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GENETIC ANALYSIS OF ISCHEMIC STROKE AND PREDISPOSING CAROTID ARTERY STENOSIS

~ A STROKE CAROL ~

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I have endeavoured in this Ghostly little book, to raise the Ghost of an Idea, which shall not put my readers out of humour with themselves, with each other, with the season, or with me. May it haunt their houses pleasantly, and no one wish to lay it.

Charles Dickens, 1843

To my family

ABSTRACT

Ischemic cerebrovascular disease (ICVD), consisting of ischemic stroke and transient ischemic attacks (TIA), is a complex disease where contribution from both the environment and genes promote pathogenesis of the disease-complex. Carotid stenosis is sometimes a predisposing factor or cause for ICVD and a complex disease in itself. Studies of adoptees and twins support the idea that genes are of importance in ICVD and family studies in CS. Two different strategies have been applied to search the genome for ICVD genes, a candidate genome approach and a genome-wide search in familial disease. During the last two decades different candidate genes coding for coagulation-proteins, lipoproteins, renin-angiotensin-aldosterone system and different inflammatory genes have been investigated with different results. Successful examples of the genome-wide search are the phosphodiesterase 4D (PDE4D) gene and the 5-lipoxygenase activating protein (ALOX5AP) gene found in an Icelandic population. These genes have later been studied as a candidate gene in different populations.

The South Stockholm Ischemic Stroke Study (SSISS) was started in 1996 to study genetic associations of ICVD and predisposing carotid stenosis (CS), the hypothesis being that genetic factors could be used to find more coherent pathogenetical subgroups to the ischemic cerebrovascular disease complex to facilitate a more individual treatment and prophylaxis. During the first phase of the project three suspected susceptibility genes were examined in a smaller cohort (<200) of patients, namely lipoprotein lipase (LPL), methylene-tetrahydrofolate reductase (MTHFR) and angiotensin-converting enzyme (ACE). We concluded that LPL and MTHFR polymorphisms did not contribute greatly to the overall risk of ICVD or CS. In the examination of ACE we found a significant difference for the presumed susceptibility allele in patients with CS compared to healthy controls and age-matched non-CS ICVD patients. We concluded that the ACE gene polymorphism is a risk factor for the development of CS.

In the last phase of the project we expanded the material to more than 1000 patients with ICVD and phenotyped the subjects in detail including subtyping by the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria. We went on to examine PDE4D and ALOX5AP, genes discovered by linkage analysis on Iceland. We were not able confirm the association between the ALOX5AP HapA haplotype and ICVD, but a non-significant risk was observed in the large artery atherosclerosis (LAA) TOAST subtype. Our PDE4D findings although non-significant considering the number of markers and phenotypes tested, were consistent with the original Icelandic association, with a trend in the whole ICVD group, strengthened in LAA and the combined group of LAA and cardio embolic (CE) subtypes.

In the last study we examined 100 polymorphisms in 47 suspected susceptibility genes and found three polymorphisms to be weakly associated with ICVD after correction for age and gender (LPL, angiotensinogen and guanine nucleotide-binding protein beta-3). These markers were differently prevalent in the subtypes examined. Factor VII, apolipoprotein E and two renin polymorphisms were significantly more frequent in patients with evidence of CS compared to non-CS patients.

In conclusion we have found weak associations to some candidate genes and by subdividing ICVD patients by presence of CS the patterns of association change. We therefore conclude that larger well phenotyped ICVD cohorts are needed.

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LIST OF ABBREVIATIONS

ACA	Anterior cerebral artery
ACE	Angiotensin-converting enzyme
AF	Atrial fibrillation
ALOX5AP	arachnoidate 5-lipoxygenase-activating protein
APOA1	Apolipoprotein A1
APOE	Apolipoprotein E
BA	Basilar Artery
CADASIL	Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy
CCA	Common carotid artery
CE	Cardio embolism
CEA	Carotid endarterectomy
CEPH	Centre d'etude du polymorphisme humaine
CHD	Coronary heart disease
CRP	C-reactive protein
CS	Carotid stenosis
CT	Computer tomography
CTA	Computer tomography angiography
CVD III	Cerebrovascular Disease III of the National Institute of Neurological Disorders and Stroke
DNA	Deoxyribonucleic acid
ECA	External carotid artery
ECG	Electrocardiogram
ECST	European Carotid Surgery Trial
ENOS	Endothelial nitric oxide synthase
E-Sel	E-selectin
FHx	Family history
FLAP	5-lipoxygenase activating protein
GWAS	Genome-wide association study
Hcy	Homocysteine
HDL	High-density lipoprotein
ICA	Internal carotid artery
ICAM-1	Intercellular adhesion molecule-1
ICVD	Ischemic cerebrovascular disease
IL6	Interleukin-6
IMT	Intima-media-thickness
IS	Ischemic stroke
LAA	Large artery atherosclerosis
LD	Linkage disequilibrium
LDL	Low-density lipoprotein
LPL	Lipoprotein lipase
LTB4	Leukotriene B4
MCA	Middle cerebral artery
MCP-1	Monocyte chemoattractant protein-1

MI	Myocardial infarction
MIF	Macrophage migration inhibitory factor
MMP	Matrix metalloproteinase
MMP3	Matrix metalloproteinase 3
MRA	Magnetic resonance angiography
MRI	Magnetic resonance imaging
MTHFD1L	methylenetetrahydrofolate dehydrogenase (NADP1-dependent)1-like
MTHFR	Methylenetetrahydrofolate reductase
NASCET	North American Symptomatic Carotid Endarterectomy Trial.
NCBI	National center for biotechnology information
NNT	Numbers needed to treat
NO	Nitric oxide
NORVIT	Norwegian Vitamin Trial
OCSP	Oxford Community Stroke Project
OR	Odds ratio
PAD	Peripheral arterial disease
PAI	plasminogen activator inhibitor-1
PCA	Posterior cerebral artery
PDE4D	Phosphodiesterase 4D
PON	Paraoxonase
RCT	Randomized controlled trial
RRR	Relative risk reduction
SAO	Small arterial occlusion
SCD	Sickle cells disease
SNP	Single nucleotide polymorphism
SSISS	South Stockholm ischemic stroke study
TAFI	Thrombin Activatable Fibrinolysis Inhibitor
TIA	Transient ischemic attack
TOAST	Trial of Org 10172 in Acute Stroke Treatment
tPA	Tissue-type plasminogen activator
WTCCC	Wellcome trust case control consortium

STAVE 1: MENDEL'S GHOST

Mendel *was dead: to begin with. There is no doubt whatever about that.* But his legacy is very much alive in the 21st century. With the end of the Human Genome Project and the International HapMap Project, future dreams of understanding our genes, ancestry and the diseases that plague us are within reach. And to think that it all started with breeding peas. But peas can grow into gigantic beanstalks if stories are true…

STAVE 2: THE FIRST OF THREE SPIRITS

STROKE OF CHRISTMAS PAST

When Hippocrates *awoke, it was so dark, that looking out of bed, he could scarcely distinguish the transparent window from the opaque walls of his chamber.*
He got up and later, as the father of Medicine, he was naturally the first to describe a stroke in the 4th century B.C. The word apoplexy, derived from the Greek "struck down by violence" was used instead of stroke, due to the fact that the person struck, developed a sudden paralysis and change in well-being. Hippocrates even recognized transitory ischemic attacks (TIA), describing them as "unaccustomed attacks of numbness and anesthesia are signs of impending apoplexias".

In the first century Rufus of Ephesus used the term karotide or karos, meaning "plunge into a deep sleep" for the frontal arteries leading to the brain, this because compression of these vessels led to stupor or sleep.

A few years later Galen described that hemiplegia was due to a lesion in the opposite side of the brain and attributed it to congestion of phlegm in the arteries.
Not until Baroque times, Jacob Wepfer identified the cause of apoplexy in his accurately named publication Apoplexia as a bleeding in the brain in patients who had died of the disease. He postulated that blockage of the arteries leading to the brain, the carotids and vertebrals, could lead to apoplexy. History went on, but it wasn't until the 1920s that apoplexy was divided into the different causes of vessel problem and the term "stroke" was born [1].

WHAT IS A STROKE?

Stroke is defined as a sudden, nonconvulsive loss of neurologic function due to an ischemic or hemorrhagic intracranial vascular event. In general, stroke, apoplexy or cerebrovascular accidents are classified by anatomic location in the brain, vascular distribution, etiology, age of the affected individual, and hemorrhagic vs. non-hemorrhagic nature.

Symptoms of a stroke are naturally related to what part of the brain that is affected, broadly defined as anterior circulation or carotid territory and posterior circulation or vertebro-basilar territory. Symptoms are in most cases distinct and include weakness (paresis), sensory disturbances (dysesthesia), vocal dysfunction (dysphasia), visual defects (hemianopsia), slurred speech (dysarthria), difficulties to coordinate limbs (ataxia), vertigo etc. These symptoms are not always due to a stroke and in the era before computer tomography (CT) the clinical findings and development of the disease decided if a stroke or another neurological disease was responsible for the symptoms.

Therefore CT scanning has revolutionized the field and the possibility to define the region of interest or the cause, hemorrhage or ischemic lesion. Computer tomography has its limits, in not being able to distinguish early lesions and infratentorial lesions. This leads to heterogeneity in diagnosing the disease in the elderly where there are a lot

of white matter hypodensities. Vertigo as an only symptom of infratentorial ischemic lesion can be hard to distinguish from non-cerebral reasons for vertiginous feelings.

