change in migraine frequency (Study III).

- To investigate if migraineurs in a Swedish population had an increased risk for stroke, and if such a risk could be explained by familial factors (Study IV).

3 MATHERIAL AND METHODS

3.1 PATIENT POPULATIONS

The data used in this thesis derives from patient populations identified during clinical work performed in the stroke ward or at the out-patient clinic at the Karolinska University Hospital in Huddinge (Study II and III), previously collected material from the South Stockholm Ischemic Stroke Study (SSISS) (Study I), as well as register-based data from the Swedish Twin Registry (Study IV). The catchment area for the stroke ward at Karolinska University Hospital covers the southern suburbs of Stockholm. In the out-patient clinic, patients come for follow-up after stroke care at the Hospital. However, during the recruitment period of participants for Study III, patients with CS and PFO were referred to the Karolinska University Hospital from the whole Stockholm area, due to the recruitment of a clinical study.

All studies were approved by the Regional Board of Ethics. In Study II and III, all patients gave their written informed consent for participation.

3.1.1 Study I

Study I is a post-hoc analysis of a previous material, collected by co-supervisor Dr. Konstantinos Kostulas. SSISS was a genetic epidemiology study, that prospectively recruited patients with ischemic stroke or TIA attending the stroke ward, or out-patient clinic at the Karolinska University Hospital, during the period 1996-2002 (N=928). Controls were residing in the south Stockholm area and were sampled from blood donors and healthy individuals recruited by the Department of Clinical Chemistry at the Karolinska University Hospital (N=602). In the post-hoc analysis, patients investigated with trans esophageal echocardiography (TEE) as part of their stroke investigation were included (N=117), and dichotomized depending on co-existence of PFO.

3.1.2 Study II

In Study II, individuals eligible for participation, were patients with TIA and ischemic or haemorrhagic stroke, admitted to the stroke ward during a six-month period in 2009 between May 1:st and October 30:th (N=276). Forty-seven patients were either dead or severely demented, at the time of the mailed questionnaire, and were thus not able to participate in the study. Information regarding the study and a questionnaire on recurrent headache episodes were mailed to the remaining patients (N=229). One reminder was sent per mail to non-responders. The response frequency was 76.4 %, resulting in inclusion of 175 patients in the final analysis, divided regarding exposure to life-time migraine headaches.

3.1.3 Study III

Starting in 2009, there has been a collaboration between the departments of Neurology and Cardiology at Karolinska University Hospital, regarding patients with ischemic stroke and PFO. This was started as a response to participation as clinical investigators in the REDUCE study. In REDUCE, patients 18-60 years old, with a CS within the last six months, were randomized to closure plus anti-platelet treatment or antiplatelet treatment only, in a 2:1 ratio. The study was closed for recruitment in February 2015 (Clinical trial, NCT 00738894). During the recruitment for REDUCE, patients deemed appropriate for participation, were called for a screening visit at the out-patient clinic at the Karolinska University Hospital. In Study III, patients screened for the REDUCE study were recruited in two different phases; May 2011 - February 2013 and February 2014 - November 2014. In all 26 individual patients were recruited; 20 patients going through closure of the PFO and 7 controls. One patient started as a control, but had his PFO closed after six months, due to a recurrent ischemic event. All but three patients were randomized to their assigned treatment, while the remaining three were closed outside of the study.

3.1.4 Study IV

In Study IV, a large population-based sample from the Swedish Twin Registry (STR) was used. STR was founded in the 1950s in order to study the importance of smoking and alcohol on cancer and cardiovascular disease. It now comprises of all twins born in Sweden since 1886. Zygosity is assigned using questions on within-pair similarities in appearance during childhood. STR is updated on a regular basis regarding current addresses and is also cross-linked to national patient registries and the cause-of-death registry. Over the years, additional data have been collected from different cohorts within the STR (189). In Study IV, twin populations from the SALT and STAGE studies were used.

3.1.4.1 SALT-Screening across the life-time study

The study was initiated in 1998 and consisted of a telephone interview, screening for the most common complex diseases, as well as collecting data on environmental and socio-economic factors. The eligible population were twins born before 1958 and living in Sweden at the start of the study. Over 44 000 twins responded, and it was completed in 2002 (190).

3.1.4.2 The SALT headache study

In 2004, PhD-student Dan Svensson wrote his thesis on the genetic influence on primary headache disorders using data from STR (191). The SALT headache study comprised of twins from SALT, born 1926-1958. The questions were mapping onto diagnostic criteria for primary headaches; migraine with and without aura, tension-type headache and cluster headache. In our analyses the population from SALT consist of 31 105 twins.

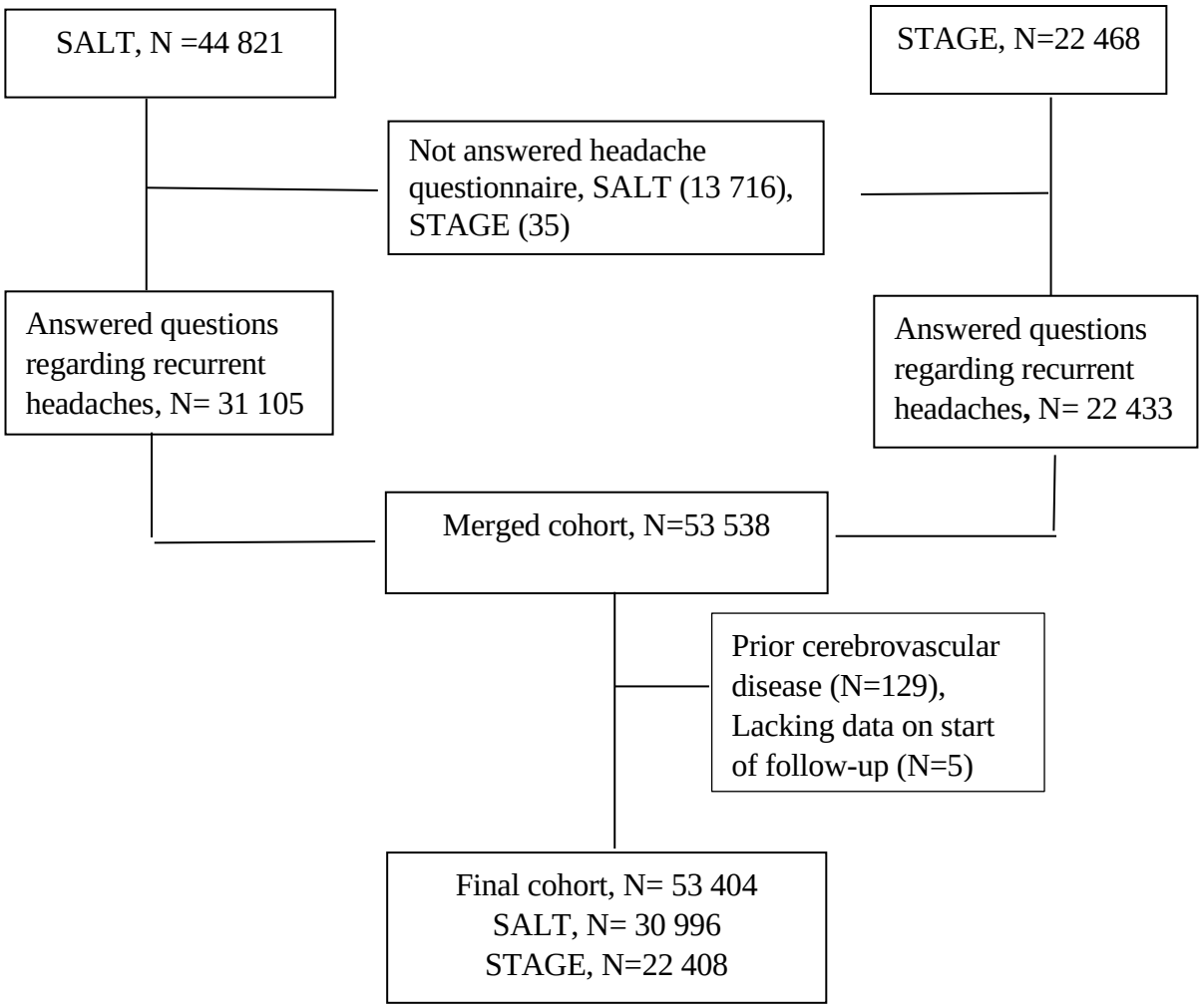
3.1.4.3 STAGE – The Study of Twin Adults: Genes and Environment

The study was initiated in 2005 and consisted of a web based interview with similar questions as in SALT, regarding common complex diseases and environmental and socio-economic factors. All twins born 1959-1985 were contacted with an invitation letter and provided with a personal login. Non-responders were reminded and eventually offered to complete the survey by phone (190). In our analyses, 22 433 twins from STAGE were included.

3.1.4.4 The merged twin cohort

In Study IV, populations from SALT and STAGE, who answered the headache questions were merged into one cohort (N= 53 538). Individuals were excluded if information on date of study entry was missing, or if a diagnosis of stroke was found in national patient registers, prior to the start of follow-up. In total, 53 404 individuals were included in the final cohort (Figure 1). One third (30.2 %) of the twins were monozygotic, while 67.9 % were dizygotic. In 2 % of the twins, zygosity was unknown. Of included participants, 38 908 twins were parts of a complete twin pair.

Figure 1. Study population in Study IV.



3.2 METHODS

3.2.1 Diagnosing migraine from questionnaires

3.2.1.1 Clinical studies

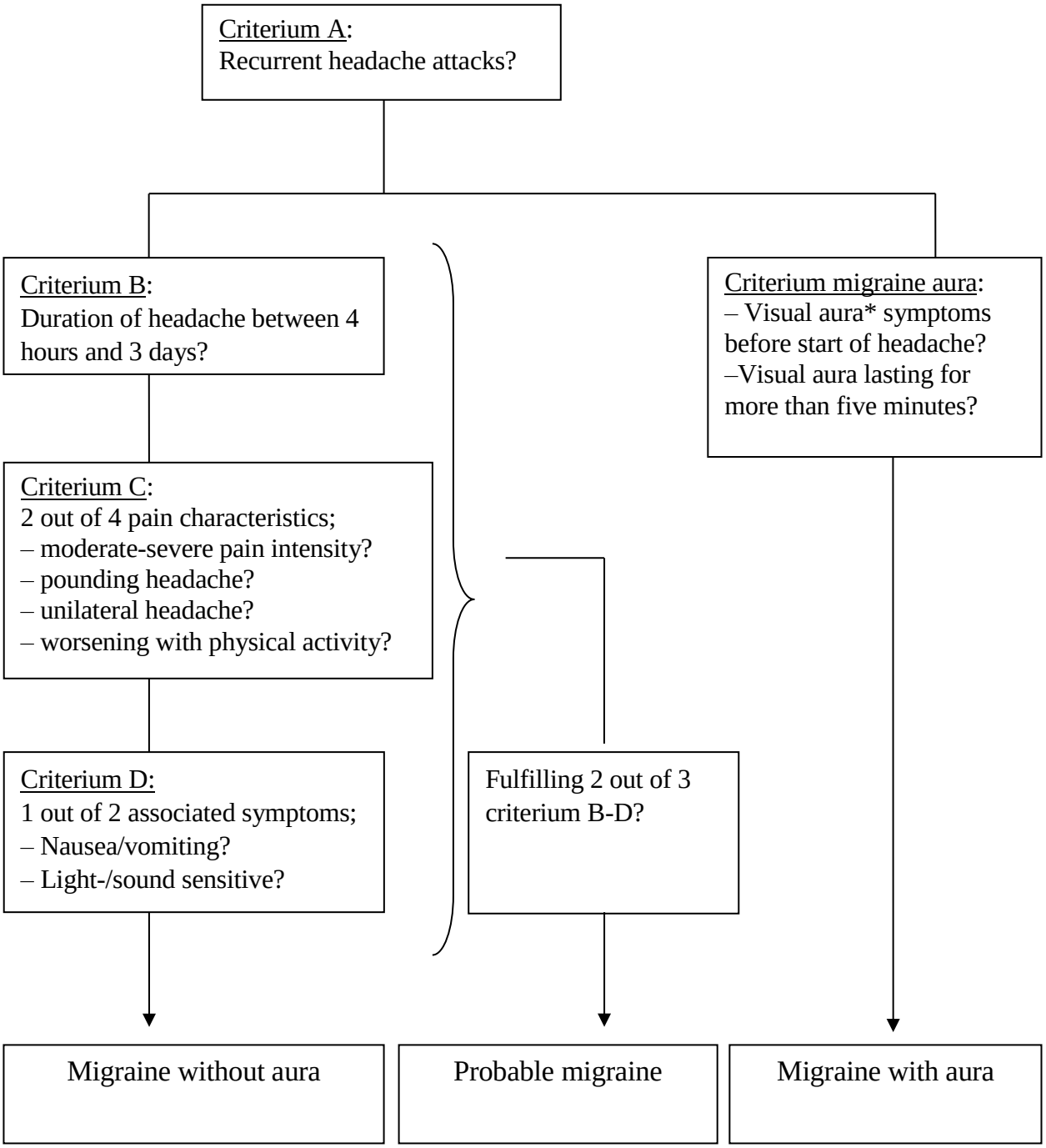
In Study II and III, a questionnaire was used to identify patients with migraine according to ICHD-II. The questionnaire had previously been validated to identify individuals with primary headaches (192). It consisted of questions regarding the diagnostic criteria for migraine and its subtypes. Patients with migraine with aura, migraine without aura and probable migraine were identified. In the statistical analysis, patients with co-existing migraine was analyzed disregarding of migraine subtype. The questionnaire is shown in Appendix 8.2.

