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Genetic, Neuropsychological and Neuroradiological
Determinants of Survival After Ischemic Stroke



ACADEMIC DISSERTATION

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UNIVERSITY OF TAMPERE

ACADEMIC DISSERTATION

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Martin Luther King Jr.: "We must accept finite disappointment but never lose infinite hope."

To Johanna and Elli

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LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original publications, which are referred to in the text by the roman numerals.

I Oksala NKJ, Heikkinen M, Mikkelsen J, Pohjasvaara T, Kaste M, Erkinjuntti T, Karhunen PJ (2007) Smoking and the platelet fibrinogen receptor glycoprotein IIb/IIIa PIA1/A2 polymorphism interact in the risk of lacunar stroke and midterm survival. *Stroke* 38:50-55.

II Oksala NKJ, Oksala A, Erkinjuntti T, Pohjasvaara T, Kunnas T, Vataja R, Kaste M, Karhunen PJ (2008) Long-term survival after ischemic stroke in postmenopausal women is affected by an interaction between smoking and genetic variation in nitric oxide synthases. *Cerebrovasc Dis* 26;250-258.

III Oksala NKJ, Oksala A, Pohjasvaara T, Vataja R, Kaste M, Karhunen PJ, Erkinjuntti T (2009) Age-related white matter changes predict stroke death in long-term follow up. *J Neurol Neurosurg Psych* Feb 23 [Epub ahead of print]

IV Oksala NKJ, Jokinen H, Melkas S, Oksala A, Pohjasvaara T, Hietanen M, Vataja R, Kaste M, Karhunen PJ, Erkinjuntti T. Cognitive impairment predicts poststroke death in long-term follow up. Submitted.

ABBREVIATIONS

A	Adenine
ADMA	Asymmetric dimethylarginine
ACE	Angiotensin converting enzyme
ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
AF	Atrial fibrillation
Ala	Alanine
ApoE	Apolipoprotein E
ARWMC	Age related white matter change
C	Cytosine
CF	Cardiac failure
CIND	Cognitive impairment no dementia
CT	Computed tomography
cNOS	Constitutive nitric oxide synthase
DNA	Deoxyribonucleic acid
DWI	Diffusion weighted imaging
eNOS	Endothelial nitric oxide synthase
G	Guanine
GpIIb/IIIa PLA2	Glycoprotein IIb/IIIa PLA2 polymorphism
HR	Hazard ratio
HDL	High density lipoprotein
ICH	Intracerebral hemorrhage
iNOS	Inducible nitric oxide synthase
LADIS	Leukoaraiosis and disability study
LAI	Lacunar infarct
LVI	Large vessel infarct
LDL	Low density lipoprotein
MI	Myocardial infarction
MCAO	Middle cerebral artery occlusion
MMSE	Mini mental status examination
MRI	Magnetic resonance imaging
MTHFR	Methylene tetrahydrofolate reductase

NO	Nitric oxide
OR	Odds ratio
PAD	Peripheral arterial disease
PCR	Polymerase chain reaction
PD	Proton density
PWI	Perfusion weighted imaging
RNA	Ribonucleic acid
SAM	Stroke aging memory cohort
SD	Standard deviation
SNP	Single nucleotide polymorphism
SAV	Subarachnoidal hemorrhage
T	Thymidine
Thr	Threonine
TNF	Tumor necrosis factor
WM	White matter
WML	White matter lesion
WMH	White matter hyperintensity

ABSTRACT

Ischemic stroke is a significant cause of disability and premature death in Finland. Development of methods to predict survival after stroke is needed to focus limited resources on patients at high risk. Considerable body of evidence supports the hypothesis that platelet fibrinogen receptor (glycoprotein IIb/IIIa $PI^{A1/A2}$), cerebral injury associated inducible (iNOS R5/4) and vasoregulatory endothelial nitric oxide synthase (eNOS 4a/b) genetic polymorphisms modulate ischemic stroke subtype. It is also evident, that there may be a gene-environment interaction between these genetic polymorphisms and history of smoking. In addition, it was hypothesized that poststroke cognitive decline associated with white matter lesions might predict poststroke survival. The association of genetic, neuroradiological and detailed neuropsychological factors on stroke phenotype and long-term survival after ischemic stroke utilizing Stroke Aging Memory cohort comprising 486 consecutive stroke patients (55-85 years old) followed up to 12 years after acute stroke was studied. GpIIb/IIIa $PI^{A1/A2}$ polymorphism showed no association with stroke subtypes or mid-term survival while we found a smoking-by-genotype association with the risk of lacunar infarcts, especially in younger (55-69 years) stroke patients, and mid-term survival. Variation in both iNOS and eNOS genes impacted upon poor long term survival. There was an interaction between female sex, smoking and iNOS R5 or eNOS 4b allele with long term survival. Of the patients 28% had mild, 18% had moderate and 54% had severe age related white matter changes (ARWMC). Severe ARWMC predicted poor overall survival and death by brain related causes. Cognitive impairment already at less severe stages without dementia was related to poor survival. Deficits in executive functions and visuospatial and constructional abilities, in particular, predicted poor outcome independently of global cognitive decline and severity of stroke. The present results indicate that smoking and genetic factors modify stroke subtype and survival and that severe ARWMCs predict poor poststroke survival in addition to deficits in several cognitive domains. Therefore, to predict poststroke survival and to identify patients at risk, one has to consider a multidisciplinary approach utilizing clinical, genetic, radiological and neuropsychological modalities.

Keywords: cognition, genetics, eNOS, GpIIb/IIIa, iNOS, stroke, survival

TIIVISTELMÄ

Aivoverenkierron estymisestä johtuva iskeeminen aivoinfarkti on merkittävä työ- ja toimintakyvyttömyyden sekä kuolleisuuden aiheuttaja Suomessa. Resurssien kohdentamiseksi on tärkeää kyetä tunnistamaan korkean riskin potilaat. On viitteitä siitä, että verihitaleiden fibrinogeenireseptorin (glycoproteiini IIb/IIIa $PI^{A1/A2}$), iskeemiseen aivovaurioon liittyvän ns. indusoituvan (iNOS R5/4) ja verisuonten säätelyyn liittyvän endoteliaalisen typpioksidisyntaasin (eNOS 4a/b) geenipolymorfiat vaikuttavat iskeemisen aivohalvauksen alatyyppeihin. On myös ilmeistä, että yksilön tupakointihistoria voi muokata näiden polymorfoiden vaikutusta. Iskeemisen aivoinfarktin jälkeen ilmenevä kognition huonontuminen liittyy valkean aineen vaurioihin. Hypoteesina oli myös, että kognitiiviset häiriöt ja valkean aineen vauriot liittyvät lisääntyneeseen aivoinfarktin jälkeiseen kuolleisuuteen. Tässä väitöstutkimuksessa selvitettiin geneettisten, neuroradiologisten ja neuropsykologisten tekijöiden vaikutusta iskeemisen aivohalvauksen tyyppiin ja pitkän aikavälin selviytymiseen käyttäen Stroke Aging Memory-kohorttia, joka muodostettiin 486 peräkkäisestä iskeemisen aivoinfarktin vuoksi sairaalahoitoon joutuneesta potilaasta (ikä 55-85 vuotta) ja joita seurattiin 12 vuotta. GpIIb/IIIa $PI^{A1/A2}$ polymorfia ei ollut yksinään yhteydessä iskeemisen aivohalvauksen tyyppiin eikä kuolleisuuteen. GpIIb/IIIa PI^{A2} lisäsi yhdessä tupakoinnin kanssa riskiä ns. lakunainfarkteihin ja kuolemaan 15 kk seurannassa erityisesti nuorehkoilla (55-69 vuotta) potilailla. iNOS- ja eNOS geenien polymorfiat olivat yhteydessä potilaiden kuolleisuuteen pitkällä aikavälillä. Naissukupuoli, tupakointi ja iNOS R5 sekä eNOS 4b olivat yhteisvaikutuksessa pitkän aikavälin kuolleisuuteen. Potilaista 28%:lla oli lieviä, 18%:lla oli keskivaikeita ja 54%:lla oli vaikeita ikään liittyviä valkean aineen muutoksia (ARWMC). Vaikeat ARWMC-muutokset ennustivat pitkän aikavälin kuolleisuutta nimenomaan aivoperäisestä syystä johtuen. Havaitsimme, että jo lievä kognitiivisten toimintojen häiriö ilman dementiaa ja erityisesti toiminnanohjauksen ja visuospatiaalisten toimintojen häiriöt ennustivat itsenäisesti kuolleisuutta. Tutkimuksen perusteella tupakointitottumukset ja geneettiset tekijät vaikuttavat aivoinfarktin tyyppiin ja kuolleisuuteen. Vaikeat ARWMC-muutokset ennustavat lisääntyntä kuolleisuutta kognitiivisten häiriöiden ohella. Kuolleisuuden ennustamiseksi ja riskipotilaiden tarkemmaksi tunnistamiseksi tarvitaankin useita erilaisia näkökulmia.

Avainsanat: eNOS, genetiikka, GpIIb/IIIa, iNOS, kognitio, selviytyminen

1. INTRODUCTION

Disturbances in cerebral blood flow due to arterial thrombosis, due to local arterial plaque or other lesion or thromboembolism originating from extracranial sources may result in cerebral injury, i.e. ischemic stroke which has several emotional, physical and health economical consequences. It remains a significant cause of disability and hospitalization in Finland affecting up to 16000 people every year (Kansanterveyslaitos 2006). The higher the age of the population, the higher is the incidence of stroke. Therefore, as the population is aging the burden of stroke is expected to increase markedly in near future.

The incidence of stroke is slightly lower in women (Sudlow and Warlow 1997). However, over the entire lifetime, women are twice as likely to die of stroke as men and therefore the overall burden of stroke is heavier in women (Bonita 1992). In Finland, for example, 62.6% of stroke mortality in 2005 occurred in women aged 65 years or older (Tilastokeskus 2005). During menopause, the incidence of ischemic stroke increases rapidly (Eaker et al. 1993) and postmenopausal women suffer more severe and fatal strokes than men (Thom et al. 2006), indicating the presence of possible unknown gender-specific factors. Classical risk factors for ischemic stroke include smoking, heart failure, atrial fibrillation (AF), advanced age, dyslipidemia, hypertension, and diabetes (Salonen et al. 1982; Breteler et al. 1994; Berger et al. 1998; Ohira et al. 2006; Kubo et al. 2008). In addition to these, genetic factors are presumed to play an important role, although their effect may be confounded by several gene-environment interactions and further modified by gender-related factors.

In addition to the classical large vessel disease, i.e. "middle cerebral artery stroke" resulting in severe clinically obvious sensorimotor manifestations, hidden cerebral small vessel disease characterized by small lacunar infarcts and white matter lesions alters the integrity of the neural networks resulting in "network stroke" with mild clinical manifestations such as cognitive deficits and tendency to fall. It can also be clinically silent and detected only by sophisticated magnetic resonance imaging techniques (Vermeer et al. 2003a). The problem is that it may be regarded as a benign entity by the clinician.

The most difficult question a clinician could encounter is "what is my prognosis?". The factors affecting ischemic stroke patient survival and stroke subtype involve classical

clinical and laboratory factors. The knowledge about genetic, neuroradiological and neuropsychological factors on the on the other hand is lacking. To be able to identify patients with increased risk of post-stroke death, novel genetic, neuroradiological and neuropsychological factors are needed to predict patient survival.

2. REVIEW OF THE LITERATURE

2.1 Pathophysiology of ischemic stroke

Ischemic stroke is a condition resulting from disturbances in cerebral circulation due to temporal or permanent occlusion of cerebral arteries. Up to 80% of all strokes are ischemic whereas the rest are hemorrhagic in nature. Up to one third of ischemic strokes are due to an extracranial source, i.e. due to a cardioembolic mechanism or embolization from stenosed carotid or vertebral arteries. The most common mechanism of extracranial carotid stenosis is due to atherosclerosis in up to 90% of cases (Cronenwett et al. 2005). Thrombotic occlusion of cerebral arteries is a result of platelet activation, their adherence to exposed vessel structures, release of proaggregatory mediators, platelet aggregation and binding to fibrinogen and accumulation of red blood cells resulting in arterial thrombus and possible microembolization to distal vasculature (Cronenwett et al. 2005) (Figure 1).

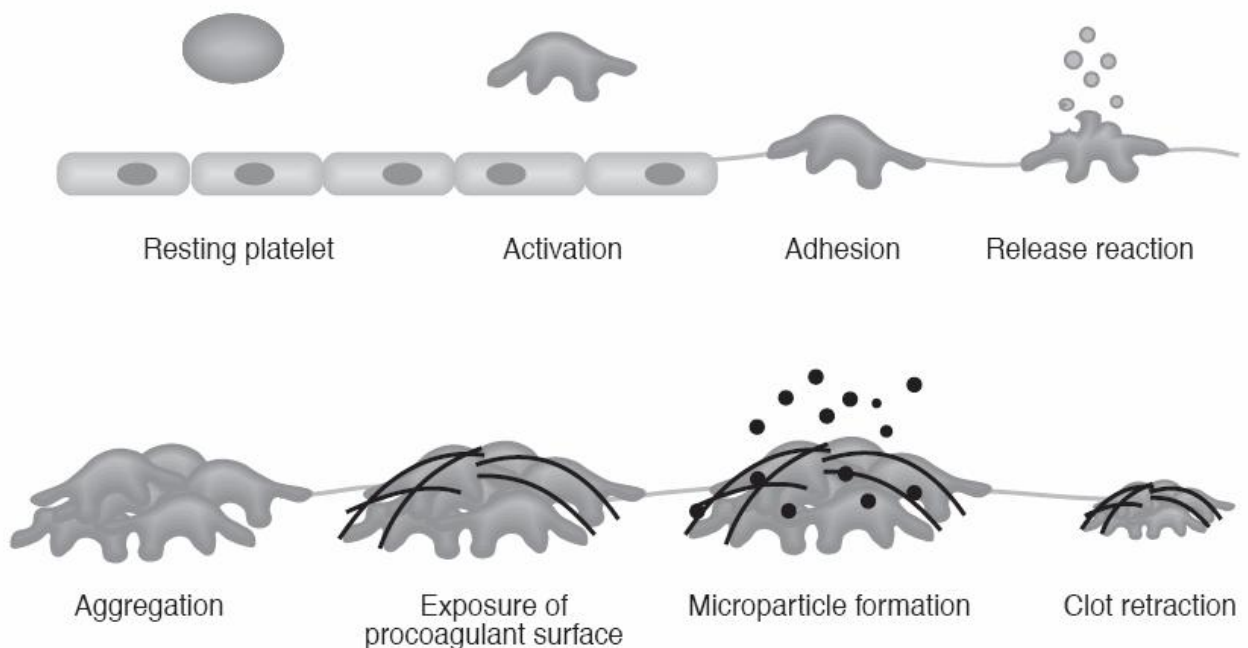


Figure 1. Schematic presentation of platelet activation, adhesion, release, aggregation and formation of thrombus (Lepäntalo 2007).

The underlying cause is rupture of an atherosclerotic plaque, endothelial injury or exposure of subendothelial collagen structures (Cronenwett et al. 2005).

In contrast to large vessel disease, intracranial cerebral small vessel disease has been shown to involve both atherosclerotic and non-atherosclerotic features depending on the location of the process, i.e. within the perforating large arteries or in the small arterioles. Approximately 11-27% of stroke patients present with lacunar infarctions (Mohr 1982; Gan et al. 1997; Marti-Vilalta and Arboix 1999) which are defined as smaller than 15 mm in size and located in the distribution territory of a penetrating cerebral arteriole. The arterioles are usually 100-400 μm in diameter and originate from a large cerebral artery without any collaterals or terminal anastomosis (Arboix and Marti-Vilalta 2009). In larger penetrating arterioles (i.e. 100-200 μm in size) atheromatous alteration of the vessel is the most common pathologic finding (Fisher 1979). Lipohyalinosis affects the smaller arterioles (i.e. <200 μm in size) and is considered an intermediate between microatheromatous alteration and fibrinoid necrosis (Fisher 1979; Mohr 1983) (Lammie et al. 1997). Embolic occlusion due to cardiac or arterial origin is a rare cause of lacunar infarctions (Fisher 1979). Nowadays lacunar infarction is considered as a focal manifestation of a diffuse and progressive vascular disease of the small-sized cerebral arterioles, i.e. small vessel disease (Wardlaw 2005). Although lacunar stroke has a low early mortality rate, in the long-term the risk of death increases from 27.4% at 5 years to 60% and 75% at 10 and 14 years (Arboix and Marti-Vilalta 2009).

Subsequent cerebral ischemia results in irreversible neuronal cell injury and death, apoptosis or reversible injury. Neuronal injury usually manifests as cognitive, sensory, motor and autonomic nervous dysfunction. In some cases however, it may be clinically "silent" and only detected by experienced physicians and imaging methods.

A dose-response relationship between smoking and the risk of stroke was discovered in an occupational cohort (Breteler et al. 1994). Smoking is a significant risk factor for thromboembolic stroke in women (Colditz et al. 1988; Bhat et al. 2008). Cigarette smoking has been found to cause a transient increase in serum fibrinogen levels possibly explaining its pro-thrombotic effect (Balleisen et al. 1985; Krobot et al. 1992; Thomas et al. 1996), and it has been demonstrated to constitute a significant risk factor for ischemic stroke in both sexes (Mannami et al. 2004; Ueshima et al. 2004; Qureshi et al. 2005). Furthermore, the risk of recurrent ischemic stroke increases linearly with fibrinogen levels (Rothwell et al. 2004). Cigarette smoke contains large amounts of exogenous nitric oxide and results in the induction of NO synthesis in activated inflammatory cells (Puhakka et al.

2006) paralleled by increased levels of inducible nitric oxide synthase (Orosz et al. 2007). Nitric oxide is involved in both normal brain physiology due to its crucial role in the vasoregulatory responses and ischemic stroke, and it also plays a role in the pathology of neurodegenerative diseases (Luth et al. 2002).