Transitory ischemic attack (TIA), a precursor of stroke just as angina is for myocardial infarction, can not be detected by CT due to the fact that the symptoms are temporally short and do not lead to visible tissue damage. TIAs can therefore be readily mistaken for non-vascular disease leading to neurological symptoms, such as migraine aura, vertigo etc. This leads to an increasing heterogeneity in diagnosing these events, which is important since they might be pre-emptive signs strokes to come.

PATHOLOGY OF ISCHEMIC CEREBROVASCULAR DISEASE

Ischemic stroke and TIA, together labelled ischemic cerebrovascular disease (ICVD) is a heterogeneous disorder when looking at its causes and outcome. Many different etiologies can lead to ICVD, of which some are inherited and some due to exogenous factors.

Different taxiologies are currently being used to define different causes or types of stroke, both based on clinical presentation; such as the Oxfordshire Community Stroke Project (OCSP) classification [2] or based on clinical investigations in conjunction with some clinical characteristics; such as the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification [3-6] or Classification of Cerebrovascular Disease III (CVD III) of the National Institute of Neurological Disorders and Stroke [7]. The most commonly used pathogenetic classification is the TOAST criteria; however this classification has been designed to classify ischemic strokes and not TIAs.

Stroke Subtypes

In general one can divide the pathological causes of ischemic stroke in three different groups based on the origin of the embolus/thrombus or location of lesion; atherothrombotic (also called large vessel disease, large artery atherosclerosis (LAA)), cardioembolic (CE) and lacunar (also called small vessel disease or small artery occlusive disease (SAO)). In general large vessel disease is atherosclerotic, cardioembolic due to emboli from the heart of different aetiologies and lacunar due to lipohyalinosis and microatheromas [8] in the small arterioles of the brain. Of course other more uncommon diseases may lead to strokes from the large arteries such as dissection and vasculitis and the small vessels of the brain; such as monogenic disorders.

Large Artery Atherosclerosis and Carotid Stenosis

Large artery atherosclerosis including carotid stenosis is the most common cause of ischemic cerebrovascular disease and, as stated above, is due to atherosclerosis of the large and medium sized precerebral and cerebral arteries. The most common location for the atherosclerotic lesions; plaques, is in the bifurcation of the carotid artery where the common carotid artery (CCA) divides into the external carotid artery (ECA, supplying the face and thyroid gland) and the internal carotid artery (ICA, supplying the frontal ⅔ of the cerebrum and the retina). Other common locations extracranially are the vertebral ostiums and the carotid siphon as well as the proximal segments of the

large intracerebral arteries (middle cerebral artery (MCA), anterior cerebral artery (ACA), posterior cerebral artery (PCA) and basilar artery (BA). The predominance of atherosclerotic lesions is different in different populations; favouring extracranial locations in Caucasians and intracranial in Asians, Africans and Hispanics [9, 10].

The atherosclerotic lesion evolves from an intima-media thickness (IMT, the two inner vessel walls) developing into a plaque which, as it enlarges, leads to a stenosis of the vessel. In some cases an ulceration of the plaque leads to thrombus formation and emboli. The stenosis can also progress to an occlusion of the vessel and through a hemodynamic mechanism leading to an ischemic event. The stenoses also have effects on the aggregation of thrombocytes by activating surface proteins on platelets by the increased shear rate [11].

Carotid stenosis (CS) is a very common phenotype of the atherosclerotic disease in the precerebral arteries and can easily be visualized by ultrasonography, angiography, magnetic resonance angiography (MRA) and computer assisted tomography angiography (CTA). However, only ultrasonography is minimally invasive and easily available in most clinical settings including the emergency room at certain hospitals. In Sweden ultrasonography is currently the required investigation for deciding upon operating a carotid stenosis.

The evidence for CS as a causal factor for ICVD is plentiful; including epidemiological studies of patients with ischemic strokes and CS as well as asymptomatic CS [12-14]; randomized controlled trials (RCT) of surgical treatment (Carotid endarterectomy, CEA) of symptomatic/asymptomatic CS [15-20].
Many different subphenotypes of carotid stenosis have been studied from its precursor IMT, to plaque surface area, plaque morphology and stenosis degree. In the RCTs investigating CEA the stenosis degree of 50 % was defined as a significant stenosis, and treatment was most beneficent for a stenosis above 70 % (NASCET, again declining at the pre-occlusive stage). The North-American (NASCET) and the European (ECST) RCT used different methods of calculating stenosis degree, based upon angiography, as shown in table 1.

Table 1. Comparison between degrees of stenosis for the two large trials.

Study	NASCET	ECST
Stenosis degree	70-99%	80-99%
	50-69%	65-80%
	30-49%	50-65%
	<30%	<50%

NASCET, North American Symptomatic Carotid Endarterectomy Trial.
ECST, European Carotid Surgery Trial

Since atherosclerosis of the precerebral anterior circulation is so easily accessible for investigation, studies of posterior circulation and intracranial circulation are less common, but there is evidence for the their importance in ischemic events of the brain.

Intracranial lesions are even harder to study, but some epidemiological studies and one RCT shows that they are extremely dangerous with a recurrence rate up to 25 % annually [21]. In the TOAST criteria atherosclerotic lesions of the aortic arch are not included as a possible atherosclerotic cause of stroke. Recent studies show that atheromas > 4mm thick increase the risk of stroke [22-26] and currently an RCT is assessing the treatment options for this condition [23].

Clinical manifestations of LAA include large cortical infarctions, hemodynamic watershed lesions and TIAs, for example amaurosis fugax (monocular blindness).

Carotid stenosis is quite easily investigated and clinically relevant. We decided to use it as an intermediate phenotype in our studies of the genetics of ischemic cerebrovascular disease.

Cardioembolism

Cardioembolic strokes are the most common strokes, approximately 30 % of all strokes [27], but if you take into account TIAs, atherosclerotic lesions are more common (LAA). The pathogenic mechanism for cardio embolic events is through emboli arising from thrombi in the heart. In ⅔ of cases these emboli go to the brain and in ⅓ to the extremities and mesenterial arteries.

The most common cause of cardio embolic events is atrial fibrillation. Atrial fibrillation is classed as an independent risk factor for IS by mechanism of formation and embolism of left atrial appendage thrombi. Approximately 60 000 strokes occur in patients with AF in the US each year [28]. The prevalence of AF increases with age reaching a peak of 5% at the age of 65 years and above. The stroke risk of stroke is at least 6 times higher in AF patients compared to healthy controls [29]. In table 5 the different morphological and etiological causes for cardioembolism are listed.

The clinical features of cardioembolic strokes are described as a rapid onset to maximum symptom levels. The lesions range from large cortical and subcortical infarctions in multiple vascular territories, sometimes including systemic emboli, to very small localized infarctions leading to specific aphasias and singular symptoms. TIAs are less common than in LAA.

Small Artery Occlusive Disease

Lacunar infarctions were first described by Fisher in the 1960s after a series of post-mortem investigations of small infarctions (<2 cm) localized in the deep brain structures (basal ganglia, thalamus and brain stem) accompanied by certain clinical lacunar syndromes. These above mentioned structures are supplied by deep penetrating end-arteries. Fisher found evidence of atherosclerosis or lipohyalinosis, postulating that this leads to occlusive disease of these arteries and thus small localized infarctions, so called lacunae. These lesions were strongly associated to hypertension in the first studies done by Fisher [30]. Later the association to hypertension was shown to be less evident [31, 32].

My own experience and other researchers is that a lacunar syndrome does not always have to be caused by a lacunar infarction but also small cortical lesions. In fact many other causes can lead to small infarction in the deep structures of the brain ranging from cardioembolism to coagulation disorders and vasculitis [33].

In the TOAST criteria lacunar infarctions are defined up to a size of 1.5 centimetres. Patients with lacunar strokes have less frequent TIAs than other subgroups of stroke [31]. Lacunar TIAs have also been described [34-36].

Other causes of ischemic cerebrovascular disease

The most common non-atherosclerotic, non-cardioembolic causes of ICVD are uncommon coagulation disorders, arterial dissection, vasculitis and monogenic disorders. The TOAST criteria recognize a multitude of disorders as shown in table 2 [4]. Explanations for the diagnostic testing can be found online at: http://stroke.mc.duke.Edu/related_links_and_software.htm.

Table 2. Other causes for ischemic stroke according to Goldstein et al.