3.2.1.2 Twin studies

The questionnaires in SALT and STAGE included questions of recurrent headache episodes and their characteristics. The first question singled out whether individuals have, or had had, recurrent headache episodes not caused by infection or other evident cause. Thereafter, additional questions covered characteristics of headache episodes according to ICHD-III criteria for migraine with and without aura. An algorithm was constructed to identify cases of migraine with or without aura, as well as probable migraine (Figure 2). The questions in SALT and STAGE were similar, though not identical. In STAGE a question on duration of headache was lacking, and therefore only a diagnosis of migraine with aura and/or probable migraine could be assigned, while for SALT all three diagnosis could be assigned. Diagnoses were overlapping, with twins fulfilling only one or multiple of the diagnoses. The statistical analyses were made according to the following constructed groups:

- Any migraine – twin fulfilling criteria for either type of migraine
- Migraine with aura – all twins fulfilling migraine with aura
- Non-aura migraineous headache – Twins with migraine without aura or probable migraine, without co-existing migraine with aura

Figure 2. Algorithm used for assignment of migraine diagnosis in Study IV according to ICHD-III.



*visual disturbance such as flickering of light, zigzag lines moving in the visual field.

3.2.2 Diagnostics and characteristics of cerebrovascular events

3.2.2.1 Clinical studies

In Study I-III, patient populations were previously sampled, or collected during clinical work at the Department of Neurology. All included patients had a diagnosis of TIA (ICD G45.1-G45.9), ischemic stroke (I63.1-I63.9) or hemorrhagic stroke (I61.0-I61.9), and we had access to medical records to verify the diagnosis if needed.

3.2.2.2 Population based twin study

In Study IV, a diagnosis of stroke was identified using the national patient register (NPR) and cause-of-death register (CDR) (193). NPR register diagnoses from hospital discharges and from outpatient visits at hospital-based clinics (194). Each record includes primary and secondary diagnoses. CDR contains diagnoses of causes of death; both major and contributory causes. Diagnoses are coded according to ICD-10 since 1998. We identified all first-time diagnosis of stroke from study entry until 2014-12-31 in these registries, using ICD-10 codes for cerebral ischemia (I63.0-I63.9 and G 46.0-G46.8) and intracerebral bleeding (I61.0-I61.9). We had however no access to medical records and we were not able to assure the correctness of the diagnosis. We chose not to have TIA as an endpoint.

3.2.2.3 Clinical description of stroke patients

In Study I-III, all patients underwent relevant investigations as part of routine stroke care; brain imaging, routine blood tests, ultrasound or radiological examinations of the vessels and cardiology work-up, as judged relevant to the stroke physician. In Study I, stroke etiology had been characterized according to TOAST in all patients, as part of the original study. In Study II and III, clinical description, radiological features of the stroke and diagnostic workup were reviewed. In Study II and III, stroke severity at stroke onset and at follow-up, was assessed using National Institute of Health Stroke Scale (NIHSS) and Modified Rankin Scale (mRS). These are common stroke scales used to assess stroke severity and functional dependency after stroke. NIHSS are shown in appendix 8.1 and mRS are shown below (Table 2).

Table 2. Modified Rankin Scale (mRS)

Modified Rankin Scale (mRS)	
0	No symptoms
1	No functional disability despite symptoms.
2	Slight disability, cannot perform all previous activities.
3	Moderate disability, but able to walk without assistance.
4	Moderate to severe disability, cannot walk without assistance, and requires help in attending bodily needs.
5	Severe disability, requires constant nursing and attention.
6	Death

3.2.3 Risk factors for cardiovascular disease

3.2.3.1 Traditional cardiovascular risk factors

Prevalence of common cardiovascular risk factors are presented in all studies. In Study I, definition of risk factors had been pre-defined in SSISS. In general, data were taken from medical history at stroke onset and from medical records regarding treatment with anti-hypertensive or hyperlipidemic drugs. In Study II and III similar methods were used for definition of risk factors; depending on the medical history from the patient at stroke onset, previous diagnosis in medical records, and the use of anti-hypertensive or hyperlipidemic drugs prior to stroke onset.

In Study IV, the questionnaires in STAGE and SALT were used to identify risk factors. Both studies screened for the prevalence of common complex diseases including cardiovascular risk factors such as hypertension and diabetes mellitus, using simple yes or no questions. We also recorded smoking habits and calculated BMI from data on length and weight. A diagnose of atrial fibrillation was taken from NPR (ICD-10 I48.0-I48.9).

3.2.3.2 Genetic markers associated with ischemic stroke.

In SSISS, genotyping was performed using Multiplex polymerase chain reaction studies and linear immobilized probe assays. The method has been described in the original study (195). The selected 100 SNPs had been shown to be associated with the development of atherosclerosis, including lipid metabolism, inflammation, thrombosis and hemostasis and platelet function. A list of the different markers is shown in Appendix 8.4. In Study II, allele frequency for all the different SNPs were calculated and compared between groups.

3.2.4 Assessment of endothelial function

ED has been discussed previously and involve impaired vascular reactivity and endothelial activation. Endothelial function can thus be assessed by measuring either vascular reactivity as a response to shear stress, or concentrations of biomarkers associated with endothelial activation. Evaluation of vascular reactivity can be done using different techniques; flow-mediated dilatation of the brachial artery (FMD) or by reactive hyperemia peripheral artery tonometry using a finger plethysmograph, and there is a wide array of different biomarkers that can be used to evaluate endothelial activation (20).

In Study III, vascular reactivity and selected biomarkers were investigated at baseline (day before the closure procedure), the day after closure, one and six months after closure. The control patients were followed with the same intervals.

3.2.4.1 Endo-PAT 2000®

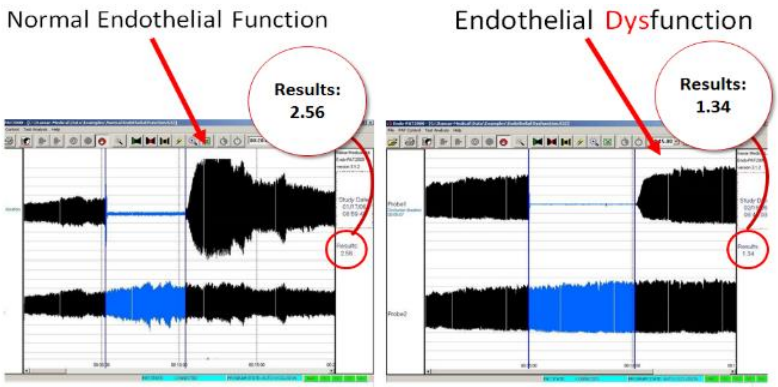
In Study III, Endo-PAT 2000® (Itamar Medical) was used to assess endothelial function. Endo-PAT 2000® is a non-invasive method estimating endothelial function. Its major advantage compared to FMD is that it is more simple and operator-independent. Studies have shown that Endo-PAT 2000® and FMD are comparable in predicting the presence of coronary artery disease (196). Endo-PAT 2000® has also

been proven to have good reproducibility (197). Figure 5 shows the device, including the set-up with the patient.

Figure 3. The Endo-PAT 2000® *device, and set-up with the patient. (Published with permission of Itamar Medical).*

The Endo-PAT 2000® measures pulse wave amplitude in the fingertip at baseline and as a response to shear stress. The changes in amplitude are used to calculate a reactive hyperemia index (RHI), showing the capacity to react with dilatation to shear stress. This in turn, corresponds with vascular endothelial function (Figure 6). The patients other arm serve as an internal control. Vascular reactivity is affected by many factors, for example temperature, time of day, smoking and coffee intake. The test was therefore taken in the morning after a fasting period (preferably overnight, but a minimum of 4 hours) and in a temperature controlled room. A RHI <1.67 correlates to ED and has been proven to predict future cardiovascular events (198, 199).

Figure 4. The Reactive Hyperemia Index (RHI). Normal vascular response to shear stress (left), as opposed to ED (right). Published with permission from Itamar Medical.



3.2.4.2 Biomarkers of ED

In Study III, venous blood was collected in serum- and EDTA-tubes and were either sent to the University laboratory at the Karolinska University Hospital, or stored in freezer awaiting further analyses. Blood samples were taken in the morning with patients fasting for a minimum of four hours.

Concentration of ET-1was analysed with ELISA technique, performed by a laboratory assistant at the Department of Cardiology. The Kit QuantiGlo® from R&D systems was used, and the concentration was presented in pg/ml. Levels of hs-CRP, vWf and Hcy were analysed at the University Laboratory using standardized methods.

3.3 STATISTICAL ANALYSIS

Baseline characteristics were presented as percentages for categorical variables. Continuous variables were presented as means with +/- SD (Study I-II, IV), or as median with inter quartile range (IQR) (Study III) depending on the distribution of data.

In Study I and II, Chi-Square or Fishers exact test was used to compare distribution of cardiovascular risk factors between stroke patients with or without co-existing PFO (Study I) or migraine (Study II). Fisher's exact test was also used to estimate odds ratios for the association between the different SNPs and stroke (Study I).

In Study III, repeated measures one-way ANOVA or Friedman's test (non-parametric test) was used to evaluate whether there was a significant change of endothelial function over time after PFO-closure. For comparison of baseline values of RHI or biomarkers of endothelial activation, between closure patients and controls, non-parametric test of Mann-Whitney or unpaired t-test was used. For comparison of RHI at baseline and after one day, Wilcoxon Signed Rank Test was used.

In Study IV, Cox proportional hazards model with attained age as underlying time scale was used to estimate hazard ratios (HR) with 95 % confidence intervals. Assessments were made for any migraine, migraine with aura, and non-aura migraineous headache and the outcome of total stroke, ischemic stroke and hemorrhagic stroke. In order to adjust for dependence between observations in the twin data, a robust sandwich estimator was used. The model was initially adjusted for gender and age, which were considered true confounding factors, affecting both the risk of having migraine attacks and subsequent stroke. In a second step, the model was adjusted for established cardiovascular risk factors that could serve as a mediator, increasing the likelihood of stroke. With the use of an interaction term, separate analyses were made for women and men, as well as attained age of 50 years or older. Within-pair analysis was made to control for familial confounding factors in twins with migraine with aura.

In all studies the significance level was set to $p<0.05$. The statistical software used was Graph Pad Instat Software (version 3.06) (Study I-II), Graph Pad Prism (Version 6.07) (Study III), SAS version 9.4 (Study IV) and STATA version 14.1 (Study IV).

4 RESULTS

4.1 PREVALENCE OF MIGRAINE AND PFO IN STUDY POPULATIONS

4.1.1.1 Age- and gender distribution in study populations

Prevalence of PFO was investigated in Study I and II, and prevalence of migraine in Study II-IV. The prevalence of migraine is affected by the gender- and age distribution of the populations, which have to be accounted for when interpreting the data. Mean age and distribution of female gender in Studies II-IV are shown in Table 3.

Table 3. Mean age and gender distribution of populations in Study I-IV.