2.2 Factors affecting ischemic stroke subtype

There is considerable evidence that cardiovascular risk factors differ in various stroke subtypes (Schulz and Rothwell 2003). Diabetes and dyslipidemia have been shown to associate with cerebral small vessel disease and lacunar strokes whereas atrial fibrillation associates with nonlacunar infarcts or large vessel infarcts (Lai et al. 2008; Tuttolomondo et al. 2008). In addition to diabetes, current smoking seems to be dominant factor for lacunar rather than for nonlacunar strokes (Ohira et al. 2006). However, diabetes also associates with large vessel disease in males (Kim et al. 2008). Hypertension and diabetes were more prevalent in patients with lacunar stroke (You et al. 1995; Jackson and Sudlow 2005) while atrial fibrillation and carotid stenosis dominated in patients with nonlacunar stroke (Jackson and Sudlow 2005). In contrast, according to another report, atrial fibrillation but not hypertension or diabetes seemed to be more prevalent in nonlacunar stroke than in lacunar stroke (Bejot et al. 2008). It must be emphasized that lacunar infarcts also occur in nonhypertensive patients (Arboix et al. 2003) although an association with hypertension has been demonstrated (Arboix et al. 2004; Khan et al. 2007b). There exists non-traditional risk factors for nonlacunar and cardioembolic stroke such as waist-to-hip ratio, history of coronary artery disease and left ventricular hypertrophy (Ohira et al. 2006). In addition, visceral fat accumulation (Nagura et al. 2004) and chronic kidney disease (Kobayashi et al. 2004) are risk factors of lacunar stroke or small vessel disease. Male gender seems to predominate in patients with lacunar stroke (Chamorro et al. 1991; Staaf et al. 2001).

Interestingly, race seems to be associated with stroke subtype with predisposition to lacunar stroke in non-white people (Ohira et al. 2006; Wolma et al. 2009). Increasing LDL-cholesterol levels associate with lacunar strokes without an effect on cardioembolic strokes (Imamura et al. 2009). Accordingly, patients with lacunar strokes seem to benefit from treatment with statins (Martinez-Sanchez et al. 2009). Despite its controversial role in cardiovascular medicine, homocysteine level seems to be a risk factor for lacunar infarcts and small vessel disease (Shimizu et al. 2002; Li et al. 2003; Hassan et al. 2004b;

Zylberstein et al. 2008). Microalbuminuria (Wada et al. 2007), blood asymmetric dimethylarginine (ADMA) (Khan et al. 2007a), serum glutathione (Shimizu et al. 2004), hyperinsulinemia (Zunker et al. 1996; Kario et al. 2001), blood white cell count (Ohira et al. 2006) and plasma fibrinogen levels (Kario et al. 1996; Marti-Fabregas et al. 2002) have been demonstrated to be associated with lacunar stroke while lipoprotein(a) and von Willebrand factor have been associated with nonlacunar stroke (Ohira et al. 2006).

2.3 Factors affecting ischemic stroke survival

Previously, several factors have been identified to modulate short, mid- and long term survival after stroke. Advanced age is a predictor of poor survival at several time points ranging from seven days to 12 years (Elneihoum et al. 1998; Williams and Jiang 2000; Counsell et al. 2002; Kaarisalo et al. 2005; Saposnik et al. 2008; Wahlgren et al. 2008; Kim et al. 2009; Kissela et al. 2009). Gender is a predictor from seven days to 1 years but there is controversy — in majority male sex seems to be a poor predictor while in some studies female sex is a predictor of poor survival (Niewada et al. 2005; Elkind et al. 2007; Saposnik et al. 2008; Wahlgren et al. 2008; Kim et al. 2009).

History of cardiovascular disease and its risk factors are important predictors of survival. History of hypertension predicts survival from the acute setting all the way to 23 years of follow up (Stead et al. 2005; Weitzman and Goldbourt 2006; Yong et al. 2007; Wahlgren et al. 2008; Jain et al. 2009). History of smoking is a predictive factor from one month to three months (Ovbiagele et al. 2006; Kissela et al. 2009). Myocardial infarction or cardiac disease predict survival at one months to 3 years (Elneihoum et al. 1998; Kaarisalo et al. 2005; Basile et al. 2008; Wahlgren et al. 2008). Atrial fibrillation has been demonstrated to predict poor survival in a time range from in-hospital setting up to 4 years of follow up (Elneihoum et al. 1998; Kaarisalo et al. 2005; Marini et al. 2005; Roquer et al. 2006; Wahlgren et al. 2008). Previous symptomatic atherosclerotic disease is a predictor of in-hospital outcome (Roquer et al. 2007) and peripheral arterial disease determines one year survival (Agnelli et al. 2006). Of other comorbidities, chronic kidney disease is a predictor of survival at one to 10 years (MacWalter et al. 2002; Tsagalis et al. 2009; Yahalom et al. 2009) and diabetes is a predictor from one month to 3 years (Elneihoum et al. 1998; Kaarisalo et al. 2005; Kamalesh et al. 2008; Kissela et al. 2009).

Stroke-related factors such as previous transient ischemic attack is a predictor at one month (Kaarisalo et al. 2005), while stroke severity predicts survival at several time points ranging from one week to 5 years (Elneihoum et al. 1998; Frankel et al. 2000; Hankey 2003; Saposnik et al. 2008; Wahlgren et al. 2008; Kissela et al. 2009). Loss of consciousness during hospital referral and duration of hospital admission are predictors at 6 years (Kim et al. 2009). Off-hour presentation is a predictor of in-hospital mortality (Reeves et al. 2009). The occurrence of infectious complications during 3 month recovery period is a predictor of 3 month survival (Kissela et al. 2009). Stroke type predicts survival at 3 months to 3 years (Elneihoum et al. 1998; Kolominsky-Rabas et al. 2001; Norrving 2003). During admission, pulse pressure and blood pressure profiles determined survival at one month to 3 months (Yong et al. 2005; Grabska et al. 2009).

Other factors include dementia which occurs most commonly three months after stroke (Tatemichi et al. 1994b; Desmond et al. 1998; Desmond et al. 2002) and prestroke dependence (Counsell et al. 2002; Dallas et al. 2008; Wahlgren et al. 2008), socioeconomic status (Li et al. 2008) and undernutrition (Yoo et al. 2008). In addition to seasonal variation (Turin et al. 2009), geographical factors have also been demonstrated to be associated with stroke outcome (Ali et al. 2009).

Of laboratory measurements, these include C-reactive protein (Muir et al. 1999; Shantikumar et al. 2009), leukocyte count (Kannel et al. 1992) and erythrocyte sedimentation rate (Zuliani et al. 2006) possibly indicating the importance of inflammation and in atherosclerosis. The prothrombotic state is a predictor of poor survival as indicated by fibrinogen concentration (Turaj et al. 2006), and degree of platelet activation (Carter et al. 1998; Yip et al. 2005). Blood glucose level at admission (Capes et al. 2001; Saposnik et al. 2008; Wahlgren et al. 2008), and low tri-iodothyronine level (Alevizaki et al. 2007) are also predictors of survival. Kidney function is an important predictor of survival. Uric acid concentration (Chen et al. 2009), microalbuminuria (Turaj et al. 2001), and serum creatinine level (Friedman 1991b) are all predictors of poststroke survival. Furthermore, impaired calculated creatinine clearance, elevated creatinine level, high urea or ratio of urea to creatinine have previously been demonstrated to function as a long-term predictor of mortality in patients hospitalized for acute stroke in a follow-up of 7 years (MacWalter et al. 2002). Estimated glomerular filtration rate has also been demonstrated to be an

independent predictor of stroke mortality at one year (Yahalom et al. 2009) and 10 years (Tsagalis et al. 2009).

2.4 Genetics of ischemic stroke

Genetic polymorphism indicates the presence of more than one genetically distinct and heritable morphs. The frequency of the least common morphs must be higher than the frequency of new mutations which makes pure mutations clearly a distinct entity. Single nucleotide polymorphisms (SNP) present as DNA sequence variations. SNP occurs when a single nucleotide, adenine (A), thymidine (T), cytosine (C) or guanine (G) in the genome differs between members of a species, i.e. there are two different alleles. SNPs are classified according to allele frequency, i.e. those with frequency below one percent are classified as minor alleles.

SNPs may be located within the coding sequences or non-coding regions of genes or in the intergenic regions. Location of a SNP within the coding sequence may not necessarily result in alteration of the protein structure since several codons, i.e. sequence series encode a given amino acid. Therefore, there exists several synonymous or silent SNPs which do not change the polypeptide sequence. Location of SNP outside the protein coding regions on the other hand may modulate transcription factor binding, alternative splicing of the genes or result in alterations in non-coding RNA. These SNPs may therefore modify the expression of genes.

The role of genetic risk factors in ischemic stroke is not clear. In acute stroke, platelet activation is enhanced (Carter et al. 1998; Yip et al. 2005). Previously, carriers of the A allele of the fibrinogen –455G/A polymorphism were predisposed to the development of multiple lacunar infarcts (Martiskainen et al. 2003). This polymorphism has been shown to be functional and be associated with serum fibrinogen levels (Thomas et al. 1996). The available data indicates that smoking, fibrinogen, and genetic factors may interact with platelet aggregation and clot formation, which is crucial in the pathophysiology of atherothrombotic stroke. Thrombotic clot formation requires the binding of fibrinogen and von Willebrand factor to platelet glycoprotein GpIIb/IIIa, a membrane integrin receptor (Newman et al. 1989) (Figure 2). The PI^{A1/A2} polymorphism of the GpIIb/IIIa, i.e. human platelet antigen-1b (HPA-1b) (Corral et al. 1997) is characterized by a single-point mutation in exon 2 of the GpIIIa gene, leading to the substitution of leucine (PI^{A1}) with

proline (PI^{A2}), which in turn results in a change in the three-dimensional configuration of the receptor (Newman et al. 1989).

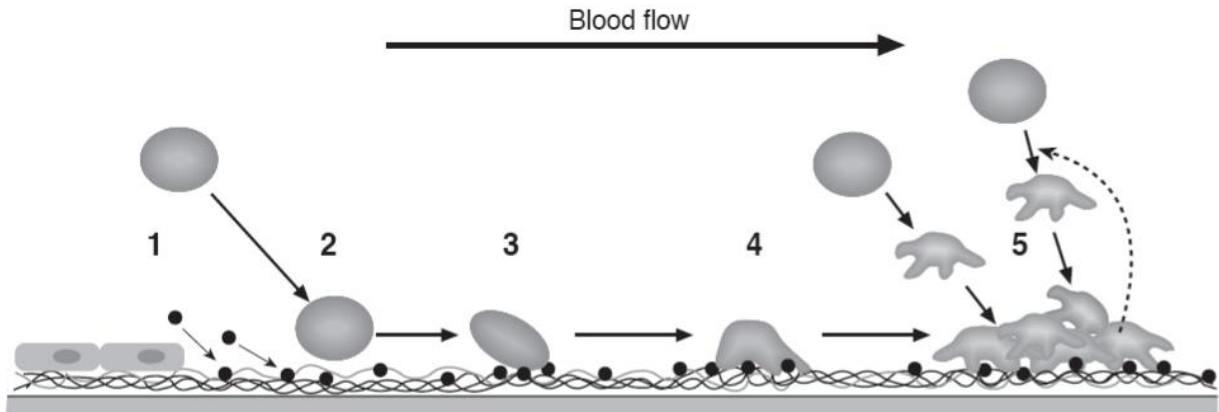


Figure 2. A traditional model of platelet activation and adhesion to immobilized collagen.

1) Binding of vWf molecules onto the collagen surface

2) Platelets moving on the surface – GPIb-V-IX complex and vWf interact

3) Tight binding of platelets on collagen by GP Ia/IIa

4) Collagen-induced activation of platelets by GP VI

5) Formation of a platelet aggregate – mediated by activated GP IIb/IIIa and its ligands vWf and fibrinogen. Activated platelets release prothrombotic agents which further activate other platelets. Reaction steps 3 and 4 occur almost simultaneously and recent studies suggest that both GP Ia/IIa and GP VI participate in adhesion and collagen-induced activation alike. Modified from Jung & Moroi (Jung and Moroi 2000; Lepäntalo 2007).

The presence of 1 or 2 PI^{A2} alleles is associated with increased binding affinity of the receptor to fibrinogen as well as with platelet aggregability in response to epinephrine, adenosinediphosphate (ADP), and collagen *in vitro* (Michelson et al. 2000). The association of PI^{A2} allele with coronary events, acute coronary syndrome (ACS), aspirin resistance and increased risk of restenosis after percutaneous coronary angioplasty in addition to its interaction with smoking on composite cardiovascular outcome (cardiac death, myocardial infarction, or refractory angina requiring revascularisation) has been demonstrated in detail (Weiss et al. 1996; Walter et al. 2001; Lopes et al. 2004; Papp et al. 2005). Contradictory results also exist since it was not associated with ischemic heart disease or cerebrovascular disease or *in vitro* platelet aggregability in Mediterranean subjects which may also be related to environmental factors (Corral et al. 1997). There is information lacking with regards to the association of the PI^{A1/A2} polymorphism with ischemic stroke. It has been shown to associate with atherothrombotic stroke in young patients (Carter et al. 1998) and in young Caucasian women in a rather small and limited study (Wagner et al.

1998). In some studies, no association with ischemic stroke has been detected (Carlsson et al. 1997; Ridker et al. 1997). In patients with stable angina pectoris, however, it has been reported that only smokers with the PI^{A2} allele are at an increased risk of subsequent cardiac events (Lopes et al. 2004). At the present, no data about its effect on ischemic stroke survival exists.

Previously, polymorphisms of the leukotriene C4 synthase (Bevan et al. 2008), tissue plasminogen activator enhancer (Jannes et al. 2004), angiotensin-converting enzyme (Szolnoki et al. 2002) and the methylenetetrahydrofolate reductase (Choi et al. 2003) have been associated with cerebral small vessel disease. On the other hand, male carriers of the tumor necrosis factor alpha gene polymorphism were less susceptible for the development of lacunar stroke (Harcos et al. 2006) and apolipoprotein E and beta-fibrinogen polymorphisms associated with large vessel disease (Kessler et al. 1997). Inflammatory factors may also play an important role as evidenced by association of interleukin-6 (IL-6) SNPs with stroke risk (Cole et al. 2008).

Endothelial i.e. constitutive nitric oxide synthase (eNOS, cNOS) is mainly located in the endothelial cells, and it plays a role in the regulation of normal vascular tone (White et al. 1998), platelet adhesion (Radomski et al. 1987), and aggregation (Radomski et al. 1990). Gender has an effect on eNOS since female sex steroids are important regulators of nitric oxide synthesis by directly increasing its production by endothelial nitric oxide synthase (Wellman et al. 1996). Patients with the 4a variant of the intron 4a/b promoter polymorphism of the eNOS gene have increased NO levels and significantly fewer lacunar infarcts (Hassan et al. 2004a). According to a recent meta-analysis, eNOS 4a/b polymorphism seems to modify the stroke subtype (Rao et al. 2009). A common polymorphism in exon 7 of the eNOS gene (894G/T) (Markus et al. 1998) or another SNP (Glu298Asp) in exon 7 (Guldiken et al. 2008) were not associated with any specific stroke subtype. However, a significant interaction between the eNOS G894T and methylenetetrahydrofolate reductase 677TT genotype and angiotensin-converting enzyme (ACE) (D/D) genotype in relation to risk of ischemic stroke has been demonstrated (Szolnoki et al. 2005). The promoter variants 922A and 786T in the eNOS gene were associated with ischemic stroke; however, this was found in young black women only (Howard et al. 2005). The effect of eNOS genotype on stroke survival is not known.

In contrast to eNOS, NO produced by inducible nitric oxide synthase (iNOS) located in microglia and macrophages at the area of injury plays a role in free radical damage and in worsening the injury after brain ischemia in mice (Loihl et al. 1999) and in rat models (Iadecola et al. 1996) of middle cerebral artery occlusion (MCAO) and also in humans (Nanetti et al. 2007). iNOS immunoreactivity and peroxynitrite formation as a consequence of its action has been demonstrated in the ischemic area (Forster et al. 1999). In addition to its effect in the ischemic area, in reperfusion of the ischemic brain, NO reacts with superoxide anion to form peroxynitrite, causing neuronal death (Oliver et al. 1990). Several agents which are capable of inhibiting iNOS activation result in increased survival after experimental ischemic stroke in rats (Margaill et al. 1997; Parmentier et al. 1999; Kidd et al. 2005) while evidence is lacking in humans. In addition, the infarct size is reduced in mice lacking functional iNOS gene, confirming a critical role for iNOS in ischemic brain injury (Zhao et al. 2000). It is possible that gender modulates the activity of iNOS since in female mice, lower expression of iNOS explains the better tolerance of MCAO than that found in male mice, a phenomenon mediated by female sex steroids (Park et al. 2006).

The iNOS gene is polymorphic at two sites, which may alter the functional activity of the gene (Bellamy and Hill 1997; Burgner et al. 1998; Morris et al. 2002). The bi-allelic AAAT-repeat located 0.75 kb upstream of the gene has been shown to alter the promoter activity (Bellamy and Hill 1997; Burgner et al. 1998; Morris et al. 2002). It has been recently demonstrated that this polymorphic site is associated with the progression of atherosclerosis especially in men aged > 55 years (Kunyas et al. 2003). No data on its effect on ischemic stroke survival exists.

Other SNPs that have been linked to stroke survival are those of thrombomodulin (Olivot et al. 2008), ApoE4 in conjunction with gender (Gromadzka et al. 2007a), interleukin-1 receptor antagonist (Gromadzka et al. 2007b), insulin-like growth factor promoter (van Rijn et al. 2006), alpha-fibrinogen Thr312Ala polymorphism in conjunction with atrial fibrillation (Carter et al. 1999) and angiotensin converting enzyme polymorphism (Catto et al. 1996; Doi et al. 1997).

Taken together, among other SNPs, there is considerable evidence that GpIIb/IIIa P1^{A1/2}, eNOS 4a/b and iNOS R4/5 might modify acute ischemic stroke phenotype and outcome.

2.5 Neuroradiology of ischemic stroke

In acute ischemic stroke, neuronal ischemia inevitably results in disturbances in membrane potential, subsequently to neuronal edema and disruption of the blood-brain barrier resulting in leakage of plasma proteins into the extracellular space. Subsequently, this is followed by diffusion of water in to the brain parenchyma during subsequent reperfusion and sometimes accompanied by transformation to a hemorrhagic infarct.

Usually, two different MRI techniques are used – diffusion-weighted imaging (DWI) and perfusion weighted imaging (PWI). DWI can be used to detect acute cerebral ischemia since it is sensitive to the motion of the water molecule protons. DWI is sensitive and relatively specific in the detection of acute ischemic stroke. PWI makes it possible to obtain information about the perfusion status of the brain and it requires the use of bolus-contrast tracking or utilization of blood oxygen level and arterial spin tagging. This technique makes it possible to estimate the cerebral blood volume and quantitative maps of cerebral blood flow. Combination of DWI and PWI has been demonstrated to be better than conventional MRI in acute stroke and because they provide information on both location and extent of infarction. The types of lesions seen in MRI can be classified as thromboembolic, watershed, lacunar and infarction due to venous thrombosis.