Disorder	Diagnostic Testing
Infectious vasculopathy:	
Neurosyphilis	**CSF**: pleocytosis, high protein; **VDRL > 1:16**; **PCR;** + serum VDRL, RPR, or FTA-ABS
Neurotuberculosis	**CSF**: pleocytosis, high protein, low glucose, **AFB; PCR;** MRI; + PPD, +chest x-ray
Typhus	Clinical, **serology**, **PCR**
Schistosomiasis	**Microscopic urinalysis, stool for O & P**
Trichinosis	**Muscle biopsy, serology**
Mycoses	**Specimen isolated in tissue**
Mucormycoses	**Specimen isolated in tissue**
Rickettsioses (eg Rocky Mountain Spotted Fever)	Clinical, **serology**
Neuroborreliosis (Lyme disease)	Clinical, **serology (ELISA)**
Neurobrucellosis	**CSF**: **culture for brucella, IgG agglutination > 1/160**
Malaria	**Peripheral blood smear**
HIV	**Histology demonstrating vasculitis;** +serology, Western blot, PCR/AIDS defining illness
Herpes encephalitis	**CSF:PCR, brain biopsy**
Inflammatory vasculopathy:	
Systemic lupus erythematosus	Clinical, **fANA test, histology**
Scleroderma	**Clinical**
Sjogen's syndrome	**Lip biopsy, anti SSA and SSB antibodies**
Polyarteritis nodosa	**Angiography, histology**
Rheumatoid arthritis	Clinical features, **+rheumatoid factor**
Takayasu's disease	**Histology**
Wegener's granulomatosus	**Histology, cANCA**
Granulomatous angiitis of the CNS	**Angiography, histology**
Degos-Kohlmeier Disease	Clinical, **gastroduodenal endoscopy**
Churg-Strauss syndrome	Eosinophilia, **histology**
Behcet's syndrome	**Clinical**
Hodgkin's lymphoma	**Histology**
Neurosarcoidosis	Clinical, **histology**

Eale's disease	**Clinical**
Relapsing polychondritis	**Clinical** (nonspecific lab testsl)
Drug induced vasculitis	**Clinical**
Vasospasm	
Drug induced (amphetamine, cocaine, heroin, LSD, PCP, ecstasy)	**Urine and serum drug screens**
Congenital connective tissue disorders	
Marfan's syndrome	**Clinical**
Ehler's-Danlos syndrome	**Clinical** (there are **specific tests for certain subtypes**, such as protein electrophoresis (I,II), urine lysyl hydroxylase (VI), skin biopsy (IV, VIIb, VIIc),
Pseudoxanthoma elasticum	Clinical, **histology (skin)**
Fabry's Disease	**Functional assay for alpha -galactosidase A**.
Osler Weber Rendu Syndrome	**Clinical**
Other noninflammtory vasculopathies	
Fibromuscular dysplasia	**Angiography**
Moya moya	**Angiography**
Homocysteinemia	**Urine and serum homocysteine**
Post-irradiation vasculopathy	History, **CT/MRI/Angiography/Histology**
Neoplastic angioendotheliosis	**Histology**
Dolichoectasia	**Angiography, CT, MRI**
Amyloid angiopathy	**Histology**
Sneddon's syndrome	**Histology**
Spontaneous arterial dissection	**Angiography**
Venous sinus thrombosis	**CT, MRI, angiography**
Hematologic	
Severe Anemia	Complete blood count, **hgb > 7 g/dl**
Thrombotic thrombocytopenic purpura (TTP)	**Clinical features, peripheral blood smear (schistocytes)**
Lupus anticoagulant	**Functional assay for lupus anticoagulant**
Anticardiolipin antibody	**ELISA for anticardiolipin antibody**
Sickle cell disease	**Hemoglobin electrophoresis**
Thalassemias	**Peripheral blood smear**
Hemoglobin SC disease	**Hemoglobin electrophoresis**
Polycythemia rubra vera	**Complete blood count, hgb > 20 g/dl or requiring phlebotomy**
Waldenstrom's macroglobulinemia	**Serum and urine protein electrophoresis (SPEP and UPEP)**
Cryoglobulinemia	**SPEP**
Essential thrombocythemia	Complete blood count, **> 600,000 platelets/mm3**
Myeloproliferative disorders	**Complete blood count, blood smear, bone marrow biopsy**
Coagulation factor deficiencies: antithrombin III, protein C, protein S, plasminogen, factor VIII (hemophilia), factor XII, C2, prekallikrein, heparin cofactor II)	**Serum factor levels by functional assay**
Activated ptn C resistance (eg Factor V Leiden)	**PCR for Factor V or functional assay for APC-R**
Platelet hyperaggregability	**Platelet aggregation studies**
Disseminated intravascular coagulation	**DIC panel**
Vitamin K therapy	Clinical**, PT**
Paroxysmal nocturnal hemoglobinuria	**Ham test, flow cytometry**
Paraneoplastic syndrome	Neoplasm, **laboratory evidence of coagulopathy**
Oral contraceptive induced hypercoagulability	History/Clinical, **laboratory evidence of hypercoagulable state**
MELAS	Clinical, **mitochondrial DNA analysis**
CADASIL	**PCR for CADASIL gene**

EPIDEMIOLOGY OF ISCHEMIC STROKE

Risk Factors

Since stroke is a multifactorial disease both genetic and environmental risks affect the disease. Previously it has been believed that conventional risk factors (e.g. smoking, hypertension, diabetes mellitus and hypercholesterolemia) constitute approximately 50% of risk and the rest of the risk is related to genetic factors and biomarkers [37]. This belief has been contested recently as data from randomized trails and observational studies show that up to 90% of coronary heart disease (CHD) patients have at least one of these risk factors [38]. Presumably ischemic stroke may not be too different.

Conventional Risk Factors

Age and Gender

Stroke incidence increases with age; with each decade above 55 years the risk doubles. Approximately 80% of strokes also occur in the elderly. Gender also affects the risk; men develop strokes at a higher rate than women up to the age of 75 [39].

Smoking

Cigarette smoking is a well-established risk factor for IS [40, 41]. A meta-analysis has shown a 50 % increase in stroke risk for smokers [42]. In a recently published study, where quantitative modeling was used, cessation of smoking lead to a marked decrease in stroke risk (RRR 87 %, NNT 4) [43]. Smoking also works in concert with genetic factors.

Diabetes Mellitus

Diabetes mellitus increases stroke risk two- to four-fold compared to non-diabetes subjects while also increasing mortality and morbidity after stroke [39]. A recent systematic review found that diabetes was an independent risk factor for IS in patients with known atrial fibrillation [44] Treatment studies in diabetes (PROactive) and hypertension (HOPE 2) have found that treatment in diabetic patients has effect on stroke risk even if the primary treatment endpoint is not met [45, 46].

Hypertension

Hypertension is the single most important modifiable risk factor for all stroke, accounting for up to 50 % of all strokes [47]. Increasing blood pressure is directly related to stroke risk, even in the normotensive range [48]. Secondary prevention of stroke by means of lowering blood pressure is very effective with a RRR of 29 % [49].

Hypercholesterolemia

It is a well-established fact that elevated total cholesterol, as well as low-density lipoprotein (LDL) cholesterol increases the risk of CHD. The Scandinavian Simvastatin Survival Study was the first study to show that lipid-lowering treatment with statins decrease stroke risk in a population of CHD patients [50].

For stroke it becomes more complicated as different studies have found different associations to cholesterol [51-54]. The same can be said about LDL. The reason for this might be random chance but also that many of these studies did not distinguish the different stroke type or subtype.

Recently the first selective secondary prevention study with statins against recurrent stroke showed that treatment decreases the risk of overall stroke and vascular death [55]. Other lipid lowering medications or diet has not shown the same effect [56]. This has given rice to the hypothesis that other pathways except the lipid-lowering effect might be the cause of the statins beneficence. Vaughan et al found both neuroprotective and anti-inflammatory effects of statin treatment [57]. This has also been shown in rodent models [58].

New Risk Factors

Silent stroke

Silent cerebral infarcts have often been detected by CT or magnetic resonance imaging (MRI) in older subjects, particularly in those with hypertension [59]. Silent cerebral infarct is a strong predictor of subsequent clinically overt stroke [60]. Treatment with ACE-inhibitors does not seem to increase risk of silent infarcts [61]. Intima-media-thickness together with atherosclerotic plaques have been shown to be associated with silent cerebral infarcts [62].

Obesity

Recently obesity by different measures has been reported as risk factors for stroke in different populations [63-68]. The measurements used have been raised body-mass-index, waist-to-hip ratio. In a Swedish observational study of 7402 middle aged men (47-55 years), followed for 28 years, 495 ischemic strokes occurred. After correction for other risk factors the association between IS and obesity persisted [66].

Homocysteine

Homocysteine (Hcy) has been described as a possible risk factor for ischemic stroke. The presumed mechanism for the increased risk is based on epidemiological and in vitro/vivo studies showing increased stress intolerance of the endothelium, thrombosis, inflammation and oxidative stress [69]. A randomized trial (VISP) has shown no effect on cardiovascular endpoints even though Hcy levels were lowered after 2 years follow up of ischemic stroke patients [70]. A sub analysis did show a borderline significant effect in patients with intermediate cobalamine levels [71].
The Norwegian Vitamin Trial (NORVIT) showed a borderline significant increase in myocardial infarction endpoints and a borderline decrease in ischemic stroke [72]. The HOPE 2 study did not show any significant effect in cardiovascular endpoints, but yet again a borderline significant decrease of stroke [73]. There are suspicions that B12 and folic acid treatment in atherosclerotic disease could affect DNA methylation and thus affect Hcy and methionine metabolism leading to an increased proliferation in the vascular walls.

Inflammation and biomarkers

Inflammation as part of the atherosclerotic pathway has been implicated in cardiovascular disease and ischemic stroke. Inflammatory biomarkers such as C-reactive protein (CRP), inteleukin-6 and heat shock proteins (Hsp) have been shown to be elevated in acute stroke and also shown to increase risk in prospective studies of IS [74-81]. Leukotriene metabolism has also lately been implicated through genetics in MI and stroke [82].

GENETICS OF ISCHEMIC CEREBROVASCULAR DISEASE AND CAROTID STENOSIS

Genetics of complex disorders

Is there a genetic component to a disease?