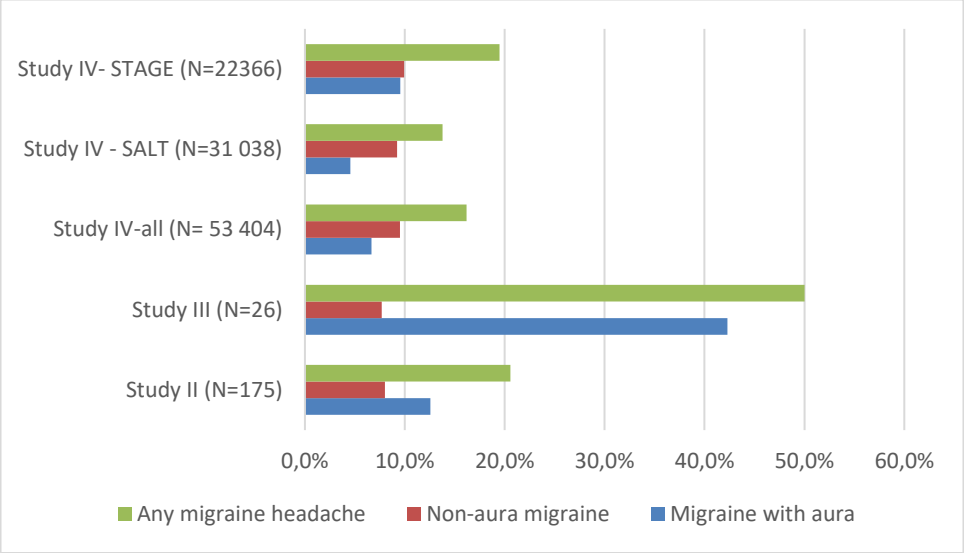
	Mean age (SD)	Female gender
Study I	59.0 (53-65) *	64.5 %
Study II	69.3 (13.0)	46.3 %
Study III	45.4 (8.5)	50 %
Study IV (all)	45.3 (11.9)	54.3 %
Study IV (SALT)	53.7 (5.8)	52.6 %
Study IV (STAGE)	33.7 (7.7)	56.6 %

*Non-normal distribution of data. Age presented as median with IQR.

4.1.1.2 Migraine prevalence

Figure 5 shows lifetime prevalence of any migraine headache, migraine with aura and non-aura migraine headache in Study II-IV. In Study II, 20.1 % had migraine, of which 61.1 % had migraine with aura. Nine percent reported active migraine. In Study III, migraine prevalence was 50 %, of which 84.6 % had migraine with aura. Migraine prevalence in Study IV was 16.2 %, of which 41.1 % had migraine with aura. The younger population in STAGE had higher migraine prevalence compared to SALT (19.5 % vs. 13.7 %) and the subtype of migraine with aura was more common among migraineurs in STAGE compared to SALT (49.0 % vs. 33.1 %). In the studies, the prevalence of migraine in females ranged between 27.2 % and 61.5 %, while in men prevalence ranged between 14.9 % and 38.5 %.

Figure 5. Lifetime prevalence of migraine headache in Study II-IV



4.1.1.3 PFO prevalence

Prevalence of PFO was investigated in Study I and II, but the method for identifying PFO varied between studies. In Study I, the population consisted of a subgroup of the initial SSISS population, examined with TEE as part of the routine stroke work-up. TEE as investigation method is mainly used in younger stroke patients, and only 12.6 % of the initial SSISS population was included in Study I. Mean age of the patients were 58.5 years old. In Study II, mean age of study participants were 69.3 years, and 61.1 % of the cohort (N=107) were examined with echocardiography as part of stroke investigation. Of these only 26.2 % were examined with TEE, while the majority was examined with TTE. The prevalence of PFO was similar in the two groups; 11.9 % in Study I and 11.2 % in Study II.

4.2 CARDIOVASCULAR RISK FACTORS CHARACTERISTICS OF STROKE

4.2.1 Cardiovascular risk factors in stroke populations

The population in Study I-II consisted of mixed stroke populations of different etiology, and presence of cardiovascular risk factors (smoking, hypertension, hyperlipidemia, diabetes mellitus and previous cardiovascular disease) were investigated in both studies, and analyzed in relation to co-existence of either PFO (Study I) or migraine (Study II). Data on peripheral artery disease were available in Study I and BMI in Study II. In both cohorts, traditional risk factors, especially smoking and hypertension, were highly prevalent (Table 4).

Table 4. Prevalence of common cardiovascular risk factors in Study I and II

	Study I (N=117) % (n)	Study II (N=175) % (n)
Ever smoking	50.4 (59)	63.4 (111)
Hypertension	41.9 (49)	68.6 (120)
Hyperlipidemia	21.4 (25)	38.3 (67)
Diabetes	12.8 (15)	19.4 (34)
Atrial fibrillation	12.8 (15)	24.6 (43)
Previous AMI[*1]	4.3 (5)	19.4 (34)
Previous CVD[*2]	18.8 (22)	21.1 (37)
BMI[*3]	-	25.6 (23.3-28.7)
PAD[*4]	3.4 (4)	-

[*1] AMI Acute Myocardial Infarction
[*2] CVD Cerebrovascular Disease
[*3] BMI presented as median with IQR
[*4] PAD Peripheral Artery Disease

In Study I, mean age was similar in patients with and without co-existing PFO and there was no significant difference in risk factors between the groups, although hypertension was less common in patients with co-existing PFO (21.4 % vs. 44.7 %).

In Study II, patients with co-existing migraine were significantly younger compared to those without migraine; mean age 63.4 years vs. 70.9 years (p=0.007). Co-existing PFO was associated with migraine (31.6 % vs. 6.8 %, p=0.007), and atrial fibrillation was less common in stroke patients with migraine (11.1 % vs. 28.1 %, p= 0.049). Otherwise there were no significant differences between the groups.

In Study III, all patients were considered cryptogenic, and the majority were thus lacking cardiovascular risk factors, although a substantial part of the patients were previous smokers (38.5 %). Mean RoPE-score was high; 7.5 out of 10 possible.

4.2.2 Genetic risk markers in patients with stroke and PFO

In Study I, allele frequency of 100 different SNPs was investigated in stroke patients with PFO compared to those without PFO, and to healthy controls. Four SNPs were significantly associated to stroke patients with co-existing PFO (Table 5). These were located in Apolipoprotein CIII, prothrombin and Selectin E, where the strongest association was found for Prothrombin 20210 G/A (p=0.005). All four SNPs, and one additional SNP in Paraoxonase 1 were also significant compared to healthy controls (Table 6).

Table 5. Frequency of minor allele, and Odds Ratio (OR) with 95% CI, in stroke patients with and without co-existing PFO.

Gene	**Polymorphism**	**Minor Allell**	**Frequency** (PFO+)	**Frequency** (PFO-)	**P**	**OR**	**95 % CI**
Selektin E	leu554phe(C/T)	T	0.125	0.027	0.050	5.17	1.15-23.21
Prothrombin	20210 G/A	A	0.125	0.005	0.005	26.43	2.63-265.80
Apolipoprot CIII	-641C/A	A	0.591	0.343	0.037	2.77	1.12-6.87
Apolipoprot CIII	-455T/C	C	0.591	0.324	0.018	3.01	1.22-7.44

Abbreviations: A = adenine; C =cytosine; G = guanine; T =thymine

Table 6. Frequency of minor allele, and Odds Ratio (OR) with 95% CI, in stroke patients with PFO compared to healthy controls.

Gene	**Polymorphism**	**Minor allell**	**Frequency** (PFO+)	**Frequency** (Controls)	**P**	**OR**	**95 % CI**
Selektin E	leu554phe(C/T)	T	0.125	0.028	0.034	4.89	1.38-17.35
Paraoxonase1	gln192arg(A/G)	G	0.455	0.259	0.049	2.39	1.02-5.60
Protrombin	20210 G/A	A	0.125	0.007	0.001	19.29	4.78-77.88
ApolipoprotCIII	-641C/A	A	0.591	0.366	0.043	2.51	1.06-5.93
ApolipoprotCIII	-455T/C	C	0.591	0.371	0.044	2.45	1.07-5.80

Abbreviations: A =adenine; C= cytosine; G= guanine; T= thymine

4.2.3 Stroke characteristics

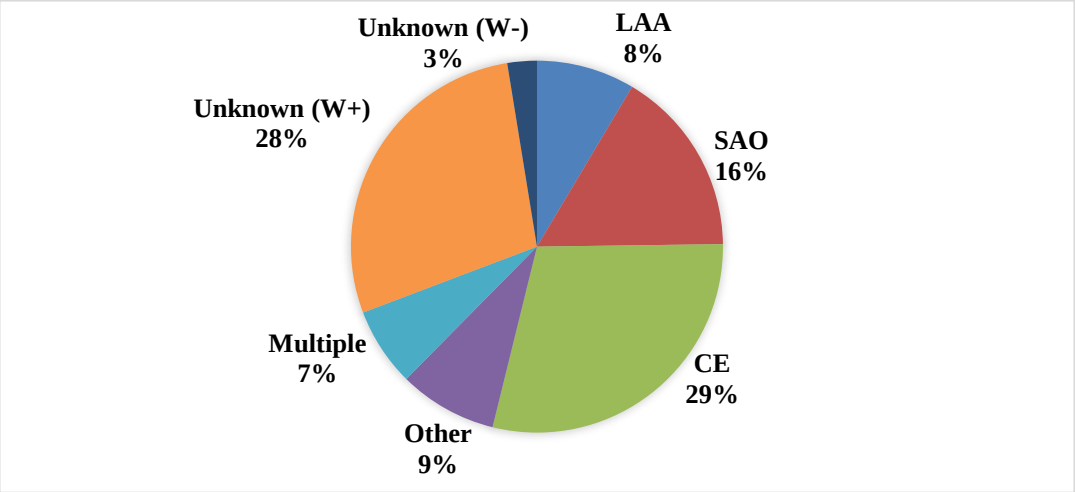
In the clinical studies, patient populations differed regarding type of stroke. Study I consisted of patients with TIA or ischemic stroke, Study II included both TIA, ischemic and hemorrhagic stroke, while Study III only included patients with ischemic stroke. The proportion of different stroke subtypes in Study I and II are seen in Table 7.

Table 7. Proportion of stroke subtype in Study I and Study II.

	Study I (N=117) % (N)	**Study II (N=175) % (N)**
TIA	24.8 % (29)	25.1 % (44)
Ischemic stroke	75.2 % (88)	68.6 % (120)
Hemorrhagic stroke	-	6.3 % (11)

In Study I, patients were classified according to TOAST (Figure 6); PFO patients were either assessed as cardioembolic or as unknown due to multiple mechanisms. Non-PFO patients were heterogenous regarding etiology, although cardioembolic stroke and unknown was more common. We did not have data on stroke severity from Study I.

Figure 6. Stroke etiology according to TOAST in Study I (N=117).



In Study II, both TIA, ischemic and hemorrhagic strokes were included. There were no significant difference regarding type of cerebrovascular event between stroke patients with or without migraine. The majority of the lesions were in the anterior circulation (70.8 %). The stroke population in Study II were assessed regarding stroke severity at onset. Most stroke events were TIA or minor strokes and the majority of patients were independent on follow-up. There were no significant differences in relation to migraine status.

The patients in Study III were all regarded as cryptogenic. Similar to Study II, the majority of the lesions were located in the anterior circulation (76.9 %). Stroke severity with mRS <4 was a pre-requisite for screening for REDUCE, and all included patients had minor sequele with NIHSS 0-1 and mRS 0-2.