In addition to changes in cerebral grey matter in the acute phase of stroke in MRI, cerebral white matter lesions (WMLs) are found in increased frequency and extent in these patients (Wen and Sachdev 2004). WMLs are known to accumulate during aging, therefore, they are also known as age-related white matter changes (ARWMCs). ARWMCs are regarded as a surrogate marker of cerebral small vessel disease, which is associated with arterial hypertension and other vascular risk factors (Inzitari 2003; Wardlaw 2005; Basile et al. 2006). The entity “small-vessel disease” encompasses the silent lacunar ischemia all the way to the presence of Binswanger’s disease, i.e. ischemic injury of the white matter. Progression of small vessel disease is paralleled by a decline in cognitive function (van Dijk et al. 2008). In general population in persons aged 65-84 years moderate to severe ARWMCs occur in up to one third of cases (Breteler et al. 1994). They are clinically important since ARWMCs associate with functional decline leading to loss of independence mainly due to decline in cognitive and motor functioning (Inzitari et al. 2007). ARWMCs also relate to risk of recurrent stroke (Pantoni and Garcia 1995; Inzitari et al. 1997; Briley et al. 2000; Vermeer et al. 2003a; Fu et al. 2005; Kuller et al. 2007). In

general population of patients aged over 65 years and without evidence of prior stroke or neurological diseases, severe ARWMCs were associated increased risk of death in 2-12 years of follow up (Fu et al. 2005; Kerber et al. 2006; Kuller et al. 2007) which were mostly due to vascular causes (Kerber et al. 2006). ARWMCs also associate with gait disturbances and incident falls which may also explain their association with stroke survival (Blahak et al. 2009; Srikanth et al. 2009).

There is body of evidence supporting the role of white matter changes as a predictor of survival although no data on acute stroke patients exists. There is also need to determine the causes of death in those with ARWMCs.

2.6 Neuropsychology of ischemic stroke

In addition to classical sensorimotor deficits, stroke patients also have cognitive deficits which range from mild to severe and often present several months after stroke (Jokinen et al. 2006). Patients with lacunar infarctions often have minor neuropsychological abnormalities of the executive functions (Grau-Olivares et al. 2007a; Grau-Olivares et al. 2007b). In addition to ischemic stroke, normal aging process also results in mild cognitive decline (Schaie 1994) and does not result in marked deterioration of daily activities. Poststroke cognitive impairment on the other hand is frequent – up to 65 to 78% of patients demonstrate cognitive decline (Tatemichi et al. 1994a; Pohjasvaara et al. 1998a) and 25 to 31% of patients demonstrate poststroke dementia (Tatemichi et al. 1994b; Pohjasvaara et al. 1998b; Barba et al. 2002; Desmond et al. 2002; Henon et al. 2003). Interestingly, cognitive decline is closely associated with the burden of white matter changes as seen in MRI (Pohjasvaara et al. 2000; Vermeer et al. 2003a; Jokinen et al. 2005; Pohjasvaara et al. 2007).

Global measures of cognition e.g. Mini Mental Status Examination (MMSE) (Friedman 1991a; Friedman 1994; House et al. 2001; Patel et al. 2002) and similar short mental status tests (Woo et al. 1992) have been related to a poor survival in poststroke follow-up studies. Similarly poststroke dementia has been related to a poor survival (Tatemichi et al. 1994b; Barba et al. 2002; Desmond et al. 2002; Henon et al. 2003). In addition, deterioration of mental speed has been related to ischemic stroke (Rasquin et al. 2002; Sachdev et al. 2004) but its effect on survival is not known. Whether a mild cognitive impairment without dementia, i.e. cognitive impairment no dementia (CIND) is related to

poststroke survival is poorly known although some convincing data exists (Tuokko et al. 2003).

Poststroke cognitive impairment consists of decline in various cognitive domains such as memory, language, visuospatial and constructional abilities (Bowler et al. 1994; Tatemichi et al. 1994a) and also in executive function (Glosser and Goodglass 1990; Horn et al. 1990; Della Sala et al. 1993). Executive functions are a set of cognitive skills responsible for the planning, initiation, sequencing and monitoring of complex goal-directed behavior (Royall et al. 2002). The primary structures mediating these functions are the prefrontal cortex and its connecting pathways with the subcortical regions (Royall et al. 2002). The memory functions are composed of several different entities but the major structures responsible for it locate in the medial temporal lobe consisting of the hippocampus and the adjacent cortical areas (Squire et al. 2004).

The independent role of impairment in cognitive domains on poststroke long-term survival has not been studied in detail although narrow neuropsychological tests have demonstrated the predictive value of cognitive impairment in long-term survival (Moulin et al. 1997). In contrary, no predictive value was detected in another study with a 3-year follow-up (Johnston et al. 2004). Deficits in cognitive domains like verbal, visuomotor and memory performance and reaction time have previously been associated with shortened survival at general population level (Portin et al. 2001; Shipley et al. 2006) but no such data exists in stroke patients.

There is considerable evidence supporting the hypothesis that cognitive deficits as determined by structured neuropsychological tests could be predictors of poststroke survival.

3. AIMS OF THE PRESENT STUDY

The aim of the present study was to investigate the association of genetic factors, white matter changes and neuropsychological deficits on stroke subtypes and survival. The purposes of the study were:

1. To test the hypothesis that genetic polymorphism (P1^{A1/A2}) of the platelet GpIIb/IIIa fibrinogen receptor and smoking modify ischemic stroke subtypes and survival.
2. To test the hypothesis that genetic polymorphisms of the vasoregulatory endothelial (eNOS 4a/b) and cerebral injury associated inducible (iNOS R4/5) nitric oxide synthases and smoking modify stroke subtypes and survival and explain gender differences.
3. To test the hypothesis that ARWMCs are associated with poor long term survival after ischemic stroke
4. To test the hypothesis that cognitive impairment predicts poor ischemic stroke survival and to characterize the effect of several cognitive domains.

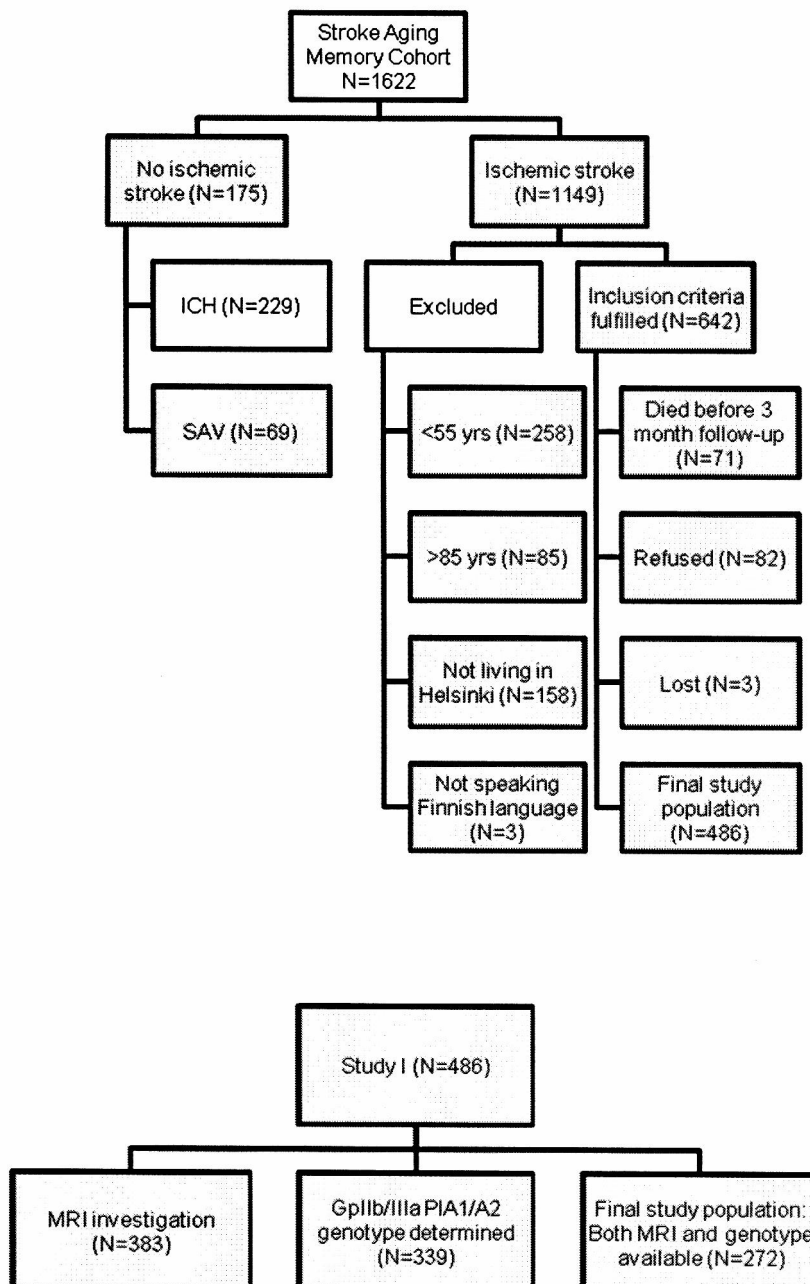
4. MATERIALS AND METHODS

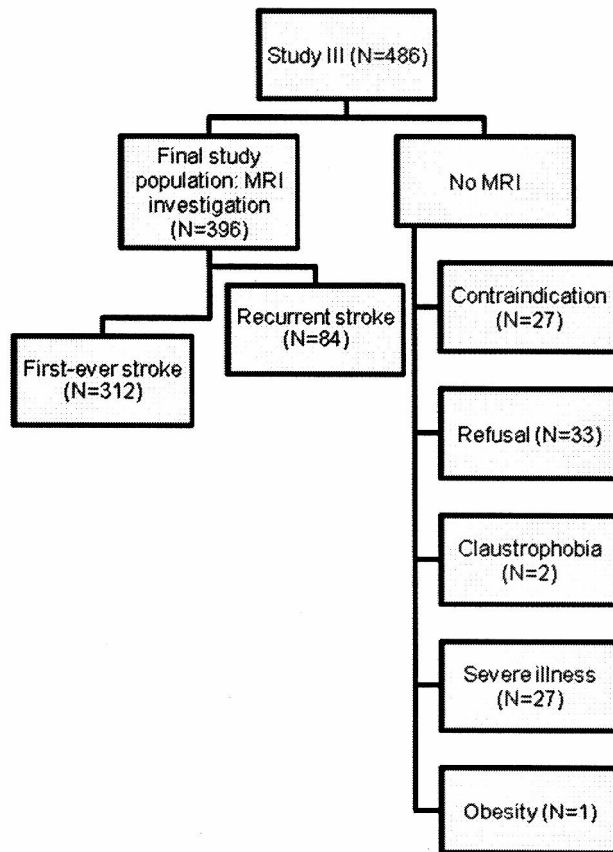
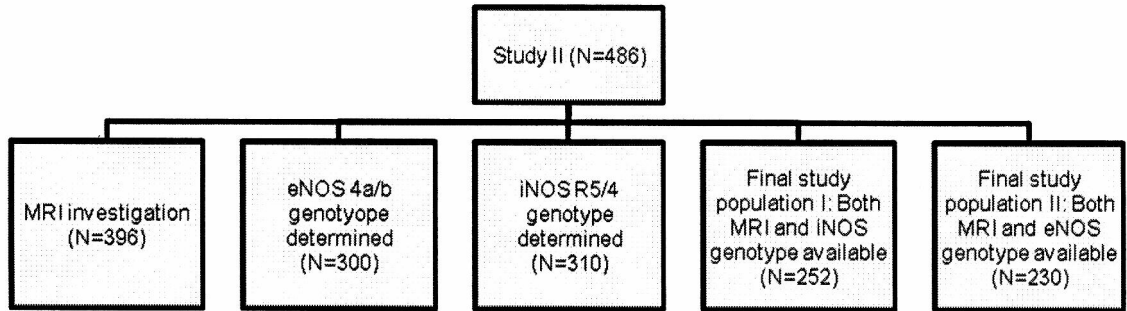
4.1 Stroke Aging Memory cohort

The Helsinki Stroke Aging Memory (SAM) cohort comprised a consecutive series of all Finnish (caucasian) patients with suspected stroke admitted to Helsinki University Central Hospital (n=1622) between 1 December 1993 and 30 March 1995, as described in detail previously (Pohjasvaara et al. 1997) (Figure 3). Patients without ischemic stroke (n=175), presenting with intracerebral (n=229) or subarachnoid hemorrhage (n=69) were excluded. Of the 1149 patients with ischemic stroke, we further excluded those younger than 55 years (n=258) or older than 85 years (n=88), those not living in Helsinki (n=158), and those not speaking the Finnish language (n=3). A total of 642 patients fulfilled the inclusion criteria and were invited to a baseline visit 3 months later. Of these, 71 died (11.1%) before the 3-month follow-up, 82 refused (12.8%) and 3 were lost (0.5%) due to undefined causes. Finally, 486 (85.1%; 246 men, 240 women) of the living patients were included in the final cohort (Mantyla et al. 1999b). Of these 409 (84.2%) participated in neuropsychological examination. Patients with severe aphasia and thus unable to participate neuropsychological examination were excluded together with those with severe sight or hearing disabilities, or reduced level of consciousness. The 85 patients who refused or were not identified were compared with the 486 patients in the cohort: the mean age of the former group was 79.2 ± 7.68 years and that of the final cohort was 71.2 ± 7.6 (p=NS) (Pohjasvaara et al. 1997). The percentage of women was 67.1% and 49.4% (p=0.023), and that of hospitalized subjects at the time of examination was 60.0% and 16.8% (p=0.0001). A detailed medical and neurological history was taken (Pohjasvaara et al. 1997). Hypertension was defined as systolic blood pressure ≥ 160 mmHg, or diastolic blood pressure ≥ 95 mmHg. Atrial fibrillation (AF) was defined by clinical criteria and cardiac arrhythmias other than AF were defined as other arrhythmias. Myocardial infarction (MI) and cardiac failure (CF) were based on clinical diagnosis. Diabetes was defined as previously documented diagnosis, current use of insulin or oral hypoglycemic medication, or fasting blood glucose >7.0 mmol/l. Peripheral atherosclerosis (PAD) was considered if the patient had claudication, >2 peripheral pulses missing, or history of amputation or peripheral arterial surgery due to atherosclerosis. Smoking habits were scored at admission as non-smokers and smokers (current or former). Laboratory analyses included total and high-density lipoprotein cholesterol, triglycerides, and fasting blood glucose. Hypercholesterolemia was defined as total cholesterol >5.0 mmol/L.

Hypertriglyceridemia was defined as serum triglycerides >2.0 mmol/L. Low HDL was defined <1.2 mmol/L. There were 388 patients (79.8%) with first-ever and 98 patients (20.2%) with recurrent stroke. Stroke severity was assessed using modified Rankin score (van Swieten et al. 1988).

The study was approved by the Ethics committee of the Department of Clinical Neurosciences, Helsinki University Central Hospital, Finland. The study was explained to the patients, and informed consent was obtained.





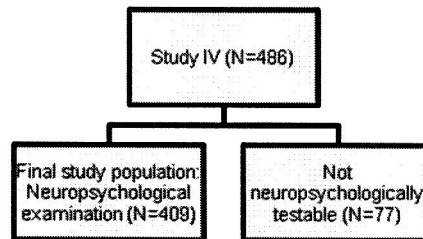


Figure 3. Patient enrollment in Stroke Aging Memory Cohort and studies I-IV.

4.2 Genetic analysis (I-II)

DNA was separated from frozen blood samples according to standard procedures. Polymerase chain reaction (PCR) for DNA amplification was carried out as described previously (Mikkelsen et al. 1999; Mikkelsen et al. 2000; Kunnas et al. 2002; Kunnas et al. 2003). The polymorphism of cytosine/thymidine in exon II of the glycoprotein IIIa gene (PI^{A1/A2}) was detected by PCR and restriction digestion. The primer sequences and PCR protocol have been described in detail previously (Mikkelsen et al. 1999). The eNOS 4a/b genotype was determined in 300 (61.7%) and the iNOS R4/5 genotype in 310 (63.8%) subjects of the original cohort which constituted the final study populations.

4.3 Magnetic resonance imaging (I-III)

Infarct subtypes were determined by MRI and defined as lacunar (LAI) if the infarct was situated in the deep white or gray matter and had a diameter of 3–9 mm. Large vessel infarct (LVI) was defined as an infarct located in the cortico-subcortical layers of cerebral hemispheres in (the territories of) the superficial branches of the anterior, middle, or posterior cerebral artery, with a diameter of \geq 10 mm (Mantyla et al. 1999b).

MRI was performed with a 1.0 T imaging equipment (Siemens Magnetom) (Mantyla et al. 1999b) as detailed previously. The protocol included transaxial T2, proton density (PD)- and T1-weighted 5 mm-thick slices (conventional spin echo-technique) and a three-dimensional gradient-echo-sequence yielding 64 3 mm-thick coronal sections. ARWMCs

were rated on PD-weighted images in accordance to the LADIS (Leukoaraiosis and Disability in the Elderly) ARWMC rating to no to mild, moderate and severe degree. The rating atlas has been detailed previously (Pantoni et al. 2005). In no to mild degree of ARWMC periventricular lesions included no more than small cap or thin lining and in other WM (white matter) areas no more than large focal lesions. In moderate degree of ARWMC the periventricular lesions included no more than large cap and smooth halo, and the other WM areas no more than focal confluent lesions. The severe degree of ARWMC included cases with extending caps or irregular halo in the periventricular area and diffusely confluent lesions or extensive WM change in other WM areas. The intra- and interobserver reliabilities for rating basic WMLs in periventricular and other WM areas were tested previously and were found to be good (interobserver agreement, weighted $\kappa=0.90$ to 0.95 ; interobserver agreement, weighted $\kappa=0.82$ to 0.84) (Mantyla et al. 1997; Mantyla et al. 1999a; Mantyla et al. 1999b). However, we did not re-test intra- and interobserver reliabilities using atlas based assessment of the present follow-up study (Pantoni et al. 2005).