A complex disorder is defined as depending on a multitude of causes and risk factors Ischemic cerebrovascular disease is a heterogeneous disease-complex and regarded as a complex disorder. When trying to analyze the genetic component of a complex disorder one first has to prove that genetics is involved in the complex forms of the disease. This is evident in the monogenic disorders leading to stroke.

The easiest way is to observe the degree of familial clustering of the disease by measuring the quantity λ_R. This is the risk to relative R of an affected proband compared with the risk in the general population, high values indicate high λ_R. One of the most common measurements is λ_S, the risk for siblings. This of course includes the shared environment of the family which means we have to add other observations such as twin studies and adoption studies to observe the genetic component and exclude the environmental component.

Twin studies have the advantage of comparing a genetic identical pair of individuals (monozygotic twins) with a pair with approximately 50% identical genetic setup (dizygotic twins). They still have the same environment if they were not split up at a young age and this is a limitation of twin studies. Even monozygotic twins are not entirely identical (T-cell receptors, B-cell receptors (immunoglobulins), somatic mutations, different mitochondria and pattern of X inactivation in females). The best way to disentangle genes and environment is to study adoptees to see if the concordance of the disease follows their biological family or their adoptive family.

Another way of measuring the genetic component (G) of a phenotype (P) is to measure its heritability, i.e. the proportion of the phenotype that is due to genetic effects. Heritability is designated h^2.

$$h^2 = \text{Variance}_G \ / \ \text{Variance}_P$$

Linkage analysis versus Association studies

The question about how to best find genetic markers for complex disease has been argued for a long time. Two methods have been used; linkage analysis and association studies. These two methods use predominantly two different types of genetic markers; microsatellites and single nucleotide polymorphisms (SNP).

Microsatellites.
Microsatellites consist of DNA repeats in tandem of di-, tri- or tetranucleotides, repeated from 1 to 50 times. They are common and evenly distributed in the genome, usually in non-coding regions. These repeats are useful since they are very polymorphic, and since they change on average once per 1000 replications. Microsatellites become polymorphic because of their repetitious nature, which makes the DNA polymerase slip during the replication, leading to different amount of repeats in different people. What microsatellites normally lack compared to other markers is their location; in non-coding regions. They are therefore used mainly to find loci of interest in the genome to be more extensively mapped with SNPs.

The microsatellites are compared to a set of normative microsatellite markers form the Centre d'etude du polymorphisme humaine (or the Center for the Study of Human Polymorphisms) in Paris, France, which has now been renamed to the Foundation Jean Dausset-CEPH. The foundation provides a genetic map of markers based on immortalised cell cultures derived from families studied by Dausset et al for HLA, leading to his 1984 Nobel Prize [83].

Single nucleotide polymorphisms.
These markers are the most common form of genetic variation and they are defined as a single base pair variation (usually bi-allelic) with a frequency of at least 1% for the rare allele. In contrast to microsatellites, SNPs are common inside genes and sometimes found as causes of genetic disorders but more commonly as candidate susceptibility variants. They are evolutionarily conserved and thus of use in mapping quantitative traits. A SNP changes on average once every 1 000 000 replications. There is on average one SNP per 0.5 to 1.2 kilo bases in the genome. A mapping project, the International HapMap Project, has completed a haplotype map of the human genome (the HapMap), describing common patterns of human genetic variation [84]. Approximately 9.5 million SNPs are currently available (NCBI dbSNP Build 127).

Linkage Analysis.
In linkage analysis the segregation of genetic markers in families is compared to the segregation of a trait. A significant co-segregation, above what is expected by chance indicates the presence of a gene of importance for the trait close to the marker. This is based on the proven hypothesis by Thomas Hunt Morgan that crossing over in the genome leads to a linkage between alleles in close proximity to each other, meaning that they co-segregate more often than expected by chance alone.

Linkage analysis is used in extended families and sib-pair studies with sibs concordant for the disease, mapping a locus following the studied disease or trait. Normally 300 to 1000 markers are screened across the genome. This has been widely utilized in monogenic disorders with frequent success, but is less effective in complex diseases.

Association analysis/LD-mapping.
In association studies the distribution of marker alleles is compared between an affected group and a general population or matched control group. This can be done in a family-based fashion where the controls are un-affected family members or by a comparison of affected to a general population. Usually the markers are based on previous knowledge

with a hypothesis that they could be relevant for the disease (susceptibility or resistance genes) and consist of different SNPs. However, recently, a gnome-wide approach to association analysis

When deciding which markers (usually SNPs) to choose one can utilize data from the HapMap Consortium and NCBI's dbSNP database. A multitude of different susceptibility genes have been screened with various levels of success in this way.

Finding association to a marker does not imply that the chosen marker is causative; it could also be in linkage disequilibrium (LD) with the "real" causative variant, located close to the typed marker. This fact can be used to map genes and select the most relevant markers.

One problem with association studies is that they depend on the control group being comparable. Epidemiologists recommend that you select the control group by thoroughly matching it for age, gender, ethnicicity and sometimes risk factors, such as BMI, smoking status etc. Statisticians want to maximize the sample and believe the population controls give them maximum power, at least in common diseases where it is difficult to know who is unaffected. Epidemiologists think the maximum power is achieved when they use the appropriate unaffected controls which can rarely be identified. In a similar way they consider the right controls have to be matched by age with the cases. The reason for all this is the scare of population stratification, i.e. subgroups in the population regarded as affected.

LD-mapping uses linkage disequilibrium which is the correlation between one particular marker allele and a marker in an adjacent locus. LD exists due to the fact that chromosomes have a shared ancestry, meaning that markers close to each other tend to be inherited together during recombinations. When a new allele arises through spontaneous mutation it is bound to a certain chromosome and groups of markers (a haplotype). As recombinations occur the allele will lose its correlation to the specific haplotype but this happens slowly (typically approximately 100 generations); a phenomenon called LD decay. LD decay is slow and thus LD between markers and inside haplotypes can be used to find the causative marker or to decrease the amount of markers needed to type, to fully map a region in the genome. LD mapping has been used to find causal mutations in genes for Mendelian diseases and recently also for complex disorders.

There are two common ways of measuring LD: the D' and r^2 methods. The D' is an absolute description of D which quantifies the difference between the observed frequencies of a haplotype A B (in two loci) to the expected frequency of the haplotype if the markers were segregating randomly. This can be expressed in this formula:

$D = P_{AB} - P_A \times P_B$

P_{AB} is the observed frequency of the haplotype AB and P_A and P_B the frequencies of the separate alleles at the two loci. D is however of less use as a numerical measure and therefore D', which is an absolute measure is used, as to be able to compare LD. D' is determined by dividing D with the maximum value D can reach given the allele

frequencies at the two loci. This mean that if D'=1 (known as complete LD) the alleles A and B have not been separated at all by recombination. However lower values of D are not very reliable, since D' is strongly dependent on sample size. Therefore the measure r^2 is more often used and chosen for quantifying LD to do comparisons. R^2 is expressed by dividing D^2 with the product of all four allele frequencies at the two loci:

$$r^2 = D^2 / (P_A \times P_a \times P_B \times P_b)$$

Thus r^2 =1 (known as perfect LD) will only occur if the markers have not been separated by recombination and have the same allele frequencies. This means that r^2 =1 gives you complete information about the second marker making it redundant. R^2 is also less dependent on sample sizes and even values over 0.33 (r^2 >0.33) are sufficiently strong to use in LD mapping.

Many factors affect LD, the strongest being mutations and recombination but also other factors have effect:
Genetic drift: increasing LD in stable populations due to decreasing amount of haplotypes.
Population growth: decreasing LD.
Admixture or migration: increasing LD in nearby markers.
Population structure: Inbreeding increasing LD without reducing variation.
Natural selection: By "hitchhiking effect" where an adjacent haplotype follows a favoured variant and by epistatic effect between two loci.
Variable recombination and mutation rates.
Gene conversion: breaking down LD.

Genome-wide association studies (GWAS). LD mapping was conceived as to be able to minimize the numbers of markers needed to genotype to map a region of the genome or the whole genome. Recently, through the advance of biotechnological methods with dense genotyping chips containing hundreds of thousands of SNPs in conjunction with the HapMap project documenting genome-wide variation and linkage disequilibrium and collections of large well phenotyped clinical samples genome wide association studies have become feasible.

Interestingly, this year (2007) has seen tremendous advances in the identification of genes in complex diseases, through the GWAS approach. The Wellcome Trust Case Control Consortium (WTCCC) screen together with a number of similar screens of specific diseases have shown that the GWAS methodology is capable of identifying novel and frequently surprising risk genes. A number of conclusions have been drawn from this experience:

1) The identified risk genes all have a limited OR, in the range of 1.2-1.5. Few examples of more strongly acting genes have been seen.
2) Success has been most evident in diseases with a reasonable genetic component, such as type 1 diabetes which has a λ_S of ~15 and Crohn's disease (λ_S 17-35).
3) Even in these conditions, sample sizes below 2 000 cases seem to loose power and risk false negative results.

4) The risk of false positive findings due to poor matching of patients and controls seems smaller than expected, allowing even the use of common sets of controls in different materials.

These experiences have highlighted the GWAS as an efficient way of identifying new risk genes in complex diseases pointing toward ever greater success when marker maps become dense and clinical materials larger.