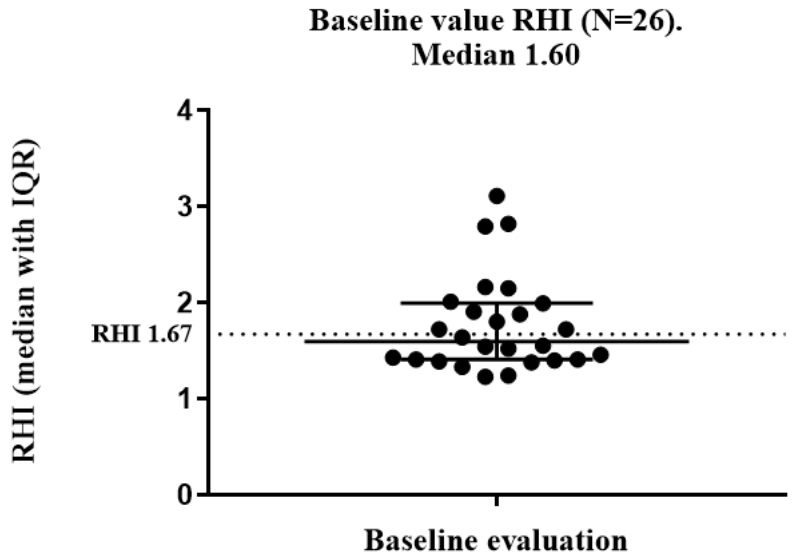
4.3 ENDOTHELIAL FUNCTION IN CRYPTOGENIC STROKE

Evaluation of endothelial function was assessed in Study III; using Endo-PAT 2000® to evaluate vascular reactivity, as well as selected biomarkers of ED; ET-1, hs-CRP, vWf and Hcy.

4.3.1 Vascular reactivity before and after PFO closure

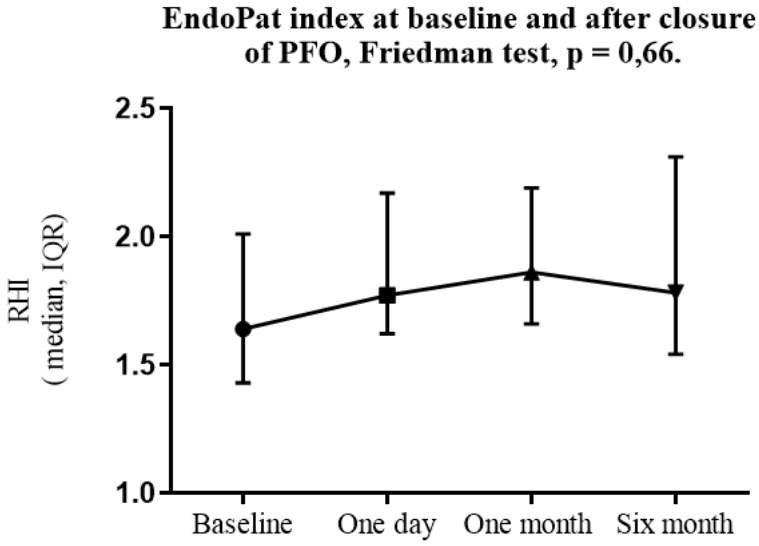
In total 26 individual patients were assessed at baseline and followed according to protocol. One patient started as a control but later had his PFO closed due to a recurrent ischemic event. In the figure below, his baseline RHI as a control patient is presented. The baseline data of RHI-score were not normally distributed, as seen in the scatterplot of baseline data (Figure 7). Median baseline RHI was 1.60 (IQR 1.41-2.00).

Figure 7. Baseline RHI of patients in Study III (N=26).



There was no significant change in RHI after closure of PFO, either from baseline to one day post closure (p = 0.46), or from baseline and over the first six month after closure (p=0.66) (Figure 8). Neither controls had a significant change from baseline to day 1 (p=0.23), or over the evaluated six-month period (p=0.32). Also, when looking at migraine patients having their PFO closed separately (n=13), there was no change over time (p= 0.87).

Figure 8. RHI at baseline and after closure of PFO.



4.3.2 Biomarkers of endothelial activation

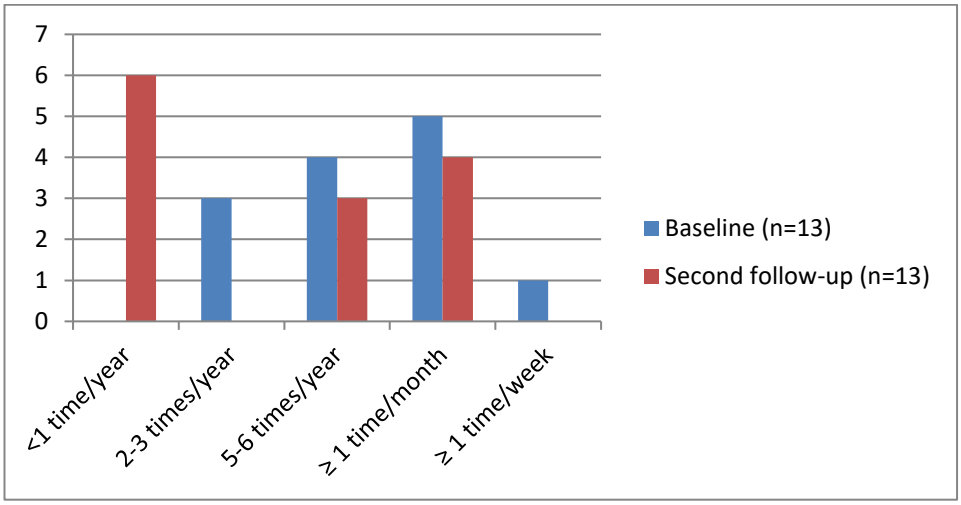
Median plasma level of ET-1 at baseline (n=26) was 2.42 pg/mL (IQR 2.26-2.55). There was no difference between closure patients and controls. Baseline data for hsCRP (n=26) was median 0.54mg/L (IQR 0.2-1.1), for Hcy (n=26) mean 11.07 (SD +/- 3.4) and for vWF (n=25) mean 0.96 (SD +/- 0.3). Closure of PFO did not result in any significant change in plasma levels of either ET-1 (p=0.17), hsCRP (p=0.57), vWF (p=0.15) or Hcy (p=0.83).

4.3.3 Migraine frequency after PFO closure

Baseline migraine was heterogenous, ranging from several times per week to 2-3 times per year. Seven patients (53.8 %) had less than 1 migraine attack per month. After closure of PFO, three patients reported a substantial increase of migraine attacks, with aura daily or several days per week, the worst period being the first month after closure. In one case, migraine transformed from migraine without aura to migraine with aura.

After the six-month period three patients had less migraine attacks than before, three patients had more, and seven patients had no change in migraine frequency. At a second follow-up (mean 43.4 month; SD +/- 13.6); 69 % (n=9) of the patients with migraine reported of less frequent migraine attacks. Six patients experienced complete cessation of migraine, or less than one migraine attack per year (Figure 9). In the case with transformed migraine, migraine with aura persisted also at long time follow-up.

Figure 9. Migraine frequency at baseline and at long-time follow-up.



4.4 MIGRAINE AS A RISK FACTOR FOR STROKE

The population based cohort consisted of 53 404 twins, consisting of both mono- and dizygotic twins. The mean follow-up time was 11.9 years and the total time of observation was 634 468.4 person-years. Mean age at the end of follow-up was 57.2 years (SD 13.9, range 27.3-80.0). The table below show baseline characteristics of the cohort depending on migraine status (Table 8).

Table 8. Baseline characteristics of cohort depending on migraine status (N=53404), given as mean (SD) or n (%).

		Migraine with aura (n=3 553)	Non- aura migraineous headache (n=5 082)	No migraine (n=44 769)
Mean age (SD):		42.5 (11.6)	44.9 (11.6)	45.6 (11.9)
Female sex:		2 811 (79.1)	3 689 (72.6)	22 484 (50.2)
Obese (BMI>30) [*2]		299 (8.4)	428 (8.4)	3 030 (6.8)
Smoking[*2]	Current:	670 (18.9)	890 (17.5)	7955 (17.8)
	Ever:	1 060 (29.8)	1 620 (17.5)	14 025 (31.3)
Hypertension[*2]		687 (19.3)	955 (18.8)	6 399 (14.3)
Hyperlipidemia[*2]		277 (7.8)	408 (5.0)	3 461 (7.7)
Diabetes[*2] (type 1 or 2)		80 (2.3)	97 (1.9)	1 120 (2.5)
Angina[*2]		105 (3.0)	117 (2.3)	1042 (2.3)
Myocardial infarction[*2]		38 (1.1)	44 (0.9)	540 (1.2)
Atrial fibrillation[*3]		107 (3.0)	158 (3.1)	1927 (4.3)
Peripheral artery disease[*2]		44 (1.2)	59 (1.2)	330 (0.7)

[*1]: Fulfill criteria for migraine with aura, migraine without aura or probable migraine.
[*2]: Self-reported data
[*3]: Data from National Patient Register

Both twins with migraine with aura and non-aura migraine headache were of female dominance, but there were no difference regarding in cardiovascular risk factors depending on aura status. However, several co-morbid conditions differed between migraineurs and non-migraineurs. Hypertension, obesity and peripheral artery disease were more common, while atrial fibrillation, myocardial infarction and diabetes was less common in migraineurs compared to non-migraineurs (Table 9).

Figure 9. Risk factors in twins with any migraine compared to non-migraineurs.

	Any migraine (N= 8635)	No migraine (N=44 769)	OR (95 % CI)	P
Obesitas	727 (8.4)	3030 (6.8)	1.27 (1.16-1.38)	< 0.0001
Current smoking	1560 (18.1)	7955 (17.8)	1.02 (0.96-1.35)	Ns
Hypertension	1643 (19.0)	6399 (14.3)	1.41 (0.66-1.50)	< 0.0001
Hyperlipidemia	685 (7.9)	3461 (7.7)	1.03(0.94-1.12)	Ns
Diabetes	177 (2.1)	1120 (2.5)	0.82 (0.70-0.96)	0.012
Angina	222 (2.6)	1042 (2.3)	1.1(0.96-1.28)	Ns
AMI[*1]	82 (1.0)	540 (1.2)	0.79 (0.62-0.99)	0.043
AF[*2]	265 (3.1)	1927 (4.3)	0.70 (0.62-0.80)	< 0.0001
PAD[*3]	103 (1.2)	330 (0.7)	1.63(1.30-2.02)	< 0.0001

[*1] AMI Acute Myocardial Infarction
[*2]AF Atrial Fibrillation
[*3] PAD Peripheral Artery Disease

During follow-up 1 297 first strokes were detected, of these 1 041 were ischemic strokes and 242 were hemorrhagic subtype. Another fourteen cases were double-diagnosed both as an ischemic and a hemorrhagic event at the same date. In all, 1 073 ischemic- and 276 hemorrhagic strokes were recorded in the material, and 38 twins had a record of both an ischemic stroke and a subsequent hemorrhagic stroke, or vice versa. Mean age for stroke diagnosis was 64.1 years (SD 8.4; range 27.3-79.4), and there was a male dominance among stroke patients (61.5 %).

There was no increased risk for stroke in twins with any migraine headache (HR 1.07, 95 % CI 0.91-1.26), or non-aura migraine headache (HR 0.97, 95 % CI 0.79-1.19). Twins with migraine with aura had a 27 % increased risk when adjusted for age and gender, however non-significant in multi-variable adjusted analyses. Estimated HR with 95 % confidence intervals for total stroke, as well as ischemic and hemorrhagic stroke are shown in Table 10.

Table 10. Estimated Hazard Ratios with 95 % CIs for total stroke, ischemic stroke and hemorrhagic stroke, whole cohort (n=53 404).

	Events (n)	Gender adjusted HR (95 % CI)	P	Multivariable adjusted[1] HR (95 % CI)	P
No migraine headache	1126	1.00 (ref)		1.00 (ref)	
Migraine with aura					
Total stroke	70	1.27 (1.00-1.62)	0.05	1.20 (0.93-1.53)	0.15
Ischemic stroke[2]	58	1.28 (0.98-1.67)	0.08	1.19 (0.91-1.56)	0.20
Hemorrhagic stroke[2]	14	1.18 (0.69-2.04)	0.54	1.16 (0.67-2.00)	0.59
Non-aura migraineous headache					
Total stroke	101	0.97 (0.79-1.19)	0.78	0.96 (0.78-1.19)	0.72
Ischemic stroke[2]	77	0.89 (0.70-1.12)	0.33	0.88 (0.69-1.12)	0.29
Hemorrhagic stroke[2]	27	1.23 (0.82-1.86)	0.32	1.24 (0.82-1.87)	0.31
Any migraineous headache					
Total stroke	171	1.07 (0.91-1.26)	0.39	1.04 (0.89-1.23)	0.59
Ischemic stroke[2]	135	1.02 (0.85-1.23)	0.82	0.99 (0.82-1.19)	0.93
Hemorrhagic stroke[2]	41	1.22 (0.87-1.71)	0.26	1.22 (0.86-1.71)	0.26

All models have attained age as underlying time scale.