4.4 Neuropsychological assessment (IV)

A comprehensive neuropsychological assessment was administered for 409 patients three months after the index stroke as detailed before (Pohjasvaara et al. 1998c; Jokinen et al. 2004; Jokinen et al. 2006). There were no statistically significant differences in gender, age, education, MMSE or modified Rankin score between these and the original 486 subjects.

In brief, global cognitive function was measured with the Mini-Mental State Examination (Weiner et al. 2008). Score ≤ 25 indicated cognitive impairment. Executive functions including attention were evaluated with the Trail making tests A and B (Reitan 1958), the Stroop color naming test (MacLeod 1991) and the Digit span subtest of the Wechsler Memory Scale (Wechsler 1945), the modified Wisconsin Card Sorting Test and the verbal fluency test with both number of animal names number of words beginning with K (Lezak et al. 2004). Data was available in 365 and missing in 44 subjects (10.8%). The assessment of memory functions included the Logical memory and Visual reproduction subtests of the Wechsler Memory Scale-revised (Wechsler 1987) as well as the Fuld Object Memory Evaluation (Fuld 1982). Data was available in 379 and was missing in 30

subjects (7.3%). Language was evaluated with the short version of the Token test (De Renzi and Faglioni 1978) the Boston naming test (Borod et al. 1980) and the verbal fluency test (Lezak et al. 2004). Moreover, overall evaluation of speech functions was based on the Boston Diagnostic Aphasia Examination (Laine et al. 1993). Data was available in 395 and missing in 14 subjects (3.4%). Visuospatial and constructional abilities were assessed with the Block design subtest of the Wechsler Adult Intelligence Scale-revised (Wechsler 1981), the clock test (recognizing and setting time), and by copying a triangle, a flag, a 3-dimensional cube, and a Greek cross (Christensen 1975). Data was available in 391 and missing in 18 subjects (4.4%). Abnormality (impaired vs. not impaired) in each domain was judged with the use of normative data from a random healthy Finnish population (2 SD or, if more than one test was used, 1 SD below the level of the norm on several tests indicated abnormality) (Ylikoski et al. 1993). The normal values were evaluated in different age groups.

Cognitive impairment without dementia, i.e. cognitive impairment no dementia (CIND) (Tuokko et al. 2003) was defined as cognitive impairment in any of the above mentioned domains after the exclusion of patients with dementia. Of the patients with MMSE \leq 25 (n=138), 94/138 (68.1%) had dementia at the 3 month follow-up. Further, of these 62/94 (66.0%) had had a prestroke cognitive decline. Assessment of prestroke cognitive decline involved interviews with the patient and a knowledgeable informant, using structured questions. Dementia was defined according to Diagnostic and Statistical Manual of Mental Disorders 3rd edition criteria. From the basic cohort of 486 patients, we first excluded those who had not been tested for dementia (n=35, 7.2%) and those who had dementia (n=115, 23.7%). Further we investigated whether patients had impairment in any of the assessed domains. 52 patients were excluded at this point because of missing values in the assessed domains, at the same time as none of the existing values indicated impairment. Finally, there were 212 patients with CIND and 72 patients with no cognitive impairment.

4.5 Survival and causes of death (I-IV)

Of the patients with MRI and GpIIb/IIIa genotype data 8.8% had died during 15 months follow-up (Study I). Long-term survival data at 21 September 2006 were obtained from Statistics Finland. The causes of death based on ICD-9 and ICD-10 classifications were also obtained and divided further into cardiac, brain related (ischemic stroke, bleeding, vascular dementia), cancer, infection, trauma, and other categories. Of patients with eNOS

genotype data 71.0%, and of the patients with iNOS genotype data 71.9% had died during the follow-up (Study II). Of the patients with MRI data on ARWMCs, 69.9% had died during the follow-up. (Study III). Of the patients with neuropsychological data, 69.2% had died during the follow-up (Study IV). The cause of death could not be determined and these were excluded for 9 cases (Study II), 8 cases (Study III) and for 8 cases (Study IV) with death certificate.

4.6 Statistical analysis (I-IV)

The investigation employed SPSS/WIN (version 12.0, SPSS Inc) software. The associations between single risk factors were analyzed using Pearson's Chi square test (dichotomous variables) and Student's t-test for independent samples (continuous variables). Statistical significance was set at $p < .05$ in all the analyses.

Study I. Logistic regression analyses with sex, age, MI, CF, arrhythmia, AF, hypertonia, PAD, diabetes, total and high-density lipoprotein cholesterol, triglycerides, presence of $PI^{A1/A2}$ polymorphism, or history of smoking as confounders were used to further explore the association of the PI^{A2} allele with lacunar and large-vessel infarcts as well as survival. They were also employed to examine the correlation between genotype and conventional risk factors. Interaction between smoking and genotype was analyzed by creating an interaction term within logistic regression analysis. To further study the possible interaction between smoking and PI^{A2} allele, we created 4 new variables: (1) non-carriers with no history of smoking, (2) PI^{A2} allele carriers with no history of smoking, (3) non-carriers with a history of smoking, and (4) PI^{A2} allele carriers with a history of smoking. Group 1 was used as a reference category. In order to study the age-dependence of the interaction, we stratified the study population to age groups of 55–69 and 70–85 years.

Study II. The effect of classical risk factors sex, MI, CF, arrhythmia, AF, hypertonia, PAD, diabetes, hypercholesterolemia, low HDL, hypertriglyceridemia, and history of smoking on survival were first analyzed by Kaplan-Meier log rank test. In Cox regression survival analysis and binary logistic regression analysis of the infarct subtypes the following covariates were used: sex, age, MI, CF, arrhythmia, AF, hypertonia, PAD, diabetes, absolute total and high-density lipoprotein cholesterol, triglycerides and history of smoking. Results are presented as hazard ratio (HR) with 95% confidence intervals. Interactions between sex, genotype, and smoking were analyzed by creating an interaction term within

the Cox-regression analysis using the same covariates. In stratified analyses the study subjects were categorized as follows: (1) non-smokers with the iNOS R4/4 or the eNOS 4a/b or 4a/a genotype, (2) non-smokers with the iNOS R5/4 or R5/5 or the eNOS 4b/b genotype (3), smokers with the iNOS R4/4 or the eNOS 4a/b or 4a/a, and (4) smokers with the iNOS R5/4 or R5/5 or the eNOS 4b/b genotype using the same covariates. All the analyses were also performed including patients with first-ever stroke only. Since the results were similar in both analyses, all the stroke cases were included in the study.

Study III. The effect of risk factors (sex, age, MI, CF, AF, other arrhythmias, arterial hypertension, PAD, diabetes, hypercholesterolemia, hypertriglyceridemia, low HDL), poor modified Rankin score (3-5 vs. 0-2) and ARWMC degree on survival were first analyzed using Kaplan-Meier log-rank test. The cumulative hazard function was also used and according to these analyses, the proportional hazards assumption was met for each parameter included in further models. When analyzing survival using cardiac death as an endpoint, the proportional hazards assumption was not met due to convergence of survival and hazard curves. Due to low number of cases at the end of follow-up (12 years), log-rank analysis was also performed at 10 years of follow-up. In Cox-regression proportional hazards survival analysis, in the forced entry model (Model 1) the potential predictors were used as covariates (sex, age, MI, CF, AF, other arrhythmias, arterial hypertension, PAD, diabetes, total and high-density lipoprotein cholesterol, triglycerides, history of smoking and poor modified Rankin score). The final model (Model 2) was constructed using likelihood-ratio for selection of significant variables. The probability for variable entry was set at $p < .05$ and for removal at $p > .10$. Since there were no differences in survival analyses between moderate and mild ARWMC categories, these categories were combined in Cox regression analyses. All the analyses were also performed including patients with first-ever stroke only. Since the results were similar in both analyses, all the stroke cases were included in the study.

Study IV. The effect of different neuropsychological domains (executive functions, memory, language, visuospatial/constructional abilities), CIND and copredictors such as global cognitive function i.e. MMSE ($MMSE \leq 25$), education (low education < 6 years), and poor modified Rankin score (3-5 vs. 0-2) on long-term survival were first analyzed using Kaplan-Meier log rank analysis. The median estimated years of survival are presented. The cumulative hazard function was also used and according to these analyses, the

proportional hazards assumption was met for each parameter included in further models. In Cox-regression proportional hazards survival analysis, in the forced entry model (Model 1) only the demographic background variables were used as covariates (age, sex, years of education). Another model (Model 2) was constructed adding MMSE \leq 25 and poor modified Rankin score (3-5 vs. 0-2) as clinical covariates. All the analyses were also performed including patients with first-ever stroke only. Since the results were similar in both analyses, all the stroke cases were included in the study.

5. RESULTS

5.1 Factors affecting ischemic stroke phenotype (I-II)

In univariate analyses, arrhythmia ($p < 0.001$) and AF ($p < 0.001$) associated with LVI while smoking associated with LAI ($p = 0.040$). In logistic regression analysis, smoking remained the only significant independent factor associated with LAI (OR 1.87, $p = 0.033$) (Table 1) and there was a smoking-by-genotype interaction between PI^{A2} polymorphism and history of smoking on risk of LAI (OR 2.10, CI 0.90–4.89, $p = 0.087$).

Table 1. Effect of the Association Between Multiple Risk Factors and Platelet GpIIb/IIIa PIA1/PIA2 Fibrinogen Receptor Polymorphisms on Stroke Phenotype Among 272 Patients With Ischemic Stroke (Stroke Aging Memory Cohort)

Variable	Valid n*	LVI (n=156)					LAI (n=165)				
				OR	95% CI	P			OR	95% CI	P
Mean age, y	272	71.0	7.6 SD	1.01	0.98–1.05	0.521	70.9	7.5 SD	1.02	0.99–1.06	0.204
Female sex	139	74	53.2%	0.73	0.41–1.32	0.303	83	59.7%	0.90	0.49–1.63	0.727
Myocardial infarction	48	33	68.8%	1.51	0.70–3.25	0.296	27	56.3%	1.05	0.51–2.17	0.894
Heart failure	56	34	60.7%	0.98	0.47–2.04	0.951	30	53.6%	0.74	0.36–1.51	0.407
Arrhythmia	59	42	71.2%	1.80	0.55–5.86	0.330	32	54.2%	0.99	0.31–3.14	0.985
AF	43	33	76.7%	1.65	0.41–6.55	0.479	24	55.8%	0.92	0.25–3.37	0.899
Peripheral artery disease	37	23	62.2%	1.05	0.49–2.24	0.905	21	56.8%	0.76	0.36–1.62	0.484
Hypertension	130	70	53.8%	0.83	0.48–1.42	0.493	84	64.6%	1.45	0.84–2.51	0.185
Diabetes	61	34	55.7%	0.84	0.43–1.64	0.599	32	52.5%	0.78	0.40–1.51	0.456
Smoking	145	86	59.3%	1.25	0.71–2.22	0.442	97	66.9%	1.87	1.05–3.31	0.033
Chol, mmol/L	272	5.6	1.3 SD	1.03	0.81–1.31	0.828	5.6	1.2 SD	1.03	0.81–1.31	0.824
HDL chol, mmol/L	272	1.2	0.6 SD	1.04	0.68–1.59	0.860	1.2	0.7 SD	1.11	0.70–1.76	0.660
Triglycerides, mmol/L	272	1.5	0.7 SD	1.01	0.67–1.53	0.950	1.5	0.8 SD	0.95	0.63–1.44	0.822
PI^{A2} allele (A2/A1, A2/A2)	76	43	56.6%	0.95	0.53–1.72	0.869	46	60.5%	1.03	0.57–1.88	0.913

The logistic regression model included sex, age, myocardial infarction, heart failure, arrhythmia, AF, hypertonia, peripheral artery disease, diabetes, total and HDL cholesterol (chol), triglycerides, presence or absence of the PI^{A2} allele, and history of smoking as confounders.

*Eighteen percent of patients had both infarct types.

Smokers carrying the PI^{A2} allele had an increased risk of LAI (OR 2.50, $p = 0.064$) when compared to the non-smokers who were not carriers of the $PIA2$ allele (Table 2). This effect was especially significant in younger stroke patients (55–69 years), among whom the interaction (OR 5.81, $p = 0.024$) was greater than the effect of smoking alone (OR 3.12, $p = 0.039$) (Table 2). Neither iNOS R5/4 nor eNOS 4b/B genotype associated with LVI or LAI and there were no interactions between smoking and either iNOS R5/4 or eNOS 4b/b genotypes on stroke subtypes (data not shown). Furthermore, there were no interactions between NOS genotypes and sex on infarct subtypes (data not shown).

Table 2. Effect of the Interaction Between Smoking and Platelet GpIIb/IIIa PIA1/PIA2 Fibrinogen Receptor Polymorphisms on Stroke Phenotype Among 272 Patients With Ischemic Stroke (Stroke Aging Memory Cohort)

	Valid n	PIA ² −, Smoking−	PIA ² +, Smoking−	OR	95% CI	<i>P</i>	PIA ² −, Smoking+	OR	95% CI	<i>P</i>	PIA ² +, Smoking+	OR	95% CI	<i>P</i>
Whole population	272*	83	43				113				33			
LVI	156	47, 56.6%	23, 53.5%	0.79	0.33–1.87	0.586	66, 58.4%	1.08	0.56–2.11	0.812	20, 60.6%	1.21	0.50–2.95	0.676
LAI	165	45, 54.2%	22, 51.2%	0.67	0.29–1.55	0.344	74, 65.5%	1.50	0.77–2.91	0.230	24, 72.7%	2.50	0.95–6.60	0.064
Age 55–69 years	122†	34	17				53				18			
LVI	67	19, 55.9%	8, 47.1%	0.65	0.15–2.76	0.555	28, 52.8%	0.87	0.31–2.39	0.784	12, 66.7%	1.50	0.40–5.59	0.548
LAI	73	16, 47.1%	10, 58.8%	1.56	0.34–7.14	0.570	34, 64.2%	3.12	1.06–9.24	0.039	13, 72.2%	5.81	1.26–26.80	0.024
Age 70–85 years	150‡	49	26				60				15			
LVI	89	28, 57.1%	15, 57.7%	0.84	0.25–2.78	0.773	38, 63.3%	1.53	0.57–4.08	0.400	8, 53.3%	1.07	0.29–3.96	0.920
LAI	92	29, 59.2%	12, 46.2%	0.56	0.18–1.77	0.326	40, 66.7%	0.86	0.33–2.24	0.754	11, 73.3%	1.75	0.39–7.91	0.465

The logistic regression model included sex, age, myocardial infarction, heart failure, arrhythmia, AF, hypertonia, peripheral artery disease, diabetes, total and HDL cholesterol, and triglycerides as confounders.

*Forty-nine, †18, and ‡31 patients had both LAI and LVI strokes.

5.2 Factors affecting ischemic stroke survival (I-IV)

5.2.1 Classical factors (I-IV)

In univariate analysis, age ($p < 0.0001$), smoking ($p = 0.030$), and AF ($p = 0.040$) predicted death at 15 months (Study I). In logistic regression analysis age (OR 1.10, $p = 0.008$) and smoking (OR 5.13, $p = 0.006$) remained independent risk factors in the model for post-stroke death at 15 months (Study I) (Table 3).

Table 3. Association of Multiple Risk Factors and Platelet GpIIb/IIIa PIA1/PIA2 Fibrinogen Receptor Polymorphisms for Survival at 15 Months Among 272 Patients With Ischemic Stroke (Stroke Aging Memory Cohort)

Variable	Valid n	Alive at 15 Months (n=248)		Deceased at 15 Months (n=24)		OR	95% CI	P
Mean age, y	272	70.1	7.8 SD	75.3	6.6 SD	1.10	1.03–1.19	0.008
Female sex	139	126	90.6%	13	9.4%	2.00	0.69–5.77	0.201
Myocardial infarction	48	40	83.3%	8	16.7%	1.72	0.60–4.97	0.315
Heart failure	56	47	83.9%	9	16.1%	1.34	0.46–3.91	0.587
Arrhythmia	59	50	84.7%	9	15.3%	0.77	0.08–7.75	0.826
AF	43	35	81.4%	8	18.6%	2.75	0.25–30.31	0.409
Peripheral artery disease	37	30	81.1%	7	18.9%	2.02	0.71–5.68	0.185
Hypertension	130	119	91.5%	11	8.5%	1.25	0.50–3.13	0.627
Diabetes	61	53	86.9%	8	13.1%	2.37	0.90–6.26	0.081
Smoking	145	127	87.6%	18	12.4%	5.13	1.61–16.36	0.006
Chol, mmol/L	272	5.6	1.2 SD	5.3	1.2 SD	0.77	0.49–1.21	0.257
HDL chol, mmol/L	272	1.2	0.6 SD	1.1	0.4 SD	0.92	0.32–2.58	0.869
Triglycerides, mmol/L	272	1.5	0.7 SD	1.6	0.6 SD	1.32	0.67–2.59	0.424
PIA ² allele (A2/A1, A2/A2)	76	66	86.8%	10	13.2%	1.80	0.70–4.62	0.221

The logistic regression model included sex, age, myocardial infarction, heart failure, arrhythmia, AF, hypertonia, peripheral artery disease, diabetes, total and HDL cholesterol, triglycerides, presence or absence of the PIA² allele, and history of smoking as confounders.

According to Kaplan-Meier analysis, predictors of poor long term survival were smoking, myocardial infarction, CF, AF, other arrhythmias, PAD and poor modified Rankin score (Study II-IV). In Cox regression survival analysis utilizing several models advanced age, CF, AF, PAD, smoking, diabetes and poor modified Rankin score were associated with poor long-term survival (Study II-III) (Table 4). Predictors of poor survival due to death from brain-associated causes of death were advanced age (HR 1.05, 95% CI 1.01-1.09, $p=0.009$), AF (HR 1.82, 95% CI 1.08-3.07, $p=0.026$), PAD (HR 2.17, 95% CI 1.19-3.95, $p=0.012$) and poor modified Rankin score (HR 2.75, 95% CI 1.67-4.54, $p<0.0001$) (Study III).

Table 4. Cox regression analysis on the association of multiple risk factors with poor long-term survival (all cause death endpoint) among patients with ischemic stroke (Stroke Aging Memory cohort).