In the largest GWAS effort so far, seven complex disorders were genotyped with a gene-chip containing 500 568 SNPs in 2000 cases and 3000 controls [85]. Four of these complex disorders are of interest from a stroke perspective, namely hypertension, type 1 and type 2 diabetes and coronary artery disease (for results see **Genetics of multifactorial ischemic cerebrovascular** disease below).

Genetic epidemiology of ischemic cerebrovascular disease.

Twin-, Family- and Sib studies

Studies of twins, families, adoptee and siblings in ischemic stroke, TIA and carotid stenosis/atherosclerosis have been performed. The first twin study in stroke was based on death certificates (stroke death) in a Swedish twin cohort of 10 900 twin pairs in the 1970s and showed no difference between concordance rates for stroke death in monozygotic compared to dizygotic twins [86]. The limitations of this study were the short follow up time (12 years) and the fact that only 19 pairs were concordant for stroke. In the 1990s Brass et al found a four-fold increase in stroke risk in monozygotic twins compared to dizygotic [87, 88]. A follow up was done 6 years later with a decrease in the relative risk to 1.63, which was not significant. This can be interpreted that genetic influences are comparatively small in an older population. Furthermore stroke was self reported. A Danish Twin study recently confirmed the initial data from Brass (concordance rates were similar, approximately 0.2), but in this study stroke death and stroke hospitalisation were measured [89]. A meta-analysis of the 4 twin-studies showed that monozygotic twins were more likely to be concordant for stroke (unspecified type) than dizygotic twins (OR 1.65, p=0.003) [90]. Regarding atherosclerosis a Finnish twin study found smoking to be a predictor and not blood pressure, cholesterol, BMI or genetic factors [91].

Family studies have shown an inherited risk for stroke. In the Framingham Offspring Study, parental history of coronary heart disease, TIA or stroke predicted stroke [92]. This effect was stronger in males than females and yet stronger for coronary artery disease (male RR 2.4, female RR 1.4, CHD RR 3.3). The risk was also observed in siblings. A Finnish prospective study found that the genetic risk varied from different types of stroke and yet again gender with the lowest effect in males with IS (RR 1.5). The inherited risk was also stronger in a younger population (25 to 50 years compares to 50 to 65 years) [93]. A Swedish study based on hospitalization of stroke found that there was an increased risk for siblings to get a stroke and this was more prominent in women [94]. In a recent British meta-analysis it was found that the heritability of stroke subtypes differs from each other. This was due to that other causes were more heritable and since the different stroke subtypes have different risk factor profiles they have a different heritability. Cardioembolic strokes having the least family history of stroke and LAA strokes significant family history of myocardial infarction (OR 1.63, p=0.05).

Hypertension was the most heritable risk factor [95]. Family history of stroke, in a young Swedish stroke cohort (<70 years), was positive for large artery atherosclerosis and lacunar stroke but no risk was associated with family history of cardioembolic stroke [96]. Wiklund et al investigated sib pairs in the US, Canada and northern Sweden and found that there was a lack of aggregation between subtypes of ischemic stroke [97]. They concluded that the genetic component of ischemic stroke is not related to different subtypes but to overall ischemic stroke. A systematic review of family history and stroke found that generally there was a major heterogeneity between studies addressing genetic epidemiology [90].

Families with atherosclerosis of the carotid arteries have been studied. In these studies IMT has been shown to be strongly heritable (h^2=0,61) and carotid stenosis degree to a certain extent (h^2=0,47) [98, 99]. In a prospective family study where 1 000 subjects were followed up for 10 years Visvikis et al found that 30 % of IMT was due to genetic factors [100].

There is only one current study of adoptees in regards to stroke. In this Danish study data supported genetic factors as influencing death by vascular causes (CHD and stroke) [101, 102].

Finally a multinational population study with two Swedish centers [103, 104] found that there is evidence that genetic factors contribute to stroke. In some cases genetic factors are more important than environmental factors [105].

In ischemic stroke the λ_S ranges from 1.5 to 4 in different studies depending on age at onset and gender [94, 106-108].

Animal models

In the 1990s rat models were used in attempts to find stroke loci in the genome. Two separate groups independently, using different rat breeds, found a linkage to chromosome 5. Three loci were found and the second contained the genes for and the atrial natriuretic peptide (ANP) and Brain Natriuretic peptide (BNP) [109, 110]. This locus was also independent of hypertension in one group of rats. One of the candidate genes, ANP, was later studied in humans and was found to increase risk of stroke. One polymorphism in ANP had an even stronger effect together with the natriuretic peptide receptor A, showing that epistatic effects can be important [111, 112]. Earlier studies have shown increased levels of ANP in IS [113]. A later study could not confirm the initial finding regarding ANP alone in IS or its subtypes [114].

Monogenic Disorders

Monogenic disorders are of interest to study in multifactorial complex stroke as they can be used as candidates by them selves, helping to understand the pathogenesis of stroke. They can be used to observe how the phenotype of monogenic disorders is modified by risk factors and other genes and, finally, by not being underestimated and under diagnosed in a clinical setting. Three monogenic disorders can be used as examples for these facts.

CADASIL

This is the classic example of a monogenic stroke disorder, where stroke is one part of the phenotype. CADASIL stands for Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy and has four main symptoms: ischemic strokes, dementia, migraine with aura and mood disturbances. It was described under different names in the 1950s. A study of a French family led to the positional cloning of the Notch 3-gene, which subsequently has been confirmed in more than 400 families. Even DeNovo mutations have been described [115, 116]. The gene codes a transmembrane receptor. The different mutations are stereotype and lead to truncations in the EC-domain which in turn lead to increased accumulation of protein in SMC with a small vessel disease as the result. There is distinctive variation in the CADASIL phenotype, which is not explained by the mutation site. New evidence suggest that the coexistence of conventional cardiovascular risk factors, such as smoking, may hasten disease progression [117].

Sickle cell disease

Sickle cell disease (SCD) results from homozygosity of a unique beta-globin mutation. It shows a phenotypic heterogeneity with stroke only affecting 6% to 8% of individuals. An impressive study has recently been performed in 1 398 individuals with SCD, where 108 SNPs in 39 genes were typed and analyzed using a Bayesian network [118]. Thirty-one SNPs in 12 genes were found to interact with hemoglobin to modulate the risk of stroke, including three genes in the transforming growth factor-beta pathway and P-selectin. A predictive model was constructed and could predict stroke occurrence in a second population with 98% accuracy.

Fabry Disease

Fabry disease is a X-linked inherited lysosomal storage disorder, due to partial or complete deficiency of the enzyme alpha-galactosidase A. This results in progressive accumulation of glycosphingolipids in vulnerable cells (endothelial cells, smooth muscle cells in the vascular system, myocardial cells, renal epithelial cells, corneal epithelial cells, dorsal root ganglia, cells of the autonomic nervous system). The classic phenotype of Fabry disease has an estimated incidence of about 1 in 50,000 males. In Sweden there are approximately 40 confirmed Fabry patients in five known families.

Later-onset cardiac and renal variants with residual alpha-Gal A activity due to missense and splicing mutations have been identified in individuals who lack the early manifestations of classically affected patients, i.e., angiokeratoma, acroparesthesias, hypohidrosis, and corneal/lenticular abnormalities. Spada and colleagues reported the screening of 37 104 consecutive newborn males for Fabry disease by determination of the alpha-Gal A activity in dried blood spots. They found that 1 in approximately 3 100 males, had deficiency of enzyme activity and alpha-Gal A mutations [119].

Molecular modeling studies and in vitro over expression of the missense mutations revealed structural characteristics and residual enzymatic activities consistent with mutations identified previously that cause the later-onset phenotype. Thus a ratio of 11:1 of patients with the later-onset to classic phenotype was found.

Rolfs et al. (2005) investigated the frequency of unrecognized Fabry disease in a cohort of stroke patients [120]. Over a period of almost 4 years, 721 German adults aged 18 to 55 years suffering from acute cryptogenic stroke were screened for Fabry disease. In men, the plasma alpha-galactosidase activity was measured followed by sequencing of the entire alpha-GAL gene in those with low enzyme activity. By contrast, the entire alpha-GAL gene was genetically screened for mutations in women even if enzyme activity was normal. In 21 of 432 (4.9%) male stroke patients and 7 of 289 (2.4%) women, a biologically significant mutation was found within the alpha-GAL gene. The mean age of onset of symptomatic cerebrovascular disease was 38.4 years in male stroke patients and 40.3 years in the female group. The higher frequency of infarctions in the vertebrobasilar area correlated with more pronounced changes in the vertebrobasilar vessels such as dolichoectatic pathology.

All this data show us that a previously very uncommon and under diagnosed disease may in fact be more important than initially believed.

Genetics of multifactorial ischemic cerebrovascular disease

A problem with genetic studies of ICVD or IS is that the phenotype can differ between studies. Most studies only include patients with stroke, sometimes not defined at all, and seldom TIAs are included. Since most stroke patients do not have Mendelian disorders as cause of their disease one can hypothetically presume that candidate genes/polymorphisms of importance for ICVD should be relatively common in the general population. Some of the earliest examples of such polymorphisms are the angiotensin converting enzyme gene (ACE) and activated protein C (APC) resistance due to the Leiden mutation in Factor V [121, 122]. These kinds of polymorphisms, i.e. the widely spread in the population, are the ones often chosen for evaluation in genetic association studies.

I will try to summarize this extremely large field with its multitude of studies and thereafter specifically summarize the primary candidate genes we chose to study. The genes and polymorphisms will be divided into the following groups: hypertension and vasodilation, coagulation and hemostatic factors, lipid metabolism, homocysteine metabolism and inflammation and atherosclerosis.