[1]Adjusted for gender, current smoking, obesity, hypertension, hyperlipidemia, diabetes, previous myocardial infarction, previous angina, atrial fibrillation and peripheral artery disease.

[2] 14 cases were diagnosed as both ischemic and hemorrhagic at the same date without knowledge of the primary event. These are accounted for in both the ischemic and hemorrhagic group.

For migraine with aura, within-pair analysis was performed to assess whether the association to stroke was confounded by familial factors. The analysis was based on the 2 142 twin pairs discordant for migraine with aura. The HR was attenuated in the within pair analysis (1.09, 95 % CI 0.81-1.46) (Table 11).

Table 11. Hazard Ratio with 95 % CI for stroke in within-pair analysis.

Stroke (1 297)	HR (95 % CI)	P
Migraine w. aura	**Co-twin control**	
Gender adjusted	1.09 (0.81-1.46)	0.588
Multi-variable adjusted*	1.11 (0.82-1.51)	0.497

4.4.1.1 Stratified analyses by gender and attained age.

Risk estimates for stroke related to migraine with aura were higher in women than in men, although the interaction term between gender and migraine was non-significant. For non-aura or any migraineous headache there were no differences between men and women with regard to stroke risk (Table 11).

Table 11. Estimated Hazard Ratios with 95 % CI for stroke, stratified by gender

	Events (n)	Women HR (95 % CI)	P	Events (n)	Men HR (95% CI)	P
No migraine headache	393	1.00 (ref)		733	1.00 (ref)	
Migraine with aura[1]	50	1.33 (0.99-1.78)	0.06	20	1.16 (0.74-1.80)	0.52
Non-aura migraineous headache[2]	57	0.94 (0.71-1.25)	0.66	44	1.02 (0.75-1.38)	0.92
Any migraineous headache[3]	107	1.09 (0.88-1.35)	0.45	64	1.06 (0.82-1.36)	0.67

All models have attained age as underlying timescale.

[1] Interaction term migraine with aura - gender, p= 0.60

[2] Interaction term non-aura migraineous headache - gender, p=0.63

[3] Interaction term any migraineous headache - gender, p= 0.87

The majority of stroke events occurred in twins older than 50 years of age; 1 227 cases compared to 70 cases in those under 50 years of age. The risk estimates for stroke in twins with migraine with aura, were higher in twins ≤ 50 years, but again the interaction term was non-significant. The estimated HR for stroke by attained age, depending on migraine status are shown in Table 12.

Table 12. Estimated Hazard Ratios with 95 % CI for stroke, by attained age ≤ and > 50 years of age.

	Events (n)	Attained age ≤50, Gender adjusted HR (95 % CI)	P	Events (n)	Attained age >50, Gender adjusted HR (95 % CI)	P
No migraine headache	58	1.00 (ref)		1068	1.00 (ref)	
Migraine with aura[1]	9	1.85 (0.92-3.73)	0.09	61	1.22 (0.94-1.59)	0.13
Non-aura migraineous headache[2]	3	0.46 (0.14-1.45)	0.18	98	0.99 (0.80-1.22)	0.91
Any migraineous headache[3]	12	1.06 (0.57-1.98)	0.45	159	1.07 (0.91-1.27)	0.40

All models have attained age as underlying time-scale.

[1] Interaction term migraine with aura-attained age, p= 0.26

[2] Interaction term non-aura migraineous headache -attained age, p= 0.20

[3] Interaction term any migraineous headache -attained age, p= 0.98

5 DISCUSSION

The work on this thesis have evolved over the past eight years, alongside the clinical work, taking care of stroke patients, with and without co-existing migraine and/or PFO. During this time the scientific knowledge in the field have expanded greatly. Large prospective studies and meta-analyses regarding the risk for stroke and cardiovascular disease in migraineurs have been published, still the question of why there is an increased risk is unknown. For the association between stroke and PFO, three RCTs have been published, as well as analyses of pooled data. In conclusion, the risk for recurrent stroke related to PFO, was lower than expected, making the individual studies underpowered. However, pooled data and longtime follow-up, give some support in favor of closing PFO, in selected patients with cryptogenic stroke. As for PFO and migraine, additional studies have failed to show benefit of PFO closure in migraine, and there is today no place for closure on this indication.

In the following sections, I will discuss the results presented in this thesis in a broader context, and in relation to other published studies.

5.1 PREVALENCE OF MIGRAINE AND PFO

5.1.1.1 Migraine prevalence

The prevalence of migraine was investigated both in a population based study and in selected stroke populations. In Study IV, the overall prevalence of migraineous headache (16.2 %) was in line with previous population based studies, reporting prevalence estimates of migraine in the general population, ranging between 10-16 % (56, 200-202). About 40 % of the patients had migraine with aura. The population in STAGE had a higher prevalence of migraine compared to SALT (19.5 % vs 13.8 %), which probably reflects the different age spectrum of the cohorts. As discussed previously, migraine prevalence follow a U-shaped curve, with higher estimates in early adulthood and midlife (54).

In the stroke populations of Study II, life-time prevalence of migraine was 20.1%, while 9 % had active migraine, compared to 50 % in Study III. However, these two populations differ in age distribution and co-morbid conditions, since age < 60 years and co-existing PFO was a pre-requisite for initial screening to Study III. This probably explain the difference; the aspect of age has been addressed previously, as well as the known association between migraine and PFO.

Migraine prevalence in stroke populations have been investigated in several case-control studies. The prevalence of migraine there ranged between 8-30 % depending on the age and gender distribution of the cohorts, while the selected control populations had a prevalence of migraine between 4-16 % (101, 103, 203-207). The higher prevalence was seen in younger populations (204, 206). Our results in Study II, are thus in-line with data from previous stroke-populations, but also comparable to the estimated prevalence found in large population based studies on the general population.

In our material, migraine was more common in younger patients; 42.9 % in those ≤ 50 compared to 18.6 % in those over 50 years of age. Though, only 14 patients were under 50 years of age at stroke onset, and the material is too small to draw any conclusions.

In both of our stroke populations, the majority of the migraine patients had migraine with aura; 61.1 % and 84.6 % respectively. In the general population, the presentation of migraine aura increases with age, which may explain our results (54). However, in Study II, migraine with aura was the most common subtype in all patients regardless of age. Previous studies in younger stroke populations have also found similar proportions of migraine with aura among migraineurs; both in the work by Chang *et al* and Abanoz *et al*; 68.9 and 60.6 % of migraineurs in the stroke population reported migraine with aura, which was suggested to be associated with an increased risk for stroke in this group (204, 206). The high proportion of migraine with aura in Study III, is in line with previous studies of individuals with PFO and migraine with aura.

5.1.1.2 Prevalence of PFO

The prevalence of PFO was low, both in Study I and Study II. In Study I, only a selective sample of the patients were examined with TEE, and we have no knowledge of the frequency of PFO in the remaining cohort, nor in controls. In Study II, the majority of the patients were examined with TTE, which is less sensitive in finding a PFO compared to TEE, and this might explain the low prevalence of 11.2 %. Also, the populations in Study I and II consisted of mixed stroke populations, and not cryptogenic strokes only. Previously, the prevalence of PFO in mixed stroke populations has varied substantially, with estimates ranging between 6-30 % (208), and are thus in line with the prevalence in our studies.

5.2 STROKE CHARACTERISTICS

5.2.1.1 Patients with stroke and co-existing PFO

In Study I, the criteria for inclusion in the post-hoc analysis, was that TEE was performed in the diagnostic work-up. In routine clinical praxis, TEE is mainly performed in younger patients, looking for minor sources of cardioembolism, or it is performed in the acute setting, but then mainly if there is a high suspicion of a cardioembolic source, such as a thrombus or endocarditis. This is probably reflected in the young median age of 59 years, where 74.3 % of the patients in Study I were < 65 years of age. This also reflects the relatively small proportion of LAA and SAO as etiology of stroke, while cryptogenic stroke (unknown, complete work-up) and cardioembolic stroke (including patients with PFO as a potential source of cardioembolism) stood for almost 60 % of the stroke cases.

The patients of Study III, were all considered as CS with co-existing PFO. Mean RoPE-score was 7.5, making it reasonable to assess the PFO to be stroke related. RoPE score above 7 have previously been suggested as a cut-off to distinguish a PFO as related to the stroke instead of incidental (209). All patients had stroke with a good functional outcome, which most likely was biased by the screening criteria for the reduce trial (mRS<4).

5.2.1.2 Patients with stroke and co-existing migraine

In Study II, stroke severity at baseline was assessed using NIHSS, and level of independence at home after discharge was assessed from medical records. Most patients, regardless of migraine status, had TIA or minor strokes, and a good functional outcome after stroke. This may be caused by selection bias, where patients with major strokes were less likely to participate in the study. Migraine has though been associated with TIA and minor strokes, as well as strokes of good functional outcome (129, 210).

5.3 ARE CARDIOVASCULAR RISK FACTORS MORE COMMON IN MIGRAINE?

Previous studies have shown that migraineurs have more cardiovascular risk factors, like smoking and hypertension, compared to non-migraineurs. However, data are inconsistent. In Study IV, migraineurs were more prone to be obese and to have hypertension or peripheral artery disease, while both previous myocardial infarction, presence of atrial fibrillation and diabetes mellitus was less common in those with migraine. Hypertension is one of the most important risk factors for stroke, and it has been associated with migraine in several population based studies (133-135). However, other studies have shown the opposite association (211-213). Hypertension has also been associated with other primary headaches (214, 215), and the association might thus not be specific to migraine.

Both peripheral artery disease and obesitas have also previously been associated with migraine, but for obesitas results have been inconsistent (134, 139). In our study, diabetes mellitus was less common in migraineurs, which is in line with previous studies showing an inverse relationship between the two conditions. The inverse relationship has been shown both for type 1 and type 2 diabetes mellitus, but the reason for this is unknown (216, 217). Atrial fibrillation was also less common in Study IV, as well as among stroke patients with co-existing migraine in Study II. There are no previous reports regarding this, unless in the setting of induction of a migraine attack after ablation treatment of atrial fibrillation (218). The prevalence of atrial fibrillation increases with age, and this might explain the differences between migraineurs and non-migraineurs in our studies (219). Apart from atrial fibrillation, PFO was also associated with migraine in Study II. Otherwise, there were no other differences in risk factors depending on migraine status.

In conclusion, previous studies are not consistent regarding the association between migraine and different cardiovascular risk factors, and whether this could be associated to a development of atherosclerosis and subsequent risk for cardiovascular disease in migraneurs, is still unclear. Migraine has also repeatedly been shown to be an independent risk factor for stroke, and on the contrary the risk has been highest in those with a low risk factor profile for cardiovascular disease (124). Also, in our longitudinal follow-up, controlling for cardiovascular risk factors did not change the risk estimates for stroke. This is in line with the hypothesis that non-atherosclerotic mechanisms may be more important in the association between migraine and stroke (129, 210).

Regardless, in migraineurs, cardiovascular risk factors should be screened for and treated appropriately.

5.4 CAN OTHER FACTORS EXPLAIN THE RISK FOR STROKE IN RELATION TO PFO?

A large minority of the general population have a PFO and a stroke may have devastating effects on the individual's quality of life. It's therefore of interest to identify the patients with the highest risk for stroke, related to PFO.

5.4.1.1 Genetic risk markers associated with PFO

The most probable mechanism associating PFO with stroke is paradoxial embolism. This implies formation of embolus on the venous side, but a deep venous thrombosis is only found in a small set of patients with stroke and PFO (161). However, with this rationale; pro-thrombotic mutations affecting the risk for venous embolism, could impact the risk for stroke with co-existing PFO. In Study II, we investigated 100 different SNPs, previously associated with ischemic stroke, including the most common pro-thrombotic mutations for venous thromboembolism; Factor V Leyden and pro-thrombin (20210 G/A). Indeed, we found that the common pro-thrombin mutation, was associated with PFO and stroke. This mutation, has previously been associated with PFO and CS (160, 220-222), but it has also been associated with cryptogenic stroke in general, regardless of co-existence of PFO (223). Factor V Leyden has also previously been associated with CS and PFO (220, 221), though not in our material.