Variable	Study II (All)			Study II (Men)			Study II (Women)			Study III		
	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p
Age	1.08	(1.05-1.10)	<0.0001	1.06	(1.03-1.10)	<0.0001	1.09	(1.05-1.13)	<0.0001	1.07	(1.05-1.09)	<0.0001
CF	1.68	(1.20-2.39)	0.004	1.29	(0.75-2.21)	0.355	1.94	(1.11-3.37)	0.020	1.39	(1.17-2.15)	0.003
AF	1.32	(0.65-2.69)	0.445	1.40	(0.46-4.32)	0.556	1.98	(0.72-5.39)	0.184	1.68	(1.24-2.27)	0.001
PAD	1.35	(0.90-2.02)	0.148	1.71	(1.02-2.86)	0.042	0.80	(0.36-1.77)	0.573	1.39	(1.11-2.26)	0.011
Smoking	1.49	(1.07-2.07)	0.017	1.11	(0.69-1.79)	0.678	2.08	(1.29-3.35)	0.003	1.60	(1.23-2.08)	<0.0001
Diabetes	1.42	(1.00-2.02)	0.049	1.40	(0.87-2.23)	0.172	1.80	(0.99-3.27)	0.002	1.44	(1.08-1.92)	0.013
Rankin score	N.A.			N.A.			N.A.			1.65	(1.26-2.14)	<0.0001

Myocardial infarction (MI), cardiac failure (CF), atrial fibrillation (AF), peripheral arterial disease (PAD), poor modified Rankin score (3-5).

5.2.2 Genetic factors

In interaction analysis, there was a PI^{A2} genotype-by-smoking effect on post-stroke survival at 15 months (OR 2.78, 95% CI 0.89-8.61, $p=0.077$) (Study I). In logistic regression analysis, smokers carrying the PI^{A2} allele were at a higher risk of dying within 15 months after stroke (OR 8.86, $p=0.010$) than were smokers not carrying the PI^{A2} allele (OR 5.06, $p=0.027$) (Study I) (Figure 4). Utilizing Kaplan-Meier and Cox regression models, no effect of GpIIb/IIIa PI^{A2} on long-term survival was observed and there was neither an interaction with smoking on long term survival (data not shown).

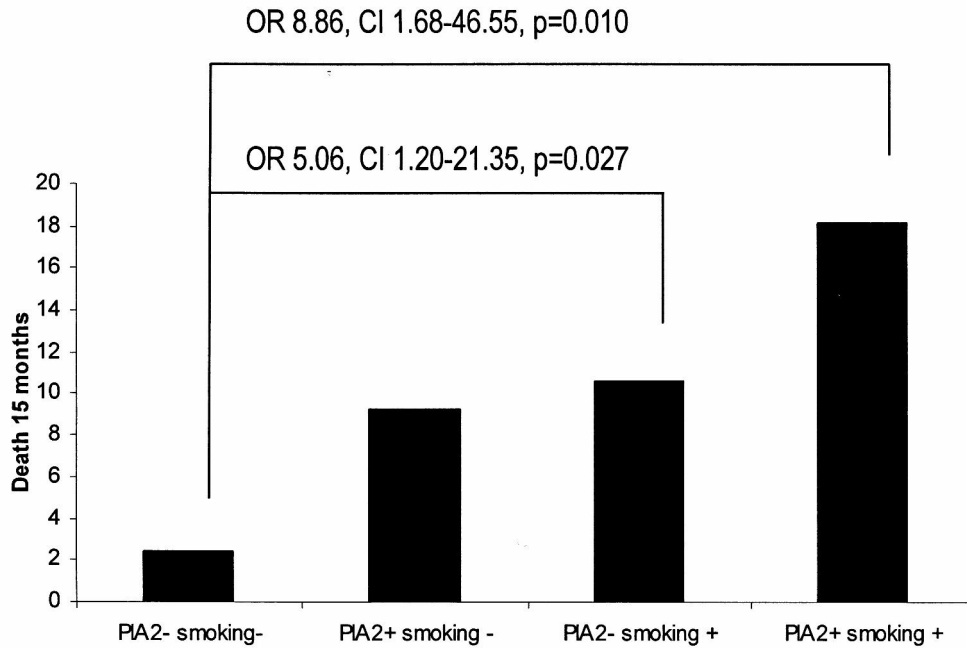


Figure 4. The effect of smoking and platelet GpIIb/IIIa PI^{A1/2} fibrinogen receptor polymorphisms on poststroke survival at 15 months. The percentage of patients with different genotype combinations and history of smoking who died within 15 months is presented (Study I).

Poor survival was found to relate to the eNOS 4b/b genotype (HR 1.44, 95% CI 1.03-2.01, $p=0.033$) (Study II). This association of poor survival with the 4b/b genotype was observed in women (HR 1.69, 95% CI 1.01-2.82, $p=0.046$), and there was a tendency in men (HR 1.52, 95% CI 0.94-2.46, $p=0.089$) (Study II). There was an interaction between smoking and the eNOS 4b/b genotype (HR 1.53, 95% CI 1.10-2.12, $p=0.011$) as well as a nearly significant one between smoking and the iNOS R5/4-genotype (HR 1.52, 95% CI 0.96-2.40, $p=0.073$) with regard to poor survival (Study II). These interactions remained significant only among women (HR 3.74, 95% CI 1.64-8.53, $p=0.002$, HR 2.39, 95% CI 1.34-4.22, $p=0.003$, respectively) and were not observed among men (HR 1.28, 95% CI 0.83-1.97, $p=0.266$, and HR 1.05, CI 0.60-1.86, $p=0.856$, respectively) (Study II). More interestingly, we found a strong interaction between smoking, female sex, and the iNOS R5/4-genotype (HR 3.23, 95% CI 1.51-6.90, $p=0.002$), but not the eNOS 4b/b genotype ($p=0.156$), in relation to poor outcome (Study II). No interactions were found between the iNOS and eNOS genotypes (Study II).

In Cox regression analysis, smoking carriers of the eNOS 4b/b genotype (HR 1.81, $p=0.018$) as well as of the iNOS R5/4 genotype (HR 1.82, $p=0.023$) had the highest risk of

poor survival when compared with non-smoking non-carriers (Study II) (Figure 5). In women, smoking carriers of the iNOS R5/4 genotype (HR 4.23, $p=0.001$) and the eNOS 4b/b genotype (HR 3.14, $p=0.003$), as well as smoking non-carriers of the iNOS R5/4 genotype (HR 1.75, $p=0.039$) were at an increased risk for poor survival when compared to non-smoking non-carriers (Study II) (Figure 5). The difference between smoking female iNOS R5/4 genotype carriers and smoking non-carriers was also significant (HR 2.57, $p=0.029$) (Study II) (Figure 5). The difference between smoking female patients with the eNOS 4b/b genotype and smoking patients with the 4a/b or 4a/a genotype was significant only in females (HR 3.06, $p=0.034$) (Study II) (Figure 5). Neither eNOS nor iNOS genotype were associated with specific causes of death (Study II).

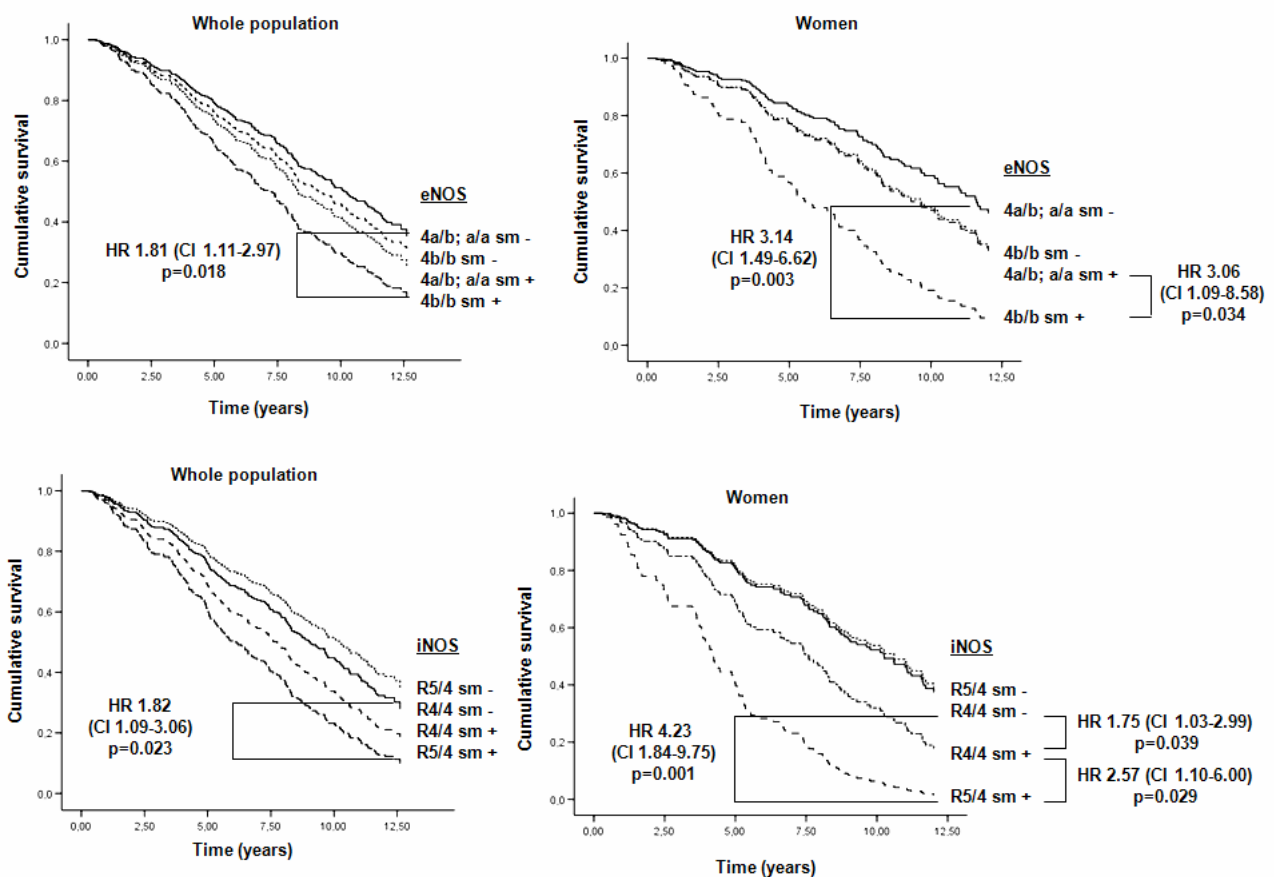


Figure 5. The effect of eNOS 4a/b and iNOS R5/4 polymorphisms on cumulative survival after stroke during long-term follow-up in Stroke Aging Memory (SAM)-cohort. Survival function according to Cox regression analysis with multiple covariates (Study II).

5.2.3 Neuroradiological factors

In the Kaplan-Meier log rank analysis, severe ARWMCs predicted poor survival after stroke compared with patients with moderate ($p=0.040$) and mild ARWMCs ($p<0.0001$). Severe ARWMCs predicted poor survival compared with mild-to-moderate ARWMCs ($p<0.0001$) (Figure 6).

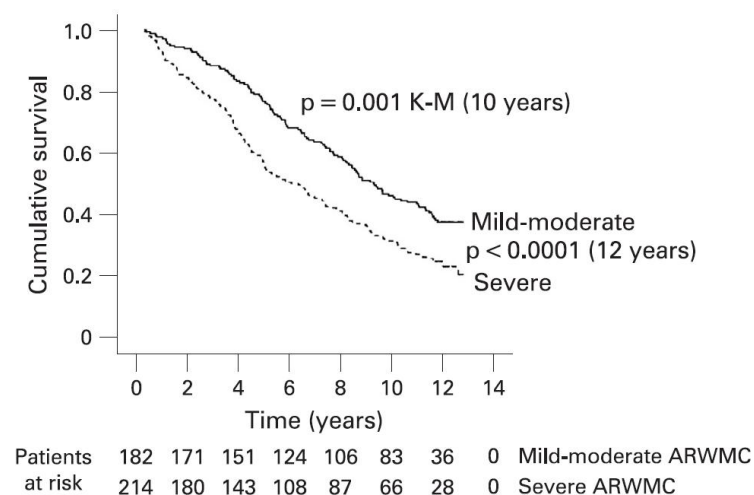


Figure 6. The effect of age related white matter changes (ARWMC) detected on MRI on overall post stroke survival (endpoint: all cause death) in Stroke Aging Memory cohort. Kaplan-Meier log rank analysis.

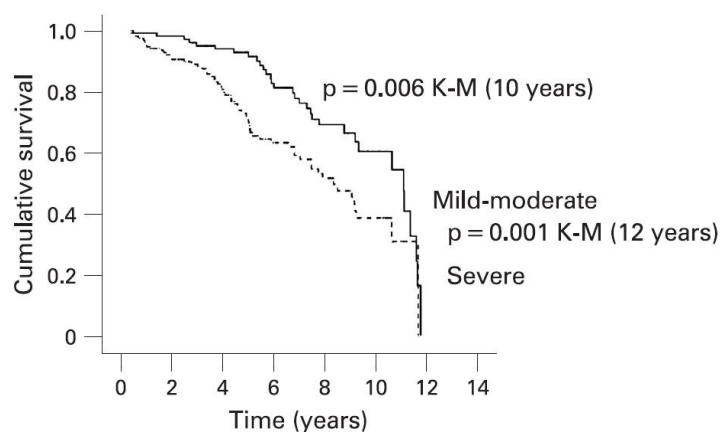
In univariate analysis, brain related causes of death ($p=0.001$) and specifically due to infarction ($p<0.0001$) were associated with severe ARWMCs (Table 5). In Kaplan-Meier analysis with brain-associated cause of death as endpoint severe ARWMCs predicted poor survival compared to mild-moderate category ($p=0.001$) (Figure 7). No associations with other causes of death were found and ARWMC category was not associated with cardiac death ($p=0.513$).

Table 5. The association of MRI-based age related white matter change (ARWMC) category with causes of death (Stroke Aging Memory cohort).

Variable	Valid n	ARWMC				p
		Mild+moderate (n=113)		Severe (n=164)		
		n	%	n	%	
Cardiac	85	40	(35.4)	45	(27.4)	0.513
Brain	91	32	(28.3)	59	(36.0)	0.001
infarct	70	20	(17.7)	50	(30.5)	<0.0001
bleeding	11	7	(6.2)	4	(2.4)	0.396
dementia	10	5	(4.4)	5	(3.0)	0.717
Infectious	12	7	(6.2)	5	(3.0)	0.493
Traumatic	11	3	(2.7)	8	(4.9)	0.136
Cancer	36	17	(15.0)	19	(11.6)	0.684
Other	34	12	(10.6)	22	(13.4)	0.068

Kaplan-Meier log rank test

The cause of death was unknown for 8 subjects: 2 mild to moderate and 6 severe ARWMC



Patients	111	100	81	53	35	13	0	0	Mild-moderate ARWMC
at risk	158	124	86	52	31	10	0	0	Severe ARWMC

Figure 7. The effect of age related white matter changes (ARWMC) detected on MRI on overall post stroke survival (endpoint: brain-related cause of death) in Stroke Aging Memory cohort. Kaplan-Meier log rank analysis.

To account for potential confounders a multivariate Cox regression analysis utilizing two models (Model 1 and 2) was used. Severe ARWMCs predicted poor survival (HR 1.34, 95% CI 1.03-1.73, p=0.029). In multivariate Cox regression analysis using brain-associated causes of death as endpoint (Model 1 and 2), severe ARWMCs remained as predictor of poor survival (HR 1.80, 95% CI 1.10-2.96, p=0.019).

5.2.4 Neuropsychological factors

In Kaplan-Meier log-rank analysis, deficits in executive functions ($p < 0.0001$), memory ($p = 0.009$), visuospatial and constructional abilities ($p < 0.0001$) and language ($p = 0.004$) predicted poor survival. Furthermore, MMSE ≤ 25 ($p < 0.0001$) and CIND ($p = 0.003$) predicted poor poststroke survival (Figure 8).

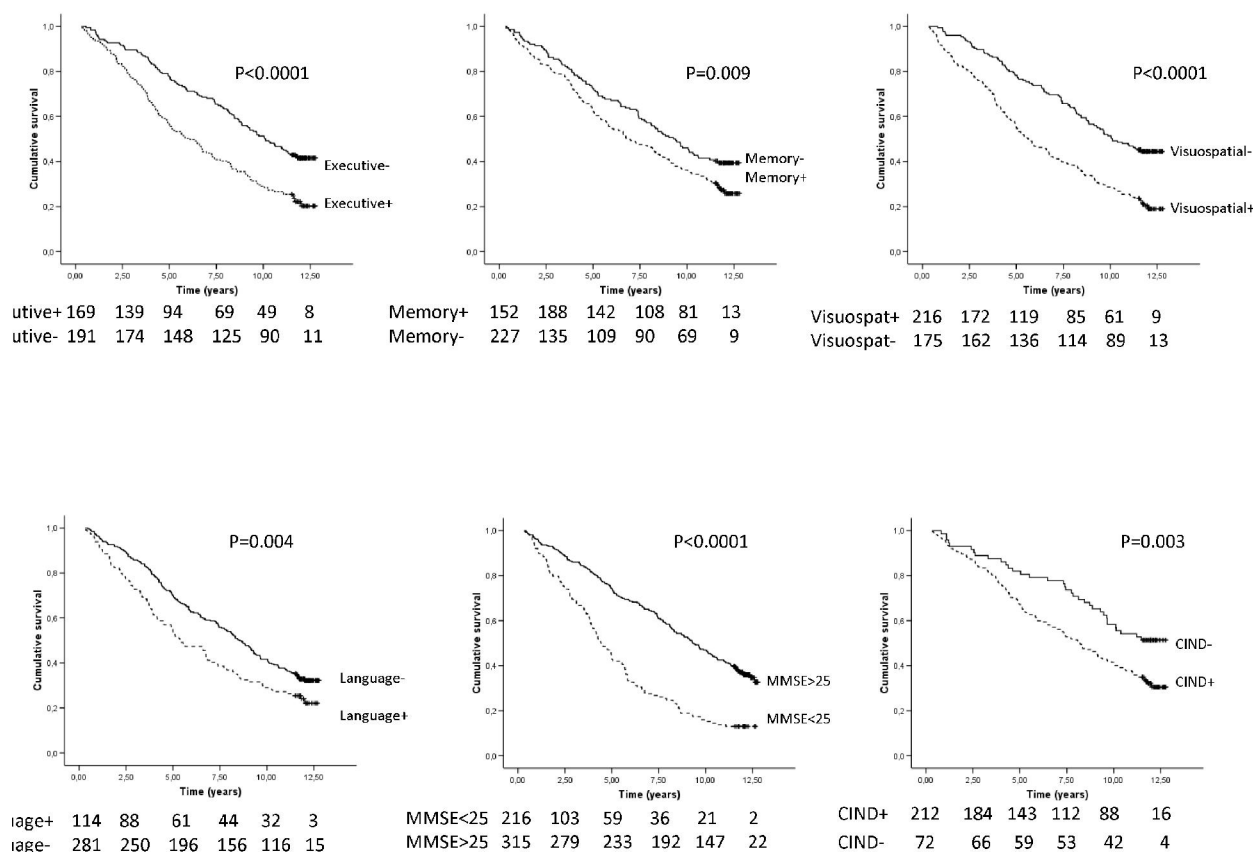


Figure 8. The effect of education and different cognitive deficits (groups with given deficits are marked with + -sign) on overall poststroke survival (endpoint: all cause death) in Stroke Aging Memory cohort. Low edu: low education (< 6 years). Kaplan-Meier log rank analysis.