Hypertension and vasodilation

An insertion/deletion polymorphism in the angiotensin converting enzyme (ACE) gene has been studied extensively. Homozygosity for the D-allele have been shown to be associated with elevated blood pressure and also been reported as a risk factor for cardiovascular disease [123]. In one of the first stroke studies it was reported that this polymorphism was associated to lacunar strokes but not large artery atherosclerosis (carotid stenosis) [121]. Later studies have not been able to confirm this [124, 125], except in hypertensive patients [126] and patients with early atherosclerotic changes [127, 128]. Indirect proof of an association to a history of IS shows that this gene is of importance to ischemic stroke with carotid stenosis [129]. Tabara et al had previously shown that IMT was associated to the D-allele in healthy men [130].The role of ACE has later been cemented with the meta-analysis by Sharma showing the D-allele to be associated with stroke under a regressive model [131] and a recent lager meta-analysis showed approximately the same effect, OR 1,21 (CI 1,08-1,35) [132].

Other genes in the Renin-Angiotensin-System have been reported in stroke. A point mutation (M235T) in the Angiotensinogen (AGT) gene was not associated with hypertension, IS or carotid atheroma in one study [133], but to lacunar strokes in later studies. The polymorphism has been shown to interact with the D/D genotype of the ACE gene [134-136].

In another study it was shown that a polymorphism in the angiotensin II receptor type 1 gene (AGTR1) had synergistic effects with the ACE I/D polymorphism and was associated to myocardial infarction [137].

As mentioned above, Rubattu et al found the human ANP gene to be of interest in IS after performing linkage studies in rats [111]. Earlier studies have shown increased levels of ANP in IS patients. A try to replicate the initial finding was negative in total IS and in a sub analysis of subphenotypes [114].

Nitric oxide (NO) has been shown to be an important mediator of vasodilation in small vessels and affect the function of the endothelium. In knockout mice the absence of endothelial NO synthetase (ENOS) makes the rats very sensitive to ischemia of the brain. An early study showed a functional variant of ENOS to increase risk of vascular disease and myocardial infarction in smokers [138]. This has not been shown in Caucasians with IS [139] or carotid disease [140], but in a Chinese IS cohort [141] and in patients with hypertension developing carotid disease [142]. A recent study found the ENOS to be associated to stroke in two independent cohorts [143].

A polymorphism in the G-protein alpha-subunit has been shown to be associated with hypertension and obesity [144-146] and later with IS independently of hypertension and other risk factors [147].

In the recently published genome-wide association study of different complex diseases, a hypertension cohort was analysed [85]. No markers were found to reach the designated significance level ($p<10^{-7}$) but some reached moderate p-values (6 markers, $p=10^{-5}$), but they do not cover previously known hypertension loci or genes. The reasons for this could be smaller effects of susceptibility genes than anticipated, possible misclassification in control samples or that the SNPs were poorly tagged for hypertension.

Coagulation and hemostatic factors

Multitudes of polymorphisms in genes involved in coagulation have been examined in IS. The identification of activated protein C resistance has been the sole most important genetic cause of venous thromboembolic disease. In the first study to investigate the Factor V Leiden mutation by Catto et al no association was found between the suspected susceptibility allele and ischemic stroke [148]. Recent meta-analyses did however show a modest increase in risk when carrying the risk allele (OR 1,33 CI 1,12-1,58) [132, 149].

A functional polymorphism of the Factor VII gene was shown to be protective in IS [150] and this was confirmed in the above mentioned meta-analysis [149].

Regarding the prothrombin gene (Factor II) G20210A polymorphism results have been ambiguous until the meta-analysis could show a small increase in risk (OR 1,44 CI 1,11-1,86) [132]. The different studies had a lot of different ischemic stroke phenotypes. Initial analysis of the polymorphisms in HPA-1, -2, -3, -5, fibrinogen, von Willebrand and collagen receptors on thrombocytes could not be shown to be associated with risk for ischemic stroke [151]. The P1 A1/A2 polymorphism in the glycoprotein IIIa gene [149, 152, 153] and a polymorphism in Factor XIII [154-156] have shown different results. A recent study did however find a increased risk for IS in the glycoprotein 1b-alfa gene (Kozak-polymorphism) [157, 158] and another polymorphism in younger women [159]. In the later study an association with glycoprotein Ia and IIb was shown. A polymorphism in the promoter region of thrombomodulin, which is involved in activation of protein C and decreases thrombin activity, has been shown to be associated to CS in patients with ischemic stroke [160]. The plasminogen activator inhibitor-1 gene (PAI-1) has also been extensively studied in IS and been shown to be associated with myocardial infarction and stroke, showing differing results and a meta-analysis found a strong association but also strong heterogeneity [161-167]. The tissue-type plasminogen activator (tPA) -7351C/T polymorphism has been associated with ischemic stroke showing an increased risk in lacunar stroke (T allele), which was refuted in a second study [168, 169]. A protective effect (together with PAI-1 4G-homozygeous subjects) was shown in another study [162]. The same group found the thrombin activatable fibrinolysis inhibitor (TAFI) activation peptide to be elevated in ischemic stroke patients and associated with TAFI gene haplotypes, but the haplotypes were not associated with ischemic stroke [170].

Fibrinogen consists of three genes (alpha-, beta- and gamma fibrinogen) localized on chromosome 4q28. Epidemiological reports have shown elevated levels of fibrinogen increases risk of MI, peripheral arterial disease (PAD) and IS. This would indicate that fibrinogen is important for the development of thrombi associated to atherosclerosis and is thus a relevant candidate gene. The fibrinogen gene cluster contains several candidate polymorphisms, one being the SNP -455 G/A localized in the 5'-region of the beta -fibrinogen gene which has been shown to be associated to MI [171] and unspecified stroke [172]. In the later study a difference of risk between genders was found. This polymorphism is functional and has been shown to increase levels of fibrinogen as well as being associated to smoking (gene-environment effect). Another polymorphism in the beta-fibrinogen gene has been shown to be associated to IS with large artery disease (carotid stenosis), but the p-value was low (and not significant if recalculated with Fischer's exact test) [173]. Our own study could not find an association with the -455 G/A polymorphism and ICVD (unpublished).

As mentioned above a multitude of genes involved in the coagulation system have been investigated and sometimes shown to be associated to IS. Recently a Finnish study found some of them to interact with each other [174].

Lipid metabolism

Many non-familial polymorphisms in different genes involved in lipid metabolism have been investigated. The most well investigated gene is apolipoprotein E (APOE), a known risk factor for Alzheimer's disease [175], where the epsilon4 allele has been associated to IS [176] and large artery disease [173, 177-179]. MCarron et al found an a

protective effect of the epislon4 allele in their cohort study of IS patients [180] but a meta-analysis found the epsilon4 allele to increase risk [181]. The APOE 158Cys (e2) allele showed a protective effect in patients with ICVD with CS. In other studies of stroke the epsilon2 allele has sometimes been shown to increase risk and sometimes decrease risk [182, 183], probably depending on population differences or mere variation due to random chance.

Different polymorphisms in apolipoprotein A1 (APOA1) have been shown to affect the vessel wall in patients with high cholesterol [184], but have not been shown to be associated with carotid atherosclerosis in young IS patients [185]. Recently an Apolipoprotein A5 gene promoter region polymorphism was shown to confer susceptibility for development of ischemic stroke [186].

Lipoprotein lipase (LPL) is involved in the uptake of cholesterol to the endothelial cells in the arterial wall and polymorphisms in this gene have been shown to be associated to low levels of High-density lipoprotein (HDL) in premature arthrosclerosis and cardiovascular disease but various results have been shown in IS. A recent study found the S447Term polymorphism to be associated with a protective effect in atherothrombotic stroke [187]. The LPL S447Term polymorphism has been studied extensively and a protective effect for the LPL 447Term allele has also been shown in Chinese [188], elderly Finnish [189] but not in Greek [190] and a strong opposite effect in an American study [191]. However, the American study did not show an association in the primary analysis but only after extensive statistical correction.

The Paraoxonase 1-gene has been shown to be associated to carotid atherosclerosis in younger patients [192], non-smokers [193] and IS in a Japanese population [194]. PON 1 activity has been associated to the polymorphism [195]. Recently other polymorphisms in the PON 2 gene have been investigated in IS [196, 197].

Homocysteine metabolism

High homocysteine (Hcy) levels in serum have been shown to correlate with increased risk of stroke [198]. A mutation in the gene coding methylenetetrahydrofolate reductase (MTHFR) shows marked lower enzymatic activity of the enzyme in homozygous carriers of the C677T polymorphism and higher Hcy levels [199]. The first study in IS did not find an association to the susceptibility allele [200]. This has been confirmed in other studies including a meta-analysis [201, 202] but a more recent larger meta-analysis found a increased risk (OR 1,24 CI 1,08-1,42) [132]. This polymorphism has been shown to affect the risk of developing carotid artery changes [203]. In a recent GWA study [85] an intronic SNP in methylenetetrahydrofolate dehydrogenase (NADP1-dependent)1-like (MTHFD1L), a mitochondrial isozyme which is involved in methionine synthesis just like MTHFR, was associated with CHD. However this gene has not been studied in IS.

Inflammation and atherosclerosis.