We also tested for other SNPs, associated with stroke through different mechanisms; homocysteine metabolism, inflammation, lipid metabolism and hypertension and vasodilation. We found two SNPs in the Apolipoprotein CIII associated with PFO, and one border-significant SNP located in Selectin E. Apolipoprotein CIII regulates lipoprotein metabolism and is associated with hypertriglyceridemia, but it may also activate a pro-inflammatory response in endothelial cells, leading to ED and atherosclerosis. (224, 225). Selectin E is an adhesion molecule, facilitating interactions between leukocytes and endothelial cells. Elevated levels are associated with ED and the selected SNP have previously been associated with atherosclerosis (226, 227). Neither Apolipoprotein CIII or Selectin E have previously been associated with CS or PFO. All four SNPs, as well as one SNP in the Paraoxonase gene, were associated with PFO compared to controls. However, we don't have any data on the prevalence of PFO in the control population and these may therefor mainly be assessed as a risk marker for an increased susceptibility to ischemic stroke in general.

5.4.1.2 Endothelial function in cryptogenic stroke and PFO

In study III, the main objective was to investigate endothelial function in CS and PFO, and to evaluate whether it was affected by closure. The method for evaluation was Endo-PAT 2000® which had been set up and used at the Department of Cardiology. Endo-PAT 2000® has previously been shown to identify patients with cardiovascular disease and to predict future cardiovascular events (228, 229). The majority of patients

were non-smokers and lacked cardiovascular risk factors, and in case of any hypertension this was well controlled with medication. All patients were also on anti-thrombotic- and statin treatment as routine secondary stroke prophylaxis, which both have been shown to improve endothelial function (230, 231). Despite of this, median RHI indicated impaired endothelial function in the majority of patients. This could be related to the recent stroke. Previous work by Sherbakov *et al.*, showed that endothelial function, assessed by Endo-PAT 2000, was impaired in the acute setting regardless of stroke subtype (232). Our patients though, were assessed in the convalescent phase after the stroke. Compared to historic controls of healthy individuals, the results in our patients indicate impaired endothelial function (232, 233). In a comparative study, Sunbul *et al*, investigated endothelial function in patients with cryptogenic stroke, with or without co-existing PFO and in healthy controls. They found an association with ED and CS, regardless of co-existing PFO. However, cardiovascular risk factors, known to impair endothelial function, were more prevalent in their patients compared to our cohort (15, 234). They used FMD to assess endothelial function which makes comparisons difficult.

A major disadvantage in interpreting the results, is that we did not have stroke-free controls, with or without PFO for comparison. Nevertheless, the results may be seen as hypothesis generating, and warrants for further investigation. Regarding the investigated biomarkers of ED, we did not find an increased level of these at baseline. However, also here we did not have a control group to compare with.

5.4.1.3

The initial idea of investigating endothelial function over time after PFO closure, came from the clinical observations of changed migraine frequency, or new onset of migraine headache, after PFO closure. This has mainly been observed during the first months after closure, and correlates in time with the reendothelialization of the device.

Changes in serum concentrations of substances, no longer escaping degradation in the pulmonary system, would also likely be seen within the first months after closure. In our study, there was no significant change; neither in vascular reactivity or in selected biomarkers in the first six months after closure. Previously, work by Lopez *et al*. found a reduced proteomic expression of substances involved in inflammation and coagulation signaling at 3 months' follow-up after PFO closure, and Deng *et al*, found reduced levels of Hcy after PFO closure (98).

5.5 DOES PFO CLOSURE AFFECT MIGRAINE FREQUENCY?

Considering the complex pathophysiology of migraine, where environmental factors together with genetic determinants combined, impact the migraine phenotype, it's not likely that PFO closure would cure migraine, and there is no support for that theory today. There is however animal data, supporting the idea that micro-embolisms through a PFO could induce migraine attacks, and thus explain a part of migraine attacks in individual patients. From Study III, we provide observational data of migraine frequency after closure. In our study, almost 70 % of the migraine patients (n=9) reported improved-, or cessation of migraine, at a second long-time follow-up. This is

consistent with a former study by Takaya et al, where 63 % stopped having migraine attacks at a follow-up three years after closure (235). Improvement of migraine may have several explanations besides closure of PFO; medications used for secondary prevention (i.e. statins and anti-thrombotic treatment) may have a beneficial effect with reduction of migraine days. Likewise, life changes with avoidance of stress after stroke, can improve migraine frequency, and it can also be the natural course of migraine in the individual patient.

5.6 DOES MIGRAINE INCREASE THE RISK OF STROKE?

In Study IV, the risk for stroke in migraineurs was investigated in a Swedish population-based twin cohort. As discussed before, there are a multitude of studies that have investigated this before, and at least migraine with aura is regarded as an independent risk factor for stroke. To our knowledge, our study is the first using a symptom based approach in identifying migraineurs, in a population based cohort with a prospective twin design, which also allows controlling for familial factors.

The incidence rate of first-stroke in the whole cohort was 204 cases/100 000 person-years, which is in line with incidence rates reported for high-income countries in epidemiological research (236). In comparison with previous studies, our results show a much weaker association for stroke in relation to migraine. We only found an increased risk for those with migraine with aura, while for any migraine there was no increased risk at all. In the multivariable model, all analyses were non-significant.

The association between migraine and stroke has previously foremost been associated with female gender and younger age, and many studies have also been restricted to these groups (100, 104-106, 109, 138, 206). Five prospective studies have like us, investigated the risk for stroke in a population of both genders, with varying results. Three studies (Merikangas et al. (108), Velentgas et al. (107) and Hall et al. (153)) showed an increased risk, while Stang et al. (237) only found an increased risk in those with migraine with aura, and Monteith et al. (126) did not find any increased risk for stroke, unless in the combination of migraine and current smoking.

In our study, migraine was identified by symptom based questions, the first being if they have/had had recurrent headaches. This approach may detect migraineurs regardless of the severity, or frequency of headache attacks. In comparison with the studies showing an increased risk for stroke related to any migraine; both Hall et al. and Velentgas et al. used patient records and/or prescription of triptans to identify migraineurs, and Merikangas et al. used self-reported physician diagnosis of migraine. However, many patients with migraine never seeks medical attention for their headaches (238), and migraineurs in these studies might thus be experiencing more frequent, or severe migraine attacks, factors that have been associated with an increased risk for stroke (105, 127). This difference might explain the weaker association in our study, unfortunately in our study, we did not have information regarding migraine frequency or whether migraine was active or not.

In the analyses of migraine and the risk of ischemic and hemorrhagic stroke respectively, all results were non-significant. Although, compared to hemorrhagic stroke, risk estimates were slightly higher for ischemic stroke related to migraine with aura. This is in line with previous studies, where the highest risk has been seen for migraine with aura and ischemic stroke. Likewise, in stratified analysis by gender and attained age of 50 years, there were higher risk estimates among females and those under 50 years of age, which also are in line with the conclusions of the meta-analysis by Schurks et al (113). There were though few endpoints in twins with attained age younger than 50 years, and we can therefore not draw any firm conclusions from these data.

In conclusion, in the scientific literature migraine and especially migraine with aura, have previously been recognized as an independent risk factor for stroke with an approximately doubled associated risk to ischemic stroke, and a less clear association to hemorrhagic stroke. In contrast, we only found a modest increased risk associated with migraine with aura, and for the large body of migraineurs without aura, we did not find any increased risk for stroke.

5.6.1 May an increased risk depend on familial factors?

The twin design of Study IV allows controlling for familial factors, by using within pair analysis. The analysis relies on complete twin pairs, discordant for the exposure, and thus allows controlling for factors that are constant within twin pairs, e.g. early childhood environment and genetic factors. In the analysis of migraine with aura in relation to stroke, the risk estimate attenuated in the within pair analyses (HR 1.27 to HR 1.09). This suggests that familial factors can attribute to the increased risk, but there was a large drop in sample size and we cannot draw any firm conclusions from these data.

The results are interesting in respect of more recent evidence from animal studies linking migraine with a phenotype being more susceptible to trigger CSD and also more vulnerable to ischemic injury. In a small retrospective case-control study, patients with migraine were also more likely to have a perfusion-diffusion mismatch on MRI, performed within 72 hours after admission (239).

5.7 LIMITATIONS

There are methodological aspects and limitations of each of the included studies, which have to be considered when interpreting the results. The methodological aspects have in part been covered in the previous discussion, in relation to each study. However, below follows some additional viewpoints regarding the limitations of the studies.

In Study I and II, the major limitations are the retrospective design and the small population sample, which makes it difficult to draw any firm conclusions. The evaluation of migraine, might be influenced by recall bias due to the retrospective design. The studies were also too small to be able to stratify analyses according to migraine and/or stroke subtype. In Study I, another major limitation is the fact that it is a mixed stroke population, and the PFO may be incidental rather than related to the stroke. We also lack data on prevalence of PFO in the control group, which make the comparison of genetic markers in patients versus controls irrelevant in regards of co-existing PFO. In Study II, the results regarding stroke characteristics may be an effect of selection bias, since patients with the most severe sequele after stroke were excluded from the questionnaire, and even those with moderate sequele might be less inclined to participate in the study.

Study III, was an experimental study in order to explore differences over time after closure. It was not designed to evaluate endothelial function at baseline, and a major limitation is the lack of a control group. The results might instead be seen as hypothesis generating. The evaluation of endothelial function is also difficult to compare with previous studies, due to the wide variety of methods used in other studies, both for evaluating vascular reactivity, as well as biomarkers of endothelial activation. This is a general consideration regarding previous studies evaluating endothelial function, both in migraine and stroke.

In Study IV, we have a large population based cohort, but a limitation of the study is the fairly short follow-up time. Mean age at the end of study was low (57 years), and far younger than the mean age for stroke in the general population. On the other hand, it is in younger and middle-aged individuals that migraine probably contributes the most to the stroke risk. Other limitations are that we were unable to verify the stroke events by medical records, and in a few cases, twins were diagnosed both as an ischemic and a hemorrhagic stroke at the same date, making these accounted for in both groups in the sub-analyses. Also, younger twins in the material may have developed migraine during the follow-up, and any strokes in these twins might have attenuated the effects. We were also unable to identify twins with migraine auras without subsequent headaches. These are fairly rare cases, although they have the migraine subtype most associated with stroke, and misclassification of these twins, might also have attenuated the results.

6 CONCLUSION AND FUTURE PERSPECTIVES

The project in this thesis have explored different aspects of migraine and PFO in relation to stroke, mainly ischemic stroke. In conclusion, we found that:

- A common pro-thrombotic mutation, Prothrombin (20210 G/A), was associated with TIA/ischemic stroke and co-existing PFO. The mutation increases the risk of venous thromboembolism and may be related to the risk for stroke through paradoxial embolism.

- Another two mutations in Apolipoprotein C III and one mutation in Selectine E, showed a weak association with ischemic stroke and PFO. The mutations have not previously been associated with stroke and PFO.

- There was no increased migraine prevalence in our mixed stroke cohort, but the majority of the patients had migraine with aura. Cardiovascular risk factors were highly prevalent in stroke patients, regardless of migraine diagnosis. A diagnosis of PFO were more common in migraineurs, as expected from previous studies.

- A majority of patients with cryptogenic stroke and co-existing PFO had impaired endothelial function, despite lack of cardiovascular risk factors and ongoing anti-thrombotic and statin treatment. There was no change over time after closure.

- In a Swedish population based twin cohort, there was no association between any migraine and stroke, although a modest risk increase was seen for migraine with aura in gender- and age adjusted analyses.

- The increased risk for stroke related to migraine with aura might be impacted by familial factors.

6.1 FUTURE PERSPECTIVES

6.1.1 Aspects of migraine in stroke

Our results from Study IV, supports the idea that the increased risk for stroke in relation to migraine is mainly restricted to the subset of patients with migraine with aura, and also shows that familial factors might contribute to the risk. Given our results, the large body of migraineurs with migraine without aura, may not have an increased risk for stroke, and even in those with migraine with aura, the absolute risk for stroke is probably low. Future studies may attempt to further establish which migraineurs that are at an increased risk, and whether this relates to the frequency of migraine attacks. In Study IV, the questionnaire in STAGE but not in SALT, included questions regarding

more frequent headaches (at least monthly) as well as use of triptan treatment, and a second, longer follow-up in this population can be of interest.