In Cox proportional hazards regression analysis (Model 1, including age, sex and years of education), deficits in executive functions (HR 1.65, $p < 0.0001$), memory (HR 1.31, $p = 0.042$), language (HR 1.33, $p = 0.036$) and visuospatial and constructional abilities (HR 1.82, $p < 0.0001$) were significant predictors of poor poststroke survival. Deficits in executive functions (HR 1.38, $p = 0.021$) and visuospatial and constructional abilities (HR 1.53, $p = 0.004$) remained as significant predictors after addition of MMSE ≤ 25 and poor modified Rankin score as covariates (Model 2). MMSE ≤ 25 (HR 1.32-1.46) and poor modified Rankin score (HR 1.54-1.61) predicted poor survival in all the models (Table 6).

Table 6. Cox regression analysis on the association of multiple risk factors and cognitive deficits with poor long-term survival (all cause death endpoint) among patients with ischemic stroke (Stroke Aging Memory cohort).

Variable	Model 1 (Forced entry)			Model 2 (Forced entry)			Model 1 (Forced entry)			Model 2 (Forced entry)			
	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p	
Age	1.08	1.06-1.10	<0.0001	1.06	1.04-1.08	<0.0001	Age	1.07	1.05-1.09	<0.000	1.06	1.04-1.08	<0.0001
Female sex	0.70	0.54-0.91	0.007	0.73	0.56-0.95	0.018	Female sex	0.71	0.54-0.92	0.010	0.71	0.55-0.93	0.014
Education	0.96	0.93-0.99	0.013	0.98	0.95-1.01	0.271	Education	0.97	0.93-1.00	0.027	0.98	0.95-1.01	0.188
MMSE≤25	not included			1.45	1.07-1.96	0.017	MMSE≤25	not included			1.44	1.06-1.94	0.019
Poor Rankin	not included			1.71	1.29-2.27	0.017	Poor Rankin	not included			1.56	1.17-2.08	0.003
Memory	1.31	1.01-1.70	0.042	1.20	0.92-1.57	0.182	Executive	1.59	1.23-2.06	<0.0001	1.33	1.01-1.74	0.040
Age	1.07	1.06-1.09	<0.0001	1.06	1.04-1.08	<0.0001	Age	1.07	1.05-1.09	<0.0001	1.06	1.04-1.08	<0.0001
Female sex	0.73	0.57-0.95	0.018	0.75	0.57-0.98	0.036	Female sex	0.64	0.49-0.82	0.001	0.68	0.52-0.89	0.005
Education	0.96	0.93-0.99	0.012	0.98	0.95-1.01	0.175	Education	0.97	0.94-1.00	0.041	0.98	0.95-1.01	0.227
MMSE≤25	not included			1.46	1.09-1.97	0.012	MMSE≤25	not included			1.28	0.94-1.75	0.116
Poor Rankin	not included			1.60	1.21-2.12	0.001	Poor Rankin	not included			1.55	1.17-2.06	0.002
Language	1.33	1.02-1.73	0.036	1.11	0.82-1.48	0.509	Visuospatial	1.82	1.40-2.36	<0.0001	1.53	1.14-2.04	0.004

In Cox proportional hazard regression analysis with age, sex and years of education as covariates, CIND remained an independent predictor of survival (HR 1.63, CI 1.12-2.39, $p=0.012$).

According to Kaplan-Meier log rank test, the probability of dying due to cardiac causes alone showed no association with deficits in any of the cognitive domains. Death due to brain related causes on the other hand was more likely in those with deficits in executive functions ($p=0.019$), language ($p=0.001$), visuospatial/constructional abilities ($p<0.0001$). No associations between cognitive domains and infectious, traumatic or other causes of death were found.

6. DISCUSSION

6.1 The effect of genetic variation on stroke subtypes and survival (I-II)

In the present study, the prevalence of PI^{A2} allele (PI^{A1/A2} or PI^{A2/A2}) carriers was 28% and it has previously been shown to vary between 28-36% in sudden cardiac death, myocardial infarction and stroke cohorts which is in line with our observation (Corral et al. 1997; Wagner et al. 1998; Mikkelsen et al. 1999; Mikkelsen et al. 2000; Grove et al. 2004; Saidi et al. 2008b; Saidi et al. 2008a). The observed PI^{A2} allele frequency of 14.2% is in line with that observed previously in a large follow-up study (Ridker et al. 1997).

Previously, no evidence of an association between the PI^{A2} allele and stroke was found and in contrast to our results, no evidence of an interaction between PI^{A2} allele and smoking was found on any vascular events (Ridker et al. 1997). However, in that study the subjects comprised those with both ischemic and hemorrhagic strokes and no stroke subtypes were analyzed separately which makes the interpretation and extrapolation of the results difficult. However, in several papers, including case-control studies, HPA-1b (PI^{A2}) allele was associated with increased risk of stroke (Szolnoki et al. 2003; Saidi et al. 2008b; Saidi et al. 2008a). These studies on the other hand lacked the analysis of survival and the possible modifying effect of smoking. The varying effect of PI^{A2} alone may be attributed to different cohort setting (i.e. case-control) resulting in different statistical power. In addition, these cohorts contained significantly younger patients than the present study (approx. 60 vs. 70 years). The possible effect of age on the effect of PI^{A1/A2} is supported by the finding that PI^{A2} allele was associated with increased risk of stroke in young women (Wagner et al. 1998) and in both sexes aged <50 years (Carter et al. 1998). However, the possible interaction with smoking habits was not studied. Our results of increased risk of lacunar stroke in younger (55-69 years) smokers carrying the PI^{A2} allele are in line with these results. The effect of PI^{A2} allele on long term survival in stroke patients has not been studied before and therefore our negative results need to be confirmed in independent cohorts.

The observed frequencies of the eNOS 4a and 4b alleles are in line with previous findings in Caucasian populations (Pulkkinen et al. 2000; Kunnas et al. 2002) although no comparable data on acute hospitalized stroke patients exists. The allele frequencies of the

iNOS R4 and R5 alleles are also in line with previous findings in Caucasian populations (Kunnas et al. 2003).

In contrast to present finding of no effect of eNOS 4a/b SNP on stroke subtypes, stroke patients with the 4a variant of the intron 4a/b promoter polymorphism of the eNOS gene have increased NO levels and significantly fewer lacunar infarcts (Hassan et al. 2004a). In addition, according to a recent meta-analysis of 526 manuscripts involving 7533 cases and 9835 controls, eNOS 4a/b polymorphism seemed to modify the stroke subtype (Rao et al. 2009). The reason that any effect of these genotypes on stroke subtype was not detected may simply be due to insufficient power to detect this weak effect.

The role of neither iNOS nor eNOS on long term survival after stroke is unknown. It is suggested that the iNOS R4/5 genotype, which alters iNOS promoter activity (Morris et al. 2002) results in an increased activity of iNOS and subsequently increase nitrotyrosine formation, enlargement of the ischemic lesion (Moro et al. 2004), and an inflammatory response mediated by nitric oxide during stroke (Hirabayashi et al. 2000). These speculations must be interpreted with caution since no definitive information exists about the effect on NO production in humans. In mouse models of ischemic stroke, iNOS plays a role in the late enlargement and determines the size of the infarct area in males without an effect in females and iNOS disrupted animals (Loihl et al. 1999), a phenomenon possibly modulated by female sex steroids (Park et al. 2006). This occurs at least partially through the suppression of iNOS expression (Coughlan et al. 2005) and of injury-induced proinflammatory cytokine release (Gibson et al. 2005). To date, no human data supporting this exists. In humans, R5 allele has been linked to higher mean values of coronary stenosis and larger areas of fatty streaks and complicated lesions than did R4/4 carriers in middle aged men in a pre-hospital sudden death cohort (Kunnas et al. 2003).

Approximately half of the present study population consisted of postmenopausal female patients aged 55–85 years whose levels of both progesterone and estrogen are low. This raises an important question of whether women with polymorphism of the iNOS gene are dependent on progesterone and estrogen. It must be emphasized that for humans, no clinical evidence exists on the protection against recurrence of stroke or mortality in postmenopausal women with cerebrovascular disease by hormone replacement therapy (Pines et al. 2002).

Present results of impaired survival with the eNOS 4b/b genotype are in accordance with the finding that the eNOS 4a allele has been found to protect against isolated lacunar infarct mediated by functional changes in eNOS promoter activity resulting in increased NO levels (Hassan et al. 2004a). The data on the effect of the 4a allele on cardiac morbidity and mortality is conflicting—according to a meta-analysis, homozygosity for the rare 4a allele results in moderately increased risk of coronary heart disease (Casas et al. 2004; Casas et al. 2006). In contrast, it has previously been shown with middle-aged men who had died suddenly of acute myocardial infarction, that the eNOS 4a allele associates with significantly lower risk of myocardial infarction when compared with patients homozygous for the b allele (Kunnas et al. 2002).

Although these findings on the influence of genetic alterations on ischemic stroke may not be of value for clinical practice, the present findings warrant future studies on gene-environment interactions.

6.2 The effect age related white matter changes on long term survival (III)

The present results are supported by previous findings in a small cohort of noninstitutionalized elderly subjects with a history of previous stroke in which severe ARWMCs predicted poor survival (HR 2.02) at a median follow-up of 23 months (Fu et al. 2005). In that study, severity of ARWMCs was also associated with increased risk of recurrent stroke. Similarly, in patients with transient ischemic attack, presence of ARWMCs doubled the risk of future stroke (van Swieten et al. 1992) and similarly in patients with symptomatic carotid stenosis (Streifler et al. 2002). Another study demonstrated the predictive value of ARWMCs independently of existing neurological deficits in a follow-up of one year (Briley et al. 2000). The effect of severe ARWMCs on survival and morbidity have been demonstrated in elderly people in a follow-up of 4.2 (Vermeer et al. 2003b) to 8.4 years (Inzitari et al. 1997). The problem with these studies is varying imaging technology, either computed tomography (CT) or MRI. At the present, the exact MRI correlates of leukoaraiosis detected in CT are not known.

The present study adds to the existing knowledge by providing long term survival data. The major difference in the present cohort is lower proportion of patients with hypertension and double proportion of patients with atrial fibrillation or history of smoking (Fu et al. 2005). Similarly but in older people without history of stroke or other neurological disease

severe white matter lesions were associated with poor survival in long term follow-up of 11.8-12 years (Kerber et al. 2006; Kuller et al. 2007).

Interestingly, in the present study, ARWMCs were associated with death due to brain related causes and death due to ischemic stroke. The present observations are supported by a previous study in which more than half of the deaths in patients with severe ARWMCs were caused by vascular causes (Kerber et al. 2006). No evidence of deaths due to trauma or pneumonia were found. In previous study, however, in addition to death, ARWMCs were related to pneumonia and falls resulting in fracture requiring hospitalization (Briley et al. 2000). In another, population-based study white matter lesions were independently associated with incident falls and gait disturbances (Srikanth et al. 2009). Similar results were obtained from the LADIS study also (Blahak et al. 2009). Future studies analyzing hospitalizations in the present cohort during long term follow-up are warranted.

ARWMCs can be regarded as surrogates for small vessel disease (Inzitari et al. 2007) (Inzitari 2003; Inzitari et al. 2007; van Dijk et al. 2008). It would be expected that the rate of death due to cerebral bleeding events and dementia would be elevated in severe ARWMC category because there is an association between cerebral amyloid angiopathy making the vessels friable, cerebral bleeding and white matter lesions (Schutz et al. 1990; Smith et al. 2004; Ritter et al. 2005; Chen et al. 2006; Maia et al. 2006). Hypertension also associates with this complex interaction (Inzitari et al. 1990). However, in the present cohort there was no association between bleeding events and dementia in different ARWMC categories. The advancement of ARWMCs can be decelerated by effective treatment of hypertension (Schiffrin 2005). Therefore, future studies elucidating the effect of antihypertensive strategy on poststroke survival in different ARWMC categories is needed.

According to the present findings, ARWMC severity can be clinically utilized for the identification of patients at risk for poor survival.

6.3 The effect of neuropsychological deficits on long term survival (IV)

The proportion of patients with cognitive deficits in different domains varied in between 29-60%. Deficits in verbal, visuomotor and memory functions have been associated with shortened survival at general population (Portin et al. 2001; Shipley et al. 2006). Similarly,

dementia either before index stroke (Barba et al. 2002; Henon et al. 2003) or after stroke (Tatemichi et al. 1994b; Barba et al. 2002; Desmond et al. 2002) is consistently related to poor survival which is in line with our finding of the association of MMSE with survival. In line with our results, cognitive impairment as measured by MMSE was associated with impaired survival up to 4 year follow-up (Friedman 1991a; Friedman 1994; House et al. 2001; Patel et al. 2002). The proportion of patients with low MMSE in the present study (28.6%) is in line with that in a study of hospitalized patients (House et al. 2001).

In a previous study with 8 year follow-up (Moulin et al. 1997) severe aphasia was independently associated with poor survival which is in line with present finding of the association of deficit in language domain and survival. Furthermore, in line with our results, CIND has been related to impaired survival in community based cohorts (Ingles et al. 2003; Tuokko et al. 2003; Hsiung et al. 2006). CIND is of relevance in the clinical setting since it is a potentially reversible condition potentially alerting clinicians to schedule strict poststroke follow-up regimen.

When controlling for the effect of global cognitive decline ($MMSE \leq 25$) and severity of stroke only executive functions as well as visuospatial and constructional abilities remained as independent correlates of poor survival. Executive functions, including planning, initiation, sequencing and monitoring of complex goal-directed behaviour, heavily rely on the integrity of the prefrontal cortices and their connecting pathways with the subcortical structures (Royall et al. 2002). The frontal lobe system is also crucial for visuospatial and constructive abilities which require attention and organizational skills together with the more posteriorly-mediated visuoperceptual functions.

White matter lesions associate with general cognitive function (van der Flier et al. 2005) and with cognitive decline (Inzitari et al. 2007; van Dijk et al. 2008) and executive dysfunction related with frontal lobe system (O'Sullivan et al. 2004; Tullberg et al. 2004; Carey et al. 2008). The association between white matter lesions and global cognitive function, impaired memory and executive function has also previously been demonstrated in the present cohort (Pohjasvaara et al. 2007). Together these results suggest that cerebral small vessel pathology and disruption of the frontal lobe functional system may explain impaired poststroke survival.

Although the neuropsychological tests used in the present study are not applicable for clinical usage, these findings may help clinicians develop clinically applicable neuropsychological tests for the identification of patients at risk.

6.4 Methodological considerations (I-IV)

A potential weakness of our study is the possibility of selection bias, as the cohort was formed three months after the index stroke. This may limit generalization of the results. Therefore, additional data on stroke-related deaths in Helsinki University Hospital district was obtained during the collection of the cohort from an independent organization (Statistics Finland). In this retrospective data, up to 64 percent of stroke related deaths occurred in women. While the proportions of both sexes in the present studies were equal, this suggests that some women may have died before hospital assessment at 3 months. Due to exclusion of patients, the true survival rate may be underestimated. Similarly, since patients with severe aphasia could not be assessed with comprehensive neuropsychological examination, there is also a possibility for survival bias since these patients are prone to impaired survival. Since stroke alone seems to be associated with over 70% risk of death during 10 years (Eriksson and Olsson 2001; Vernino et al. 2003), in several studies the interest has been to investigate subjects without history of stroke. In the present studies, the analyses were confirmed excluding those with recurrent strokes and the results were similar for analyses including first-ever and recurrent strokes which adds to the reliability of the results. With respect to genetic studies, there is also need to perform verification of the results in independent cohorts and in case-control settings to provide adequate power for the statistical analyses with special emphasis on gene-environment interactions. One of the strengths of the present study is that the cohort is consecutive one and that the the patients with suspected cerebrovascular ischemic event were reviewed by senior neurologists. The neuroradiological and neuropsychological and clinical characteristics of the patients were strictly evaluated and also the severity of stroke at admission was quantitated according to the modified Rankin score. The modified Fazekas rating scale (Pantoni et al. 2005) has been previously used in the large LADIS-study (Inzitari et al. 2007) and demonstrated to correlate well with the more complex Scheltens rating scale and semi-automated volumetric methods (Gouw et al. 2006). In the present study, the modified rating scale allowed large enough subgroups allowing sufficient statistical power.

A potential strength of the present study is that Helsinki stroke unit is responsible for primary stroke management of all inhabitants living in the Helsinki area. Also, the survival data is comprehensive with negligible amount of unresolved deaths. In Finland, the determination of cause of death is based on autopsy approximately 30% of all deaths in the past two decades (www.tilastokeskus.fi) which is rather high compared to other European countries. In addition, the death certificates of all the deceased, whether subjected to autopsy or not are reviewed by the district forensic physician. The official cause of death has been demonstrated to be an accurate means for evaluating disease specific mortality in Finland (Makinen et al. 2008). This adds to the reliability of the present study.

7. CONCLUSIONS AND SUMMARY

1. Smokers carrying the PI^{A2} allele had increased risk of lacunar infarcts especially among younger stroke patients. This indicates that the platelet fibrinogen receptor GpIIb/IIIa $PI^{A1/A2}$ polymorphism modulates the effect of smoking on stroke phenotype.
2. Smokers carrying the PI^{A2} allele were at highest risk of dying within 15 months after ischemic stroke. This indicates that the GpIIb/IIIa $PI^{A1/A2}$ polymorphism modulates the effect of smoking on mid-term survival.
3. $PI^{A1/A2}$ polymorphism alone had no effect on long term survival and there was no interaction with smoking on long term survival.