Inflammation is an important part of the process leading to atherosclerosis. Interleukin 6 (IL6) is involved in the acute phase of an inflammation and affects the levels of CRP and other acute phase reactants (including fibrinogen). Many polymorphisms are known in the IL6 gene, among others the promoter polymorphism -171 G/C, a VNTR

CA-repeat etc. The promoter polymorphism has been shown to be associated with elevated levels of IL6 [204]. The suspected susceptibility C allele has been shown to be associated with large cell arteritis in HLA-DRB1 *04 negative patients [205]. However this study could not find any association between the arteritis and ischemic events. The C allele has also been associated to CHD in on study [206]. In a later study it was found that homozygosity to the C-allele in conjunction with statin treatment decreased the risk of CHD and also non-significant increases of IL6, CRP and fibrinogen [207]. In a prospective study of healthy patients with carotid stenosis (i.e. stenosis without ischemic events) an association was seen for the G genotype and IMT, this was also found in another study [208, 209]. The prospective study examined functional promoter variants in MMP3 and hepatic lipase[209]. The promoter polymorphism has been shown to be associated to IS [210-212], but a meta-analysis could not conclude to its effect due to methodological differences [213].

Matrix metalloproteases (MMPs), a group of metalloproteases expressed in macrophages, including MMP3, where the above mentioned functional polymorphism can affect CHD [214], has recently been examined in asymptomatic CS. It was found that IMT in the carotid bifurcation was affected. It was also shown that this polymorphism interacts with an IL6 polymorphism [208]. A polymorphism in MMP9 has been shown to be a marker for plaque instability in CS [215].

The CRP gene has naturally also been investigated as a candidate gene for stroke. C-reactive protein has been shown to up regulate angiotensin type 1 receptors in vascular smooth muscle cells [216] and endogenous expression of C-reactive protein is increased in active (ulcerated) carotid artery plaques [217]. An early study of IS found no association to for the CRP 1059 G/C polymorphism [218]. An intronic GTn repeat in the CRP gene was investigated in SLE patients and carriers of the GT20 allele were found to be more likely to develop vascular arterial events [219]. In a later study it was demonstrated that the 3'UTR 1846C/T CRP and IL-6 -174G/C polymorphisms are significantly associated with the risk of stroke after cardiac surgery [220]. In a large Swedish study of young IS, genetic variants of the CRP gene were shown to be associated to CRP levels but not to overall ischemic stroke [221]. Recently, a Dutch study did not show an association between CRP haplotypes and measures of SAO strokes [222]. No CRP polymorphism has been studied solely in CS but in IS with LAA where no association was found [221].

The polymorphisms of the C-reactive protein (CRP), interleukin-6 (IL-6), macrophage migration inhibitory factor (MIF), monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin (E-sel), and matrix metalloproteinase-3 (MMP-3) genes have been studied in concert in a cohort of non cardioembolic or other etiology stroke and demonstrated that the IL-6 GG, IL-6 GC, MCP-1 GG, ICAM-1 EE, E-sel AA, and MMP-3 5A5A genotypes were significantly and independently associated with stroke history. The odds of stroke increased with the number of high-risk genotypes where three proinflammatory gene variants had an adjusted OR of 50.3 (CI 10.2 - 248.1) [218].

Peroxisome proliferator-activated receptor gamma 2 (PPARG) has been implicated in type 2 diabetes previously and in the recent GWA study [85, 223]. PPARG has been

shown to be expressed at high levels in foam cells in atherosclerotic plaques and recently a PPARG-agonist has been shown to decrease risk of stroke in type 2 diabetes [46, 224]. A recent study has shown a Pro12Ala polymorphism in PPARG to decrease risk or stroke in type 2 diabetes [225]. Four (including **Study V**) other multi-polymorphism studies did not find this polymorphism to be associated with overall ischemic stroke [143, 226, 227].

Leukotrienes are inflammatory mediators generated from arachidonic acid (polyunsaturated n-6 fatty acid) by the enzyme 5-lipoxygenase. Promotor alleles of the 5 lipoxygenase gene have been associated with increased IMT [228]. The arachnoidate 5-lipoxygenase-activating protein gene (ALOX5AP) coding 5-lipoxygenase activating protein (FLAP) which affects leukotriene levels mediated through elevated leukotriene B4 (LTB4) (a chemoattractant mediator), has been shown to be associated with increased risk of MI and IS [229]. Four ALOX5AP studies in stroke have been performed and published. One of these studies replicates the association of the HapA haplotype from the original study in Scottish stroke [230] and two did not show association with HapA in stroke [231, 232]. However, single marker association with one or several SNPs in ALOX5AP was observed in two of these studies [231, 233].

Phosphodiesterase 4D (PDE4D) encodes a member of the large super-family of cyclic nucleotide phosphodiesterases, which selectively degrades cyclic AMP (cAMP), a key signal transduction molecule in all cells. Two haplotypes in PDE4D, spanning the first exon of isoform PD4D7, were found to confer risk and protection, respectively, primarily in a subgroup of cardiogenic (i.e. cardioembolic) and carotid stroke (i.e. large artery atherosclerosis) patients in Iceland [234]. The haplotypes consisted of one SNP (SNP45) and one microsatellite (later a second SNP was added, SNP41).

This gene has been extensively studied after the initial report. Twelve studies have investigated genetic variants in PDE4D, with four of those reporting replication for some of the published Icelandic variants [232, 235-238], three report new signals in the gene [239-241] and four studies do not replicate the Icelandic results [231, 242-244]. Eight out of the 12 studies investigated cohorts from only 88 to 279 patients (cohorts of one single ethnicity) and were therefore severely underpowered to properly test for the two subtypes showing the strongest association in Iceland i.e. cardiogenic and large vessel disease. In the final study a meta-analysis was included that confirmed PDE4D's importance in ischemic stroke, but shows a statistical heterogeneity [236].

Direct comparison of these different replication studies are difficult due to the disparity in how the data was presented in the different studies. In the supplementary table in **Study IV** a compilation of the results for the studies testing the two PDE4D SNPs, SNP45 and SNP41, can be found. According to the International HapMap project (version 19) these 2 SNPs are non-polymorphic in the two HapMap Asian populations [84]. Three PDE4D replication studies (including **Study IV**) show a similar effect of SNP45 as published in the original study, in the whole stroke group [232, 242, 245]. Three studies found SNP 45 to be non-polymorphic but they tested an independent signal in PDE4D previously reported in the original paper (i.e. SNPs found in an adjacent LD block showing no correlation with SNP 45 and SNP 41) and found this to be nominally significant for all stroke [235, 238, 240].

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In addition to the examples mentioned above additional genes and polymorphisms have been examined regarding IS and CS with both positive: Cathepsin G [246], NADPH oxidase [247], Fatty acid-binding protein 2 (FABP2) [248], Sterol-Regulatory Element Binding Protein 1a (SREBP-1a) [249] and negative results: hemochromatosis [250]. In other studies epistatic effects (gene-gene interaction) have been described between MTHFR, ACE D/D-genotype and the Leiden mutation [251] and gene-risk factor interactions (ACE-age, APOE-BMI, Angiotensinogen-blood pressure) [130].

In all described polymorphisms the results are ambiguous and most of all, not always specific for IS, ICVD and CS. In a meta-analysis, the genetic component (candidate genes) in different complex disorders had been analyzed, showing that the first studies on each candidate gene overestimate the risk [252]. In consecutive studies on the same candidate, the risk tends to fluctuate, but as more and more studies are performed, the risk decreases almost asymptotic towards 1, meaning no risk.

The following conclusion can be drawn: that larger studies to begin with are more adequate and that the candidate genes so far studied have a minor or modest effect on the disease complex. It is more probable that a few genes together with risk factors and environment give the larger risk for disease. Therefore it is probably better to perform studies with large cohorts on whole groups of candidate polymorphisms with elaborate subphenotyping and adequate controls [253].

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STAVE 3: THE SECOND OF THREE SPIRITS

STROKE OF CHRISTMAS PRESENT

AWAKING in the middle of a prodigiously tough snore, and sitting up in bed to get my thoughts together, I *had no occasion to be told that the bell was again upon the stroke of One.* This was one of those times I had to think about why I was doing this project of mine, was the aim worthwhile…

AIM OF THE STUDIES

- To test the hypothesis that the chosen candidate genes are of importance for the risk of developing ischemic stroke and carotid stenosis predisposing ischemic stroke (**Paper I, II, III, IV, V**).

- To map parts of the human genome suspected of being of importance for ischemic cerebrovascular disease and to characterize new candidate genes.

- Search for pathogenetical subgroups to the disease complex, ischemic stroke, to facilitate a more individual treatment and prophylaxis with genetic factors as a fundament.

- To determine if carotid stenosis is a valuable subphenotype in ischemic stroke (**Paper III** and **V**).

- To investigate if common stroke susceptibility candidate genes work in concert.

MATERIALS AND METHODS

For a detailed description of materials and methods please see the separate articles.

South Stockholm Ischemic Stroke Study.

Subjects

Patients attending the stroke unit or the stroke outpatient clinic at Karolinska University Hospital, Huddinge unit in Stockholm, Sweden and they were recruited from 1996 onwards in the ongoing genetic epidemiology study, the South Stockholm Ischemic Stroke Study (SSISS).

All the patients had an ischemic stroke (IS), TIA (collectively labeled as ischemic cerebrovascular disease) or carotid stenosis diagnosis according to ICD-9 (until 1997) or ICD-10 and this diagnosis were verified through the medical histories by a stroke neurologist and neurology resident (M Crisby or V Kostulas and K Kostulas).