The mechanisms behind an increased risk for stroke are still unclear. If the aura itself leads to subsequent biological changes increasing the risk of stroke, the risk estimate could depend on the frequency of attacks with aura and not the total amount of migraine attacks. If instead an inborn effect increases the risk of ischemia, the attack frequency might not have an impact on the risk. Interestingly, in animal studies, several migraine prophylactic treatments have been shown to suppress the susceptibility to CSD, and also to improve stroke outcome (240, 241). If future studies can identify a subgroup of migraine patients with higher risk of stroke, this may guide future preventive treatments. As of now, there is now role for primary prevention with antithrombotic treatment or migraine prophylaxis, in regards of decreasing the risk for stroke only. The impact of cardiovascular risk factors on the risk for stroke in migraineurs is still uncertain. However, traditional cardiovascular risk factors should be investigated and treated accordingly to minimize the general risk for cardiovascular disease.

This thesis has focused on the association between migraine and stroke, but apart from the risk for stroke, migraine is also associated with an increased risk of myocardial infarctions and angina. Some mechanisms, hypothesized to increase the risk of stroke, i.e. association to PFO and CAD, as well as subsequent effects of CSD, cannot explain the increased risk for ischemic heart disease. The findings of increased prevalence of cardiovascular risk factors and ED in migraineurs, might instead be of greater interest in this respect. Further studies regarding the role of endothelial function in migraine are warranted, preferably in larger populations.

6.1.2 Aspects of PFO in stroke

Since the work on this thesis started the long-term results of the RESPECT study have been published, adding data in support of closure of PFO after ischemic stroke in selected patients. The Amplatzer device has also been FDA-approved for secondary prophylaxis of ischemic stroke related to PFO. The annual risk for recurrent stroke is low, assessed to approximately 1% on medical treatment, but in a young patient the accumulated risk of recurrent stroke may still be substantial. PFO closure is considered a safe procedure, though rarely, the device may result in complications, also many years after implantation (242, 243). Whether assessment of endothelial function could add predictive value in identifying patients at a higher risk for stroke, and thus better select patients who benefits from PFO closure, warrants further investigations. We are now planning for a follow-up study, with assessment of endothelial function in patients with cryptogenic stroke and PFO, compared to healthy controls with and without PFO.

7 POPULÄRVETENSKALIG SAMMANFATTNING

En stroke innebär att man får en propp eller en blödning i hjärnan eller ryggmärgen, vilket globalt drabbar över 10 miljoner människor varje år. Bara i Sverige får ca 25 000 människor en stroke varje år. Stroke är en komplex sjukdom där arv och miljö samverkar till en ökad risk. De vanligaste orsakerna till stroke är följdeffekter av åldrande, såsom åderförkalkning i blodkärlen eller hjärtsjukdomar som t.ex. förmaksflimmer. Vanliga riskfaktorer för att drabbas av stroke är rökning, högt blodtryck och diabetes, men det finns många fler. Den här avhandlingen handlar om två mindre vanliga riskfaktorer för stroke - öppetstående foramen ovale (PFO) och migrän.

PFO är en rest från fostertiden som finns hos ca 25 % av befolkningen. Det innebär att det finns en förbindelse mellan höger och vänster förmak, och därigenom kan proppar som bildas i det venösa systemet ta sig över till vänster förmak och orsaka en stroke, s.k. paradoxal embolisering. Studier har visat att PFO är mer vanligt förekommande hos patienter med stroke utan klar orsak, men det är också vanligare hos individer med migrän.

Migrän är en primär huvudvärksform som förekommer hos ca 11–13 % av befolkningen, men det är betydligt vanligare hos kvinnor än hos män. Migrän debuterar ofta i tonåren eller tidig vuxenålder. Diagnosen ställs kliniskt utifrån symtomen vid huvudvärksattackerna. I ca 1/3-del av fallen har patienterna en inledande aura av neurologiska bortfall och man skiljer därför mellan migrän med aura och migrän utan aura. Genom åren har upprepade studier visat på en ökad risk för stroke hos patienter med migrän. Risken har framför allt visat sig föreligga hos de med migrän med aura, samt vara kopplat till kvinnligt kön och ålder <45 år.

Det finns en rad olika hypoteser till varför migräniker har en ökad risk för stroke. Dels har studier visat att individer med migrän har fler riskfaktorer för stroke och hjärtkärlsjukdom vilket kan leda till utveckling av ateroskleros och ökad risk för stroke. Det kan också vara så att själva migränauran är kopplat till genetiska förändringar som medför att man har en ökad känslighet för syrebrist i hjärnan, eller att migränanfallet leder till biologiska förändringar som ger en ökad risk för stroke. Migrän är också kopplat till en ökad förekomst av PFO vilket i sin tur tros kunna orsaka stroke framför allt hos unga. Intressant nog, har man också sett att förekomsten och uttrycket av migränattacker kan förändras efter slutning av ett PFO, med en initial försämring av frekvensen hos en del, men på lång sikt en förbättring av migränfrekvensen.

Den här avhandlingen bygger på fyra delstudier som belyser olika aspekter av PFO och migrän i anslutning till stroke. I Studie I, undersökte vi om olika genförändringar, som tidigare associerats med ökad risk för stroke, var mer vanligt förekommande hos strokepatienter med samtidig förekomst av PFO. Vi fann att en vanlig mutation som ger en ökad risk för venösa proppar var kopplat till stroke och PFO. Vi fann också att 2 andra mutationer som påverkar fettmetabolism och inflammation var associerade med stroke och PFO.

I Studie II undersökte vi förekomsten av migrän i en blandad strokepopulation. Migrän identifierades utifrån en enkät med frågor avseende återkommande huvudvärksattacker och hur dessa brukar te sig. Vi fann att ca 20 % av populationen hade eller hade haft migrän. En större del än förväntat, 61 % av de med migrän, hade migrän med aura. Patienter med stroke och samtidig migrän hade en ökad förekomst av PFO jämfört de utan migrän, men för övrigt var det inga skillnader mellan strokepatienter med eller utan migrän.

I Studie III undersökte vi endotelfunktionen, dvs funktionen hos det innersta cellagret i kroppens alla blodkärl, före och efter slutning av PFO hos patienter med kryptogen stroke, det vill säga stroke utan någon klar orsak. Nedsatt endotelfunktion är kopplat till utveckling av ateroskleros och ökad risk för stroke, men det har också kopplats till möjliga biologiska effekter av upprepade migränanfall. Patienterna var, frånsett sin stroke, tidigare friska och utan riskfaktorer för stroke. Hälften av patienterna hade migrän och av dessa hade nästan samtliga (84.6 %) migrän med aura. Majoriteten av patienterna hade en nedsatt endotelfunktion vid första mätningen, men slutning av PFO påverkade däremot inte endotelfunktionen. Det är oklart om detta kan ha ett prognostiskt värde för att utvärdera risken för en ny stroke relaterat till PFO.

I sista studien, Studie IV, undersökte vi risken för stroke relaterat till migrän i en population från det svenska tvillingregistret. Över 53 000 individer inkluderades i studien och följdes under drygt 10 års tid. Förekomst av migrän identifierades från tidigare insamlade enkäter och strokefall identifierades via länkning till nationella patientregister. I vår studie såg vi ingen ökad risk för migrän överlag, däremot fanns det en något ökad risk hos tvillingar med migrän med aura. Vidare analyser pekar på att familjära faktorer kan bidra till den ökade risken. I jämförelse med tidigare studier var det dock en betydligt minskad risk för stroke hos individer med samtidig migrän.

8 APPENDIX

8.1 NATIONAL INSTITUTE OF HEALTH STROKE SCALE (NIHSS)

Instruction	Scale definition
1a. Level of Consciousness (LOC):	0 = Alert; keenly responsive. 1 = Not alert; but arousable by minor stimulation to obey, answer, or respond. 2 = Not alert; requires repeated stimulation to attend, or is obtunded and requires strong or painful stimulation to make movements (not stereotyped). 3 = Responds only with reflex motor or autonomic effects or totally unresponsive, flaccid, and areflexic.
1b. LOC, questions: - What month is it? - How old are you?	0 = Answers both questions correctly. 1 = Answers one question correctly. 2 = Answers neither question correctly.
1c. LOC, command: Close your eyes. Close and open your fist.	0 = Performs both tasks correctly. 1 = Performs one task correctly. 2 = Performs neither task correctly.
2. Best Gaze: Only horizontal eye movements will be tested. Voluntary or reflexive eye movements will be scored.	0 = Normal. 1 = Partial gaze palsy; gaze is abnormal in one or both eyes, but forced deviation or total gaze paresis is not present. 2 = Forced deviation, or total gaze paresis not overcome by the oculocephalic maneuver.
3. Visual: Visual fields (upper and lower quadrants) are tested by confrontation, using finger counting or visual threat, as appropriate.	0 = No visual loss. 1 = Partial hemianopia. 2 = Complete hemianopia. 3 = Bilateral hemianopia (blind including cortical blindness).
4. Facial Palsy:	0 = Normal symmetrical movements. 1 = Minor paralysis (flattened nasolabial fold, asymmetry on smiling). 2 = Partial paralysis (total or near-total paralysis of lower face). 3 = Complete paralysis of one or both sides (absence of facial movement in the upper and lower face).
5. Motor Arm: The limb is placed in the appropriate position: extend the arms (palms down) 90 degrees (if sitting) or 45 degrees (if supine). Tested in each arm separately.	0 = No drift; limb holds 90 (or 45) degrees for full 10 seconds. 1 = Drifts down before full 10 seconds; does not hit bed or other support. 2 = Some effort against gravity; limb cannot get to or maintain (if cued) 90 (or 45) degrees, drifts down to bed, but has some effort against gravity. 3 = No effort against gravity; limb falls. 4 = No movement.

6. Motor Leg: The limb is placed in the appropriate position: hold the leg at 30 degrees (always tested supine). Tested in each leg separately.	0 = No drift; leg holds 30-degree position for full 5 seconds. 1 = Drift; leg falls by the end of the 5-second period but does not hit bed. 2 = Some effort against gravity; leg falls to bed by 5 seconds, but has some effort against gravity. 3 = No effort against gravity; leg falls to bed immediately. 4 = No movement.
7. Limb Ataxia: Test with eyes open. The finger-nose-finger and heel-shin tests are performed on both sides, and ataxia is scored only if present out of proportion to weakness.	0 = Absent. 1 = Present in one limb. 2 = Present in two limbs.
8. Sensory: Sensation or grimace to pinprick when tested, or withdrawal from noxious stimulus in the obtunded or aphasic patient.	0 = Normal; no sensory loss. 1 = Mild-to-moderate sensory loss; patient feels pinprick is less sharp or is dull on the affected side; or there is a loss of superficial pain with pinprick, but patient is aware of being touched. 2 = Severe to total sensory loss; patient is not aware of being touched in the face, arm, and leg.
9. Best Language:	0 = No aphasia; normal. 1 = Mild-to-moderate aphasia; some loss of fluency or facility of comprehension, without significant limitation on form of expression. Reduction of speech and/or comprehension, however, makes conversation about provided materials difficult or impossible. 2 = Severe aphasia; all communication is through fragmentary expression. 3 = Mute, global aphasia; no usable speech or auditory comprehension.
10. Dysarthria:	0 = Normal. 1 = Mild-to-moderate dysarthria; patient slurs at least some words and, but can be understood with some difficulty. 2 = Severe dysarthria; patient's speech is so slurred as to be unintelligible in the absence of or out of proportion to any dysphasia, or is mute/anarthric.
11. Extinction and Inattention (neglect):	0 = No abnormality. 1 = Visual, tactile, auditory, spatial, or personal inattention to bilateral simultaneous stimulation in one of the sensory modalities. 2 = Profound hemi-inattention or extinction to more than one modality.

Adopted from National Institute of Health

8.2 HEADACHE QUESTIONNAIRE USED IN STUDY II AND III

Q 1. Have you any kind of recurrent headache episodes, not associated to infection and/or fever? (Yes/No)

Q 2. At what age did these headaches start?