The synergy between PI^{A2} allele and smoking is most probably a consequence of an interaction between platelet aggregability, fibrinogen levels, and fibrinogen receptor binding affinity, which is modified by the presence of PI^{A2} allele. Although these findings may not be of value for clinical practice, future studies replicating these results in independent cohorts are warranted with specific emphasis on gene-environment interactions.

4. Neither endothelial vasoregulatory eNOS 4a/b nor cerebral injury associated inducible iNOS R4/5 polymorphisms were associated with stroke phenotype and there was no interaction with smoking status on stroke phenotype.
5. There was a strong interaction between smoking and genetic variants of both eNOS and iNOS, predicting poststroke survival especially among postmenopausal women.

The synergy between eNOS 4b allele and smoking and iNOS R5 allele and smoking are most probably a consequence of altered vasoregulation and increased NO production at the area of ischemic infarct. Although these findings may not be of value for clinical practice, future studies replicating these results in independent cohorts are warranted with specific emphasis on gene-environment interactions.

6. Patients with severe ARWMCs are at risk of poor poststroke long term survival and death due to brain related causes. Treatment modalities slowing down progression of

ARWMCs should to be evaluated in future studies. The present findings may be utilized in the clinical practice to identify patients at risk for poor survival.

7. Cognitive impairment already at less severe stages without dementia is related to poor long-term survival poststroke. Deficits in executive functions and visuospatial and constructional abilities, in particular, predict poor outcome independently of global cognitive decline and severity of stroke. These findings may be utilized for the development of clinically applicable neuropsychological tests.

Future work will be needed to select the most promising predictors to create a holistic model for the prediction of survival.

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There is a growing need for applied research and co-operation between surgeons, basic researchers and many other specialties. However, there is lack of methodological competence among surgeons. To overcome this barrier we must humbly learn from each other and establish novel networks of knowledge. Therefore, we have to boldly proceed to explore new worlds, learn molecular biology in practice, to understand neuropsychology, neurology and neuroradiology. In other words, to boldly go where no surgeon has gone before!

The present study was carried out in the Department of Forensic Medicine, University Hospital of Tampere and Department of Neurology, Helsinki University during 2006-2008. This work is a tribute to my father, Ilkka Oksala, M.D., the Bear hunter, the legendary thoracic and cardiovascular surgeon who introduced the concepts of attitude, leadership, speed, quality, dexterity and skill into all areas of surgery and who is still fully operational despite obligatory retirement from his work as a cardiothoracic and a vascular surgeon. This work is also a tribute to my opponent, Professor Juhani Sivenius who has worked extensively to improve rehabilitation of stroke patients. This work is a tribute to my mother, Raijaliisa Oksala, the Artist, the power behind my family.

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Niku K.J. Oksala, The Artist 1:10

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9. ORIGINAL COMMUNICATIONS I-IV

I. Oksala NK, Heikkinen M, Mikkelsson J, Pohjasvaara T, Kaste M, Erkinjuntti T, Karhunen PJ. Smoking and the platelet fibrinogen receptor glycoprotein IIb/IIIa P1A1/A2 polymorphism interact in the risk of lacunar stroke and midterm survival. *Stroke* 2007;38:50-55. Reproduced with permission from the Wolters Kluwer Health, Lippincott Williams & Wilkins. *Stroke* 2007;38:50-55.

II. Oksala NKJ, Oksala A, Erkinjuntti T, Pohjasvaara T, Kunnas T, Vataja R, Kaste M, Karhunen PJ. Long-term survival after ischemic stroke in postmenopausal women is affected by an interaction between smoking and genetic variation in nitric oxide synthases. *Cerebrovasc Dis* 2008;26:250-258. Reproduced with permission from the S. Karger, AG, Basel.

III. Oksala NKJ, Oksala A, Pohjasvaara T, Vataja R, Kaste M, Karhunen PJ, Erkinjuntti T. Age-related white matter changes predict stroke death in long-term follow up *J Neurol Neurosurg Psych* 2009;000:1-5. doi:10.1136/jnnp.2008.154104. Reproduced with permission from the BMJ Publishing Group.

IV. Oksala NKJ, Jokinen H, Melkas S, Oksala A, Pohjasvaara T, Hietanen M, Vataja R, Kaste M, Karhunen PJ, Erkinjuntti T. Cognitive impairment predicts poststroke death in long-term follow up. Submitted.

Long-Term Survival after Ischemic Stroke in Postmenopausal Women Is Affected by an Interaction between Smoking and Genetic Variation in Nitric Oxide Synthases

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Key Words

Nitric oxide synthase · Stroke · Polymorphism · Survival

Abstract

Background: We aimed to study whether variations in vasoregulatory endothelial nitric oxide synthase (eNOS 4a/b) and tissue-injury-associated inducible nitric oxide synthase (iNOS R5/4) genes and smoking might explain gender differences in long-term survival after stroke. **Methods:** A total of 486 consecutive acute stroke patients, subjected to MRI, were followed up for a mean of 7.6 years. The eNOS 4a/b (n = 300) and iNOS R5/4 (n = 310) genotypes were determined by PCR. Of these patients, 213/300 (71.0%; eNOS 4a/b) and 223/310 (71.9%; iNOS R5/4) had died. **Results:** Despite the fact that women were older than men (72.3 vs. 69.5 years, p = 0.001) at recruitment, poor long-term survival was not sex-related, but instead predicted by age (p < 0.0001), cardiac failure (p = 0.004), smoking (p = 0.017), diabetes (p = 0.049), and variation in the eNOS gene locus (p = 0.033). Smoking and variations in both eNOS [hazard ratio (HR) = 1.53, p = 0.011] and iNOS loci (HR = 1.52, p = 0.073) were found to impact upon poor survival. We found a strong interaction between smoking, female sex, and the iNOS R5/4 genotype with the risk of death (HR = 3.23, CI = 1.51–6.90, p = 0.002).

Compared with nonsmoking noncarriers, postmenopausal women who had been smokers and carried either the rare iNOS R5 allele (17.1%; HR = 4.23, CI = 1.84–9.75, p = 0.001) or the common eNOS 4b allele (71%; HR = 3.14, CI = 1.49–6.62, p = 0.003) were at a higher risk of death during the follow-up. These interactions were independent of each other, and were not found among men. **Conclusions:** The interaction between smoking and genetic variants of eNOS and iNOS predicts survival after stroke, especially among postmenopausal women.

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Introduction

Despite having a slightly lower incidence of stroke over the entire lifetime [1], women are twice as likely as men to die of stroke [2]. In Finland, for example, 62.6% of stroke mortality in 2005 occurred in women aged 65 years or older (Statistics Finland; http://www.stat.fi/index_en.html). During menopause, the incidence of ischemic stroke increases rapidly [3]. Postmenopausal wom-

N.O. had full access to all of the data in the study, and takes responsibility for its integrity and accuracy.

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en suffer more severe and fatal strokes than men [4], indicating the presence of possible unknown gender-specific factors.

Smoking is a significant risk factor for thromboembolic stroke in women [5]. Cigarette smoke contains large amounts of exogenous nitric oxide (NO), and results in the induction of NO synthesis in activated inflammatory cells [6], paralleled by increased levels of inducible nitric oxide synthase (iNOS) [7]. NO is involved in both normal brain physiology and ischemic stroke, and it also plays a role in the pathology of neurodegenerative diseases [8].

At present, 3 different isoforms of NOS are known: an endothelial form, i.e. constitutive (eNOS, cNOS), a neuronal form (nNOS), and an inducible isoform (iNOS). eNOS is located principally in the endothelial cells, and it plays a role in the normal regulation of vascular tone [9], and platelet adhesion [10] and aggregation [11]. Female sex steroids are important regulators of NO synthesis by directly increasing its activity by eNOS [12]. Patients with the 4a variant of the intron 4a/b promoter polymorphism of the eNOS gene have increased NO levels and significantly fewer lacunar infarcts [13].

In contrast to eNOS, NO produced by iNOS in microglia and macrophages at the area of injury has been proposed to play a role in free radical damage, and in worsening the injury after brain ischemia in mice [14] and in rat models [15] of middle cerebral artery occlusion, and also in humans [16]. The action of NO therefore depends on the site and circumstances of production. In an autopsy study of patients who had suffered an acute ischemic stroke, iNOS immunoreactivity and peroxynitrite formation as a consequence of NO production were demonstrated within the ischemic area [17]. In reperfusion of the ischemic brain, NO reacts with superoxide anion to form peroxynitrite, causing neuronal death [18]. Several agents inhibiting iNOS activation have yielded increased survival after experimental ischemic stroke in rats [19–21]. Definitive evidence is provided by the finding that the infarct size is reduced in iNOS-null mice, confirming a critical role for iNOS in ischemic brain injury [22]. In female mice, lower expression of iNOS explains the better tolerance of middle cerebral artery occlusion than that found in male mice, a phenomenon mediated by female sex steroids [23].

The iNOS gene is polymorphic at 2 sites, which may alter the functional activity of the gene [24–26]. The biallelic AAAT repeat located 0.75 kb upstream of the gene has been shown to alter the promoter activity [24–26]. We recently demonstrated that this polymorphic site is associated with the progression of atherosclerosis, especially in men aged >55 years [27].

There are no previous data on the effect of genetic polymorphisms of NOS and smoking on long-term survival after stroke. We hypothesized that the eNOS 4a/b or biallelic iNOS R5/4 polymorphism alone or together with smoking might modify long-term survival; therefore, explaining possible gender-specific differences in an established and thoroughly characterized cohort.

Methods

Patients

The Stroke Aging Memory cohort comprised a consecutive series of 486 Finnish patients (Caucasian; aged 55–85 years; 246 males, 240 females) recruited for the study 3 months after an ischemic stroke, between 1 December 1993 and 30 March 1995, as described previously [28, 29]. Inclusion and exclusion criteria were used as previously defined [30]. A detailed medical and neurological history was taken [28]. Three months after the stroke, MRI was performed [29]. Hypertension was defined as blood pressure $\geq 160/95$ mmHg recorded twice. Smoking habits were scored at admission as nonsmokers and smokers (current or former). Laboratory analyses included total and high-density lipoprotein (HDL) cholesterol, triglycerides, and fasting blood glucose. For the univariate survival analyses using dichotomous variables, hypercholesterolemia was defined as total cholesterol >5.0 mmol/l. Hypertriglyceridemia was defined as serum triglycerides >2.0 mmol/l. Low HDL cholesterol was defined as <1.2 mmol/l. Diabetes was defined as fasting blood glucose >7.0 mmol/l.

The study was approved by the Ethics Committee of the Department of Clinical Neurosciences, Helsinki University Central Hospital, Finland. The study was explained to the patients, and informed consent was obtained.

DNA Procedures

DNA was isolated from frozen blood samples through standard procedures, and amplified and analyzed as previously described [27, 31]. The eNOS 4a/b genotype was determined in 300 subjects (61.7%) and the iNOS R5/4 genotype in 310 subjects (63.8%) of the original cohort, which constituted the final study populations. The study populations with genotype data did not differ from the remaining patients in terms of demographic and clinical characteristics. The analyzed and excluded patients did not differ in either vascular risk factors or stroke subtype.

Stroke Subtypes

Three months after the stroke, a total of 396 patients (81.5%) in the Stroke Aging Memory cohort underwent a brain MRI investigation. The iNOS genotype and MRI investigation were available for 252 (51.9%) patients in the Stroke Aging Memory cohort. The eNOS genotype and MRI data were available for 230 (47.3%) patients. The final study populations did not differ from the remaining patients in terms of demographic and clinical characteristics. Based on the MRI data, stroke subtypes were analyzed and defined as lacunar or large vessel infarct [29, 30].

Table 1. Demographic data on patients with ischemic stroke (Stroke Aging Memory cohort)

Variable	Subjects, n	All (n = 310)	Men (n = 156)	Women (n = 154)	p
Survival, years	310	7.33 ± 3.9	7.3 ± 0.3	7.8 ± 0.3	0.301
Mean age, years	310	71.1 ± 7.7	69.5 ± 7.4	72.3 ± 7.9	0.001
Myocardial infarct, n	57	57 (18.4)	42 (26.9)	15 (9.7)	<0.0001
Cardiac failure, n	62	62 (20.1)	24 (15.4)	38 (24.7)	0.044
Arrhythmia, n	71	71 (22.9)	36 (23.1)	35 (22.7)	0.942
Atrial fibrillation, n	54	54 (17.4)	29 (18.6)	25 (16.2)	0.584
Peripheral arterial disease, n	38	38 (12.3)	24 (15.4)	14 (9.1)	0.091
Hypertension, n	141	144 (46.5)	64 (41.0)	80 (51.9)	0.054
Diabetes, n	68	68 (21.9)	39 (25.0)	29 (18.8)	0.189
Smoking, n	164	164 (53.1)	112 (71.8)	52 (33.8)	<0.0001
fS-cholesterol, mmol/l	310	5.6 ± 1.2	5.4 ± 1.2	5.8 ± 1.1	0.003
fS-HDL, mmol/l	310	1.1 ± 0.3	1.1 ± 0.3	1.2 ± 0.3	<0.0001
fS-triglyceride, mmol/l	310	1.5 ± 0.7	1.5 ± 0.7	1.6 ± 0.8	0.484
iNOS (R5/4, R5/5) ^a	55	55 (17.7)	31 (19.9)	24 (15.6)	0.323
eNOS (4b/b) ^b	213	213 (71.0)	115 (72.3)	98 (69.5)	0.591

Values are presented as means ± SD, with percentages in parentheses. Continuous variables: Student's t test for independent samples (men vs. women). fS = Fasting blood serum. Non-continuous variables: Pearson χ^2 test (men vs. women).

^a iNOS genotype could be determined in 310 subjects (156 men and 154 women).

^b eNOS genotype could be determined in 300 subjects (159 men and 141 women).

Survival

Long-term survival data at 21 September 2006 were obtained from Statistics Finland. Of the original 486 patients, 347 (71.4%) had died. Of the 300 patients with eNOS genotype data, 213 (71.0%) had died, and of the 310 patients with iNOS genotype data, 223 (71.9%) had died during the follow-up. The causes of death based on ICD-9 and ICD-10 classifications were also obtained, and divided further into cardiac, brain-related (ischemic stroke, bleeding, vascular dementia), cancer, infection, trauma, and other categories. In 9 cases, the cause of death could not be determined, and these were excluded when analyzing the causes of deaths.

Statistical Analysis

The software SPSS for Windows (version 12.0, SPSS) was used. Pearson's χ^2 and Student's t test were used to compare subgroups. The effect of classical risk factors, sex, myocardial infarct, cardiac failure, arrhythmia, atrial fibrillation, hypertonia, peripheral arterial disease, diabetes, hypercholesterolemia, low HDL cholesterol, hypertriglyceridemia, and history of smoking, on survival were first analyzed by the Kaplan-Meier log-rank test. In Cox regression survival analysis and binary logistic regression analysis of the infarct subtypes, the following covariates were used: sex, age, myocardial infarct, cardiac failure, arrhythmia, atrial fibrillation, hypertonia, peripheral arterial disease, diabetes, absolute total and HDL cholesterol, triglycerides and history of smoking. Results are presented as hazard ratios (HR) with 95% confidence intervals (CI). Interactions between sex, genotype, and smoking were analyzed by creating an interaction term within the Cox regression analysis using the same covariates. In stratified analyses, the study subjects were categorized as follows: (1) nonsmokers with the iNOS R4/4 or the eNOS 4a/b or 4a/a genotype, (2) non-

smokers with the iNOS R5/4 or R5/5 or the eNOS 4b/b genotype, (3) smokers with the iNOS R4/4 or the eNOS 4a/b or 4a/a, and (4) smokers with the iNOS R5/4 or R5/5 or the eNOS 4b/b genotype using the same covariates.

Results

General Demographic Findings

The mean age of the female patients was higher (72.3 vs. 69.5 years, $p = 0.001$) than that of the males. Of the 310 patients with iNOS genotype data, 223 had died (71.9%) during the follow-up; 120 of them were males and 103 females ($p = 0.295$). Of the 300 patients with eNOS genotype data, 213 had died (71.0%) during the follow-up; 117 of them were males and 96 were females ($p = 0.295$). The mean survival after stroke was almost the same for men and women (7.3 vs. 7.8 years, $p = 0.301$; table 1).

Significantly more men than women had a history of myocardial infarction (26.9 vs. 9.7%, $p < 0.0001$), and they were more often smokers (71.8 vs. 33.8%, $p < 0.0001$). Compared to men, women were more likely to suffer from cardiac failure ($p = 0.044$) and had nearly significantly more hypertension ($p = 0.054$) and significantly more higher total cholesterol ($p = 0.003$) and HDL cholesterol ($p < 0.0001$) values (table 1).

Table 2. Cox regression analysis on the association of multiple risk factors with long-term survival among patients with ischemic stroke (Stroke Aging Memory cohort)

Variable	All (n = 310)			Men (n = 156)			Women (n = 154)		
	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p
Female sex	0.83	0.59–1.17	0.289						
Age	1.08	1.05–1.10	<0.0001	1.06	1.03–1.10	<0.0001	1.09	1.05–1.13	<0.0001
Myocardial infarct	1.18	0.82–1.75	0.347	1.14	0.71–1.83	0.583	2.50	1.16–5.36	0.019
Cardiac failure	1.68	1.20–2.39	0.004	1.29	0.75–2.21	0.355	1.94	1.11–3.37	0.020
Arrhythmia	1.04	0.55–1.98	0.905	0.87	0.30–2.55	0.812	1.17	0.49–2.81	0.721
Atrial fibrillation	1.32	0.65–2.69	0.445	1.40	0.46–4.32	0.556	1.98	0.72–5.39	0.184
Peripheral arterial disease	1.35	0.90–2.02	0.148	1.71	1.02–2.86	0.042	0.80	0.36–1.77	0.573
Hypertension	0.90	0.67–1.20	0.468	0.95	0.63–1.45	0.825	0.85	0.54–1.35	0.501
Diabetes	1.42	1.00–2.02	0.049	1.40	0.87–2.23	0.172	1.80	0.99–3.27	0.056
Smoking	1.49	1.07–2.07	0.017	1.11	0.69–1.79	0.678	2.08	1.29–3.35	0.003
fS-cholesterol	0.95	0.83–1.10	0.523	0.92	0.76–1.12	0.411	1.18	0.91–1.54	0.207
fS-HDL	1.01	0.62–1.65	0.965	1.43	0.71–2.90	0.316	0.48	0.21–1.10	0.082
fS-triglyceride	1.08	0.84–1.38	0.564	0.95	0.62–1.45	0.810	0.87	0.59–1.28	0.471
iNOS (R5/4; R5/5 vs. R4/4) ^a	1.12	0.77–1.62	0.551	0.93	0.56–1.54	0.785	1.42	0.80–2.55	0.233
eNOS (4b/b vs. 4a/b; 4a/a) ^b	1.44	1.03–2.01	0.033	1.52	0.94–2.46	0.089	1.69	1.01–2.82	0.046

fS = Fasting blood serum.