All patients had clinically relevant investigations performed. This included brain imaging with computed tomography (CT) or/and magnetic resonance imaging (MRI) as

well as ancillary diagnostic investigations including duplex ultrasonography of the carotid and vertebral arteries, echocardiography, cerebral arteriogram, magnetic resonance angiography (MRA), CT-angiography and standardized blood tests. Investigations in the final cohort used in **Study IV** and **V** are shown in the respective publication/manuscript.

All patients were residents in the south Stockholm area, the uptake area for Huddinge University Hospital/Karolinska University Hospital, Huddinge unit and originating from Sweden or other Nordic countries. For **Study IV** and **V** patients of Icelandic ancestry were excluded.

In **Study I** at total of 128 Patients with ICVD (113 IS and 15 TIA) belonging to the consecutive cohort later labeled SSISS and retrospectively selected patients as to lower the mean age of the patients. Fifty-five individuals with carotid stenosis >50% were included as a more homogeneous phenotype (of which 85% had had a previous ischemic event).

In **Study II** 126 patients with ICVD (116 IS and 10 TIA) and 70 patients with CS were included. Twenty-two per cent of the ICVD patients had a relevant carotid stenosis.

In **Study III** 130 patients were included in the ICVD group of which 34 had evidence of carotid stenosis and were excluded from the study. Sixty-eight individuals undergoing preoperative investigation for carotid endarterectomy were included as a CS phenotype. Of these patients 17 were asymptomatic (i.e. never having any symptoms). In this study the CS patients were matched against the ICVD (of whom nobody had evidence of carotid artery disease) as to compare an atherosclerotic phenotype against a non-atherosclerotic phenotype.

The patients in **Study IV** and **V** were included consecutively as part of the ongoing SSISS study. A total of 1 299 patients were included from the SSISS Biobank. Retrospective analyses of phenotype, ethnicity as well as laboratory causes (contamination of samples/loss of DNA, double samples and miss-encryption) reduced this number to 928 patients. The main causes for exclusion were; wrong phenotype (for example cerebral bleedings, vertigo, migraine aura, tumors, multiple sclerosis, iatrogenic strokes, asymptomatic CS, n=231) and non-Scandinavian ethnicicity (n=68). Genotyping in **Study IV** and **V** were performed partly in parallel between 2001 and 2005. In **Study V** all patients were examined and in **Study IV** only 685 patients.

The studies were approved by the Local Ethics Committee at Karolinska Institutet and data handling procedures were approved by the National Computer Inspection Board. All participants gave their informed consent.

Controls

All controls were residing in the south Stockholm area and originating from Sweden or other Nordic countries.

In **Study I** ninety-five ethnically matched healthy blood donors were used as controls. In **Study II** ethnically and age matched controls were selected from a panel of 350 previously healthy individuals recruited by the clinical chemistry department of Karolinska University Hospital, Huddinge unit. In **Study III** the control cohort consisted of a pool of 575 individuals comprised of the controls from the first two studies as well as healthy spouses of patients included at the Department of Geriatrics at Karolinska University Hospital, Huddinge unit. This was done to be able to match for age and gender.

In the two last studies (**IV** and **V**) we were not able to match controls for age and gender due to the increase in the subject cohort's size. Controls were recruited as part of the Dept of Neurology DNA Biobank for genetic studies and pooled with 139 of the matched controls from the panel collected by the clinical chemistry department as stated above. In total 751ethnically matched individuals were recruited and used in **Study IV** and 602 (135 from the clinical chemistry panel) in **Study V**. The decrease in subjects used in **Study V** was due to the fact that some DNA was used up. All controls gave their informed consent.

Risk factor definition

All patients were examined at the stroke unit or stroke out patient clinic by a stroke neurologist. Blood samples were drawn after overnight fasting, the first morning of the acute care period. For patients included at the outpatient clinic, data from the acute care episode was used.

Diabetes was defined as known medical history of type 1 or type 2 diabetes with pharmacological treatment. Hypertension was defined as known medical history of hypertension or treatment with hypertensive agents at onset of stroke.

Hyperlipidemia was defined as medical history of known hyperlipidemia or treatment with anti-hyperlipidemic agents. Smoking history was coded as current smoker, former smoker (cessation of smoking for at least 3 months) or never smoker. Ischemic heart disease was defined as history of myocardial infarction, angina pectoris or previous coronary artery intervention. Atrial fibrillation (AF) was based on either medical history of AF or twelve lead electrocardiograms (ECG) in the acute phase of the stroke or by 24 hr ECG monitoring/Holter monitoring. Frequencies of risk factors for the final 928 patients are shown in supplementary table 1 in **Study V** and table 3. Data collection was done retrospectively after inclusion but included a prospective collection for missing data (e.g. investigations performed after the acute stroke period such as echocardiography).

The ICVD patients conformed to the Swedish National Stroke Registry in frequencies of the registered risk factors (atrial fibrillation, diabetes mellitus, hypertension and smoking) for Karolinska University Hospital in Huddinge [254].

Table 3. Frequencies of characteristics of Swedish Ischemic Cerebrovascular Disease (ICVD) patients (a) and ICVD dichotomized according to carotid stenosis (b).

(a)	ICVD n=928	Stroke n=710	TIA n=218	LAA n=213	CE n=200	SAO n=85
Stroke	0,77	NA	NA	0,58	0,86	1
TIA	0,23	NA	NA	0,42	0,14	0
Current Smoking	0,27	0,26	0,28	0,37	0,16††	0,46
Previous Smoker	0,15	0,12††	0,26	0,2	0,14	0,15
Ischemic heart disease	0,26	0,26	0,25	0,31	0,42	0,13†
Myocardial Infarction	0,12	0,13	0,08	0,14	0,22	0,04*
Atrial fibrillation	0,17	0,19**	0,09	0,02	0,58††	0,02
Hypertension	0,51	0,51	0,51	0,51	0,48	0,6
Known hyperlipidemia	0,21	0,18*	0,3	0,32*	0,19	0,2
Diabetes Mellitus	0,19	0,21	0,16	0,23	0,15	0,21
Peripheral Arterial Disease	0,06	0,06	0,06	0,13†	0,04	0,01
Previous CVD	0,23	0,24	0,19	0,27	0,26	0,21
Cholesterol >7.0 mmol/L	0,13	0,11*	0,21	0,22**	0,08	0,14
Triglycerides >2.3 mmol/L	0,15	0,14	0,19	0,22**	0,08	0,15
LDL >4.6 mmol/L	0,17	0,15*	0,23	0,28*	0,11	0,16
HDL <1.2 mmol/L	0,47	0,48	0,43	0,48	0,48	0,53
CRP>10	0,32	0,36**	0,2	0,31	0,40*	0,25
Elevated Leucocytes	0,37	0,13†	0,04	0,12	0,17*	0,05
P-Glucose >6,5 mmol/L	0,28	0,30*	0,2	0,28	0,25	0,24
P-Fibrinogen >4 mmol/L	0,27	0,30*	0,17	0,34	0,27	0,23
Elevated Homocysteine	0,32	0,31	0,32	0,39	0,27	0,22
Corrected Homocysteine	0,24	0,24**	0,25	0,33	0,19	0,15

Table 3. Continued.

(b)	Carotid Stenosis All n=261	>80% n=208	50-79% n=53	Non Carotid stenosis All n=461
Stroke	0,61	0,59	0,70	0,79
TIA	0,39	0,41	0,30	0,21
Current Smoking	0,34	0,35	0,30	0,27
Previous Smoker	0,19	0,19	0,19	0,16
Ischemic heart disease	0,34**	0,33	0,40	0,22
Myocardial Infarction	0,13	0,13	0,13	0,10
Atrial fibrillation	0,10†	0,10	0,11	0,17
Hypertension	0,5	0,48	0,57	0,54
Known hyperlipidemia	0,38**	0,41	0,26	0,25
Diabetes Mellitus	0,23	0,24	0,23	0,18
Peripheral Arterial Disease	0,13††	0,13	0,11	0,03
Previous CVD	0,28*	0,25	0,42	0,20
Cholesterol >7.0 mmol/L	0,19*	0,22	0,07	0,12
Triglycerides >2.3 mmol/L	0,24†	0,26	0,14	0,14
LDL >4.6 mmol/L	0,25†	0,28	0,14	0,15
HDL <1.2 mmol/L	0,51	0,47	0,66	0,45
CRP>10	0,33	0,32	0,36	0,26
Elevated Leucocytes	0,22†	0,22	0,21	0,12
P-Glucose >6,5 mmol/L	0,26	0,26	0,26	0,25
P-Fibrinogen >4 mmol/L	0,33†	0,34	0,33	0,22
Elevated Homocysteine	0,46†	0,43	0,59	0,23
Corrected Homocysteine†††	0,36*	0,31	0,54	0,20

Significant differences: *p<0.05, †p<0.01, **p<0.001, ††p<0.0001
†††Correction for Elevated S-Creatinine (>104)

Phenotyping

In the first studies (**Study I, II, III**) patients were classified as ICVD with or without carotid stenosis.

In the last two studies (**Study IV** and **V**) patients were also classified into ICVD subtypes according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification by a physician (V Kostulas, K Kostulas) reviewing original imaging and data by the "classical" TOAST definition by Adams et al. [3, 5, 6] . In 2000 a computerized version of the TOAST algorithm