Q 3. How long is the duration of headache episodes? (< 3 hours, 3-6 hours, 6-12 hours, 12-24 hours, 24-36 hours, 36-48 hours, varying)

Q 4. What is the primary location of the headache? (Orbital, in the forehead, temporal, occipital, face/jaw, varying)

Q 5. On what side is the pain located? (right-side, left-side, side-changing, bilateral, varying)

Q 6. What character does the pain have? (explosive, incisive, pounding, dull)

Q 7. What intensity does the headache have when as worst? (Scale of 1-10)

Q 8. Is the headache accompanied by any of the following? (nausea, vomiting, light-, sound-sensitive)

Q9. Is the headache accompanied by any of the following? (conjunctive injection, tearing, nasal congestion, miosis, ptosis, increased forehead sweating)

Q 10. What do you do to relieve the pain? (resting, sitting, walking around)

Q 11. Is the headache increased by physical activity? (Yes/No)

Q 12. Do you have any of the following before, or together with the pain? (scintillating scotoma, paresthesia, disturbed sensation, dysphasia)

Q 13. If you have any of the symptoms mentioned above; does it come before or together with the headache? (before, together with headache)

Q 14. If you have any of the symptoms mentioned above; how long do they last? (< 5 minutes, 5-15 minutes, 15-30 minutes, 30-45 minutes, 45-60 minutes, > 60 minutes, varying)

Q 15. Is the headache triggered by one, or several of the following? (stress/anxiety, cheese, chocolate, beer, red wine, spirits, change in weather, smell/aromas, relaxation after stress)

Q 16. What kind of medication do you use to treat your headache? (no medication, acetaminophen, ASA, NSAID, triptans)

Q 17. How often do you have this headache? (< once a year, 2-3 times/year, 5-6 times/year, 1 time/month, 2-3 times/month, 1 time/week, 2-3 times/week, daily)

Q 18. Do you still have recurrent headaches? (Yes/No)

Q 19. If it has ceased; how old were you when it stopped?

Q 20. Have you had a physician diagnosis of migraine? (Yes/No)

Q 21. Have you had a physician diagnosis of tension type headache? (Yes/No)

8.3 MIGRAINE QUESTIONS USED IN STUDY IV

8.3.1 SALT

Q 1. Have you had recurrent headaches not caused by fever, infection or hangover? (Yes/No)
Q 2. Have you had recurrent headache lasting from 4 hours to 3 days, unless you treat them with pain medication? (Yes/No)
Q 3. Is the pain severity moderate or severe? (Yes/No)
Q4. Is the headache often unilateral? (Yes/No)
Q5. Is the headache side-changing between or under an attack? (Yes/No)
Q 6. Is the headache pounding or pulsatile? (Yes/No)
Q 7. Is the pain progressing with physical activity? (Yes/No)
Q 8. Do you feel nauseas or vomit during a headache attack? (Yes/No)
Q 9. Do you get sensitive for light and/or sound during an attack? (Yes/No)
Q 10. How old were you when this headache started? (X years)
Q 11. Do you get visual disturbances before the start of the headache (scintillating scotomas, zigzag-lines)? (Yes/No)
Q 12. Does the visual disturbances last between 5-60 minutes? (Yes/No)

8.3.2 STAGE

Q 1. Have you had recurrent headaches not caused by fever, infection or hangover? (Yes/No)
Q 2. Have you had recurrent headaches once a month or more? (Yes/No)
Q 3. Have you, on at least two occasions, had transient and short-lasting visual disturbance (scintillations, zigzag-lines and/or scotoma), which is followed by headache? (Yes/No)
Q 4. Can you sense when a headache is coming? (Yes/No)
Q 5. Is the headache severe or very severe? (Yes/No)
Q 6. Is the headache unilateral? (Yes/No)
Q 7. Is the headache worsened by physical activity? (Yes/No)
Q 8. Is the headache pulsating? (Yes/No)
Q9. Do you often get nauseas when you’re having headaches? (Yes/No)
Q 10. Do you often vomit when you’re having headaches? (Yes/No)
Q 11. Do you get sensitive for light when you’re having headaches? (Yes/No)
Q 12. Do you get sensitive for sounds when you’re having headaches? (Yes/No)
Q 13. Does the headache disrupt your daily activities? (Yes/No)
Q 14. Do regular pain killers take the headache away? (Yes/No)
Q 15. Have you ever consulted a physician because of headache? (Yes/No)

8.4 GENETIC POLYMORPHISMS ANALYZED IN STUDY I

Chr.	Gene Name	Polymorphism
C01	5,10-methylenetetrahydrofolate reductase (MTHFR)	ala222val (677 C/T)
C01	Natriuretic peptide precursor A (NPPA)	2238 T/C
C01	Natriuretic peptide precursor A (NPPA)	664 G/A
C01	Coagulation factor V (F5)	arg506gln (G/A)
C01	Selectin E (SELE)	leu554phe (C/T)
C01	Selectin E† (SELE)	ser128arg (A/C)
C01	Renin (REN)	C2646T
C01	Renin (REN)	G (-1111) A
C01	Angiotensin I (AGT)	met235thr (T/C)
C01	Angiotensin I (AGT)	A (-6) G
C01	Angiotensin I (AGT)	A (-20) C
C02	Apolipoprotein B (APOB)	E4154K (G/A)
C02	Apolipoprotein B (APOB)	P2712L (C/T)
C02	Apolipoprotein B† (APOB)	thr71ile (C/T)
C03	Peroxisome proliferator-activated receptor-gamma (PPARG)	pro12ala (C/G)
C03	Angiotensin II receptor, type 1 (AGTR1)	G (-535) A
C03	Angiotensin II receptor, type 1 (AGTR1)	T (-153) C
C03	Angiotensin II receptor, type 1 (AGTR1)	1166 A/C
C04	Adducin 1; alpha adducin (*ADD1)*	gly460trp (G/T)
C04	G protein-coupled receptor kinase GRK4 (*GRK4 (GPRK2L))*	R65L (G/T)
C04	G protein-coupled receptor kinase GRK4 (*GRK4 (GPRK2L))*	A142V (C/T)
C04	G protein-coupled receptor kinase GRK4 (*GRK4 (GPRK2L))*	A486V (C/T)
C04	Fibrinogen, beta polypeptide chain (FGB)	-455 G/A
C05	Integrin, alpha-2 (ITGA2)	thr246thr (873 G/A)
C05	Phosphodiesterase 4D (PDE4D)	SNP222 (A/G)
C05	Phosphodiesterase 4D (PDE4D)	SNP220 (C/A)
C05	Phosphodiesterase 4D (PDE4D)	SNP219 (C/T)
C05	Phosphodiesterase 4D (PDE4D)	SNP199 (A/G)
C05	Phosphodiesterase 4D† (PDE4D)	SNP175 (C/T)
C05	Phosphodiesterase 4D (PDE4D)	SNP148 (A/G)
C05	Beta-2-adrenergic receptor (ADRB2)	arg16gly (A/G)
C05	Beta-2-adrenergic receptor (ADRB2)	gln27glu (C/G)
C05	Coagulation factor XII†* (F12)	C (-46) T
C06	Coagulation factor XIII, A1 polypeptide. (F13A1)	P564L (C/T)
C06	Coagulation factor XIII, A1 polypeptide (F13A1)	V35L (G/T)
C06	Lymphotoxin alpha (LTA)	thr26asn (C/A)
C06	Tumor necrosis factor (TNF)	-376 G/A
C06	Tumor necrosis factor (TNF)	-308 G/A
C06	Tumor necrosis factor (TNF)	-238 G/A

C06	Lipoprotein, Lp (a) (LPA)	121 G/A
C06	Lipoprotein, Lp(a) (LPA)	93 C/T
C07	Paraoxonase 1 (PON1)	gln192arg (A/G)
C07	Paraoxonase 1 (PON 1)	met55leu (A/T)
C07	Paraoxonase 1 (PON1)	C (-108) T
C07	Paraoxonase 2* (PON 2)	ser311cys (C/G)
C07	Plasminogen activator inhibitor 1	-675 G5/G4
C07	Plasminogen activator inhibitor 1(serpine1(PAI1)	11053 G/T
C07	Nitric oxide synthase 3 (endothelial)(NOS3)	-922 A/G
C07	Nitric oxide synthase 3 (endothelial)(NOS3)	-690 C/T
C07	Nitric oxide synthase 3 (endothelial)(NOS3)	glu298asp (G/T)
C08	Beta-3-adrenergic receptor(ADRB3)	trp64arg (T/C)
C08	Lipoprotein lipase(LPL)	-93 T/G
C08	Lipoprotein lipase (LPL)	asp9asn (G/A)
C08	Lipoprotein lipase (LPL)	asn291ser (A/G)
C08	Lipoprotein lipase (LPL)	ser447term (C/G)
C11	Coagulation factor II or prothrombin (F2)	20210 G/A
C11	Matrix metalloproteinase 7 (MMP7)	C (-153) T
C11	Matrix metalloproteinase 7 (MMP7)	A (-181) G
C11	Matrix metalloproteinase 1 (MMP1)	1G (-1607)2G
C11	Matrix metalloproteinase 3 (MMP3)	K45E (A/G)
C11	Matrix metalloproteinase 3 (MMP3)	-1171 A5/A6
C11	matrix metalloproteinase 12 (MMP12)	N122S (A/G)
C11	matrix metalloproteinase 12 (MMP12)	A (-82) G
C11	Apolipoprotein A-IV (APOA4)	gln360his (G/T)
C11	Apolipoprotein A-IV (APOA4)	thr347ser (A/T)
C11	Apolipoprotein C-III (APOC3)	-641 C/A
C11	Apolipoprotein C-III (APOC3)	-482 C/T
C11	Apolipoprotein C-III (APOC3)	-455 T/C
C11	Apolipoprotein C-III (APOC3)	1100 C/T
C11	Apolipoprotein C-III (APOC3)	3175 C/G
C11	Apolipoprotein C-III (APOC3)	3206 T/G
C12	Sodium channel, nonvoltage-gated 1 alpha (SCNN1A)	ala663thr (G/A)
C12	Sodium channel, nonvoltage-gated 1 alpha (SCNN1A)	trp493arg (T/C)
C12	Guanine nucleotide-binding protein, beta-3† (GNB 3)	825 C/T
C13	Coagulation factor VII (F7)	G (-402) A
C13	Coagulation factor VII (F7)	G (-401) T
C13	Coagulation factor VII (F7)	-323 10-bp del/ins
C13	Coagulation factor VII (F7)	arg353gln (G/A)
C15	Lipase, hepatic (LIPC)	-480 C/T
C16	Matrix metalloproteinase 2 (MMP2)	C (-1306) T
C16	Cholesteryl ester transfer protein (CETP)	-631 C/A
C16	Cholesteryl ester transfer protein (CETP)	-629 C/A
C16	Cholesteryl ester transfer protein (CETP)	intron 1 Tami +/- (G/A)
C16	Cholesteryl ester transfer protein* (CETP)	ile405val (A/G)
C16	Cholesteryl ester transfer protein (CETP)	asp442gly (A/G)
C17	Glycoprotein Ib (Platelet), alpha polypeptide† (GP1BA)	G (-1691) T

C17	Glycoprotein Ib (Platelet), alpha polypeptide (GP1BA)	T (-5) C
C17	Integrin, beta-3 (ITGB3)	leu33pro (T/C)
C17	Angiotensin I converting enzyme (ACE DCP1))	A (-262) T
C17	Angiotensin I-converting enzyme (ACE DCP1))	intron 16 ins/del
C17	Angiotensin I converting enzyme (ACE DCP1))	T776T (A/G)
C17	Angiotensin I converting enzyme (ACE DCP1))	A20320G
C19	Intercellular adhesion molecule 1 (ICAM 1)	gly214arg (G/A)
C19	Low density lipoprotein receptor (LDLR)	NcoI+/- (A/G)
C19	Apolipoprotein E (APO E)	cys112arg (T/C)
C19	Apolipoprotein E (APO E)	arg158cys (C/T)
C20	Matrix metalloproteinase 9 (MMP9)	R279Q (G/A)
C20	Matrix metalloproteinase 9 (MMP9)	R668Q (G/A)
C20	Matrix metalloproteinase 9 (MMP9)	C (+6) T
C21	Cystathionine beta-synthase (CBS)	ile278thr (T/C)

Markers in Hardy-Weinberg disequilibrium (HWD); *controls, †affected.
HWD p values were < 0.05.

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