^a iNOS-genotype could be determined in 310 subjects (156 men and 154 women).

^b eNOS-genotype could be determined in 300 subjects (159 men and 141 women).

The distributions of the eNOS ($p = 0.591$) and iNOS ($p = 0.323$) genotypes were similar for both sexes. The genotype distribution of eNOS genotypes was 71.0% (b/b), 26.3% (a/b), and 2.7% (a/a). The distribution of iNOS genotypes was 82.9% (4/4), 17.1% (5/4), and 0% (5/5). The allele distributions were in Hardy-Weinberg equilibrium. There was no linkage disequilibrium between the eNOS and iNOS polymorphisms (Pearson's χ^2 $p = 0.690$).

Phenotype of Stroke

In the logistic regression analyses, the iNOS R5/4 or eNOS 4b/b genotypes were not associated with stroke subtypes determined by MRI. There were no interactions between smoking and either iNOS R5/4 (OR = 1.75, CI = 0.64–4.81, $p = 0.275$ for lacunar stroke; OR = 2.10, CI = 0.77–5.76, $p = 0.148$ for large-vessel stroke) or eNOS 4b/b genotypes (OR = 1.26, CI = 0.54–2.96, $p = 0.591$ for lacunar stroke; OR = 1.09, CI = 0.48–2.48, $p = 0.845$ for large-vessel stroke) on stroke subtypes. There were no interactions between NOS genotypes and sex in infarct subtypes.

Factors Affecting Long-Term Survival

In univariate Kaplan-Meier log-rank analysis of the classical risk factors (for a list of parameters, see 'Statistical

Analysis'), history of smoking ($p = 0.019$), myocardial infarct ($p = 0.023$), cardiac failure ($p < 0.0001$), atrial fibrillation ($p < 0.0001$), peripheral arterial disease ($p = 0.019$) and smoking ($p = 0.019$) were the only factors associated with poor survival. Diabetes tended to increase the risk of poor survival ($p = 0.071$). In these analyses, neither sex nor eNOS or iNOS genotype showed an association with survival.

In Cox regression survival analysis adjusted for classic risk factors (table 2), poor survival was found to relate to each additional year of age (HR = 1.08, CI = 1.05–1.10, $p < 0.0001$), cardiac failure (HR = 1.68, CI = 1.20–2.39, $p = 0.004$), smoking (HR = 1.49, CI = 1.07–2.07, $p = 0.017$), diabetes (HR = 1.42, CI = 1.00–2.02, $p = 0.049$), and the eNOS 4b/b genotype (HR = 1.44, CI = 1.03–2.01, $p = 0.033$). This association of poor survival with the eNOS 4b/b genotype was observed in women (HR = 1.69, CI = 1.01–2.82, $p = 0.046$), and there was a tendency in men (HR = 1.52, CI = 0.94–2.46, $p = 0.089$) (table 2).

Interaction of NOS Genotypes with Smoking and Sex

Using the same Cox regression model adjusted with the same covariates in the whole population, there was an interaction between smoking and the eNOS 4b/b genotype (HR = 1.53, CI = 1.10–2.12, $p = 0.011$), as well as a

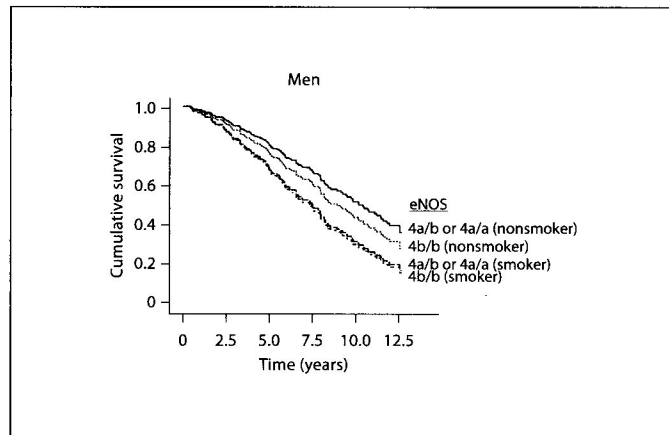
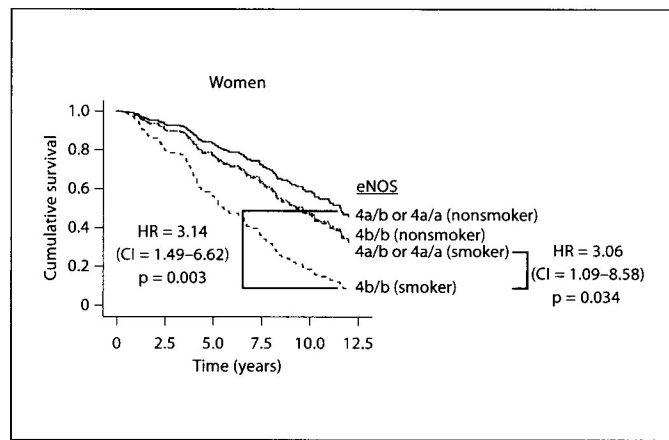
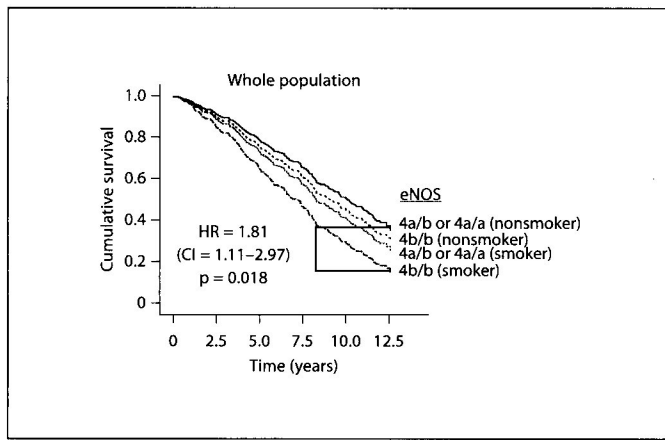


Fig. 1. The effect of the eNOS 4a/b polymorphism on cumulative survival after stroke during long-term follow-up. Survival function according to Cox regression analysis with multiple covariates (see 'Statistical Analysis' and table 2).

nearly significant one between smoking and the iNOS R5/4 genotype (HR = 1.52, CI = 0.96–2.40, $p = 0.073$) with regard to poor survival. These interactions remained significant only among women (eNOS 4b/b and smoking: HR = 3.74, CI = 1.64–8.53, $p = 0.002$; iNOS R5/4 and smoking: HR = 2.39, CI = 1.34–4.22, $p = 0.003$) and were not observed among men (eNOS 4b/b and smoking: HR = 1.28, CI = 0.83–1.97, $p = 0.266$; iNOS R5/4 and smoking: HR = 1.05, CI = 0.60–1.86, $p = 0.856$). More interestingly, we found a strong interaction between smoking, female sex, and the iNOS R5/4 genotype (HR = 3.23, CI = 1.51–6.90, $p = 0.002$), but not the eNOS 4b/b genotype ($p = 0.156$), in relation to poor outcome. No interactions were found between the iNOS and eNOS genotypes.

Consequently, we stratified the data based on history of smoking and the iNOS R5/4 or eNOS 4b/b genotype. In Kaplan-Meier log-rank survival analysis, smokers with the iNOS R5/4 genotype had nearly significantly inferior survival rates compared with nonsmoking patients with

the R4/4 genotype ($p = 0.054$) and with nonsmoking patients with the R5/4 genotype ($p = 0.016$). Smoking subjects with the R4/4 genotype had inferior survival rates compared with nonsmoking subjects with the R5/4 genotype ($p = 0.020$).

In Cox regression analysis, adjusted with classical covariates, smoking carriers of the eNOS 4b/b genotype (HR = 1.81, CI = 1.11–2.97, $p = 0.018$; fig. 1) as well as of the iNOS R5/4 genotype (HR = 1.82, CI = 1.09–3.06, $p = 0.023$; fig. 2) had the highest risk of poor survival when compared with nonsmoking noncarriers. In women, smoking carriers of the iNOS R5/4 genotype (HR = 4.23, CI = 1.84–9.75, $p = 0.001$) and the eNOS 4b/b genotype (HR = 3.14, CI = 1.49–6.62, $p = 0.003$), as well as smoking noncarriers of the iNOS R5/4 genotype (HR = 1.75, CI = 1.03–2.99, $p = 0.039$) were at an increased risk of poor survival when compared to nonsmoking noncarriers (fig. 1, 2). The difference between smoking female iNOS R5/4 genotype carriers and smoking noncarriers was also significant (HR = 2.57, CI = 1.10–6.00, $p = 0.029$; fig. 2).

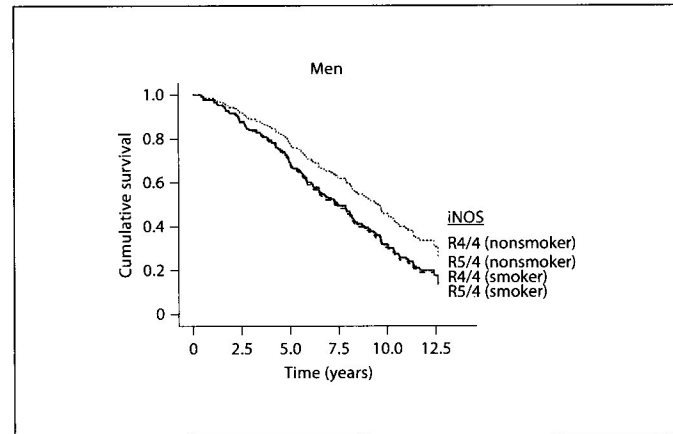
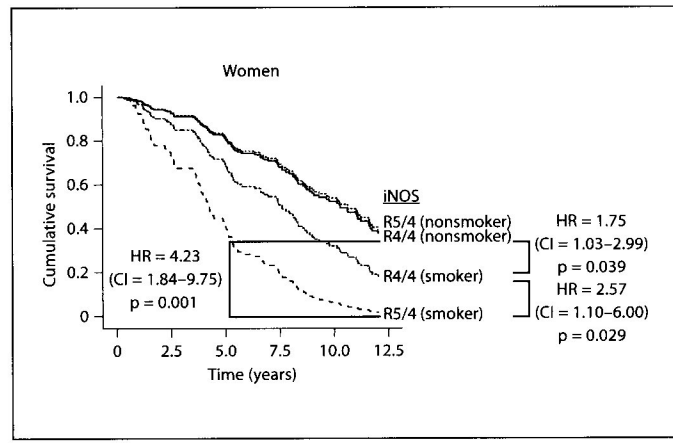
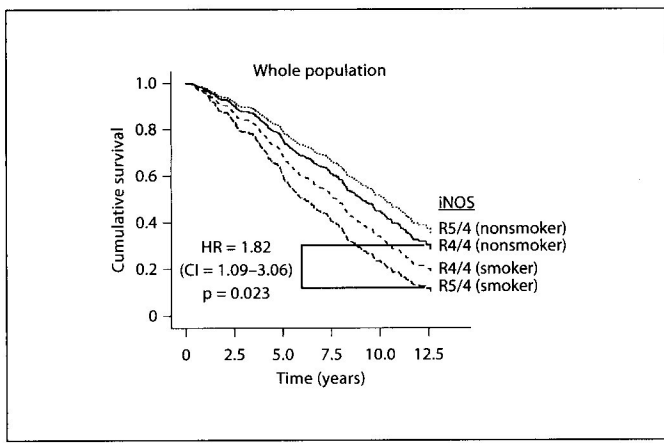


Fig. 2. The effect of smoking and iNOS R5/4 polymorphism on cumulative survival after stroke during long-term follow-up. Survival function according to Cox regression analysis with multiple covariates (see 'Statistical Analysis' and table 2).

In men, no significant effects were found. The difference between smoking female patients with the eNOS 4b/b genotype and smoking patients with the 4a/b or 4a/a genotype was significant only in females (HR = 3.06, CI = 1.09–8.58, $p = 0.034$; fig. 1).

Causes of Death of Stroke Patients

The majority of patients (68.7%) died of cardiovascular causes: 29.6% of them died of cardiac and 39.1% of cerebrovascular causes. There was a trend of men rather than women dying of cancer ($p = 0.076$; table 3). Neither the eNOS nor iNOS genotypes were associated with the causes of death.

Discussion

We found that among stroke survivors, the carriage of the eNOS 4b/b genotype alone potentiated the risk of poor long-term survival, and there was a strong interac-

tion between history of smoking at recruitment and both the eNOS 4b/b and iNOS R5/4 genotype. This interaction of genotype with smoking was evident only among women, supporting the hypothesis that the iNOS or eNOS genotype and smoking may modify long-term survival and explain gender-specific differences.

Over the entire lifetime, women have about a 16% and men only an 8% risk of dying of stroke [2]. The burden of stroke is heavier in women, which is explained at least partially by the fact that women live longer than men [32]. In the present study, however, there were as many survivors among both sexes at the time of recruitment for this university hospital series. It is obvious that there were more women among those who did not reach the hospital or otherwise dropped out of the study before the evaluation at 3 months. The frequencies of the eNOS 4a and 4b alleles are in line with previous findings in Caucasian populations [31, 33], although no comparable data on stroke cohorts exist.

Table 3. Causes of death among patients with ischemic stroke (Stroke Aging Memory cohort)

Cause of death	Valid data n	All (n = 338)	Men (n = 172)	Women (n = 166)	p
Brain-related	338	132 (39.1)	61 (35.5)	70 (42.2)	0.168
Ischemic stroke	338	110 (32.5)	51 (29.7)	59 (35.5)	0.248
Bleeding	338	12 (3.6)	5 (2.9)	7 (4.2)	0.515
Dementia	338	10 (2.9)	5 (2.9)	5 (3.0)	0.955
Cardiac	338	100 (29.6)	51 (29.7)	49 (29.5)	0.979
Cancer	338	46 (13.6)	29 (16.9)	17 (10.2)	0.076
Infection	338	15 (4.4)	6 (3.5)	9 (5.4)	0.388
Trauma	338	12 (3.6)	7 (4.1)	5 (3.0)	0.599
Other	338	43 (12.7)	23 (13.4)	20 (12.0)	0.715

All figures in parentheses are percentages. Analysis carried out with Pearson χ^2 test (men vs. women).

From the categories all, men, and women, 9, 6, and 3 cases, respectively, were excluded as the cause of death could not be specified.

As reporter gene constructs from the biallelic site of the iNOS gene (R5/4) have shown a different promoter activity of these 2 variants [26], it may be speculated that the R5/4 genotype results in an increased production of iNOS, nitrotyrosine formation, enlargement of the ischemic lesion [34], and an inflammatory response mediated by NO during stroke [35], although no definitive information exists about the effect on NO production in humans. In a mouse model of ischemic stroke, iNOS was demonstrated to play a role in the late phase of infarct enlargement in male subjects, while female mice and iNOS-gene-disrupted animals exhibited no enlargement of the infarct area and smaller infarct volumes. This suggests that female mice are protected against the deleterious effects of NO or iNOS activity [14]. In experimental models, this phenomenon has been demonstrated to be dependent on female sex steroids [23], at least partially through the suppression of iNOS expression [36] and of injury-induced proinflammatory cytokine release [37]. Previously, we have shown that among men aged >55 years, carriers of the R5 allele presented higher mean values of coronary stenosis and larger areas of fatty streaks and complicated lesions than R4/4 carriers [27]. Therefore, it is possible that the effects of the R5 allele on survival are mediated by a cardiac mechanism. However, one must interpret these results with caution since this material consisted of men who had died a sudden death [27]. Approximately half of our study population consisted of postmenopausal female patients aged 55–85 years whose levels of both progesterone and estrogen were low. This raises an important question of whether women

with a polymorphism of the iNOS gene are dependent on progesterone and estrogen. It must be emphasized that for humans, no clinical evidence exists regarding protection against recurrence of stroke or mortality in postmenopausal women with cerebrovascular disease by hormone replacement therapy [38]. A potential weakness in the present study is that the number of women smokers carrying the iNOS R5 allele is rather low, as reflected by the wide confidence intervals. Therefore, future studies replicating these results in larger cohorts are needed.

The role of eNOS polymorphisms in stroke is not clear, and it is possibly confounded by interactions with other polymorphisms. The eNOS gene is polymorphic in exon 7 (G894T), but it was not associated with stroke [39]. However, a significant interaction between the eNOS G894T and methylenetetrahydrofolate reductase 677TT genotype and angiotensin-converting enzyme D/D genotype in relation to the risk of ischemic stroke has been demonstrated [40]. The promoter variants 922A and 786T in the eNOS gene were associated with ischemic stroke; however, this was found in young black women only [41]. Our results of impaired survival with the eNOS 4b/b genotype are in accordance with the finding that the eNOS 4a allele has been found to protect against isolated lacunar infarct, mediated by functional changes in eNOS promoter activity resulting in increased NO levels [13]. The data on the effect of the 4a allele on cardiac morbidity and mortality is conflicting – according to a meta-analysis, homozygosity for the rare 4a allele results in moderately increased risk of coronary heart disease [42, 43]. In contrast, we have previously shown with middle-

aged men, who had died suddenly of acute myocardial infarction, that the eNOS 4a allele is associated with a significantly lower risk of myocardial infarction when compared to patients homozygous for the b allele [31]. This warrants future studies on environmental-genetic interactions in the cause of death after stroke.

The causes of death in the present study were similar to a previous study [44], the only difference being a slightly higher frequency of cardiac causes (30 vs. 23%) and lower frequency of traumatic causes (4 vs. 12%). In a large-scale 10-year follow-up study, the frequencies of cerebrovascular and cardiovascular causes were also similar [45].

To our knowledge, the present study is the first to demonstrate an interaction between genetic polymorphisms of NOS genes and smoking on long-term survival after stroke. Our results suggest that genetic variation in NOS is one of the factors affecting long-term survival after stroke, and that there is a strong interaction between smoking and these genetic variants, predicting survival

after stroke, especially among postmenopausal women. To our knowledge, our series is unique with a long-term follow-up of 7.6 years (mean). However, future studies replicating these preliminary findings, especially in women in independent stroke populations, are warranted.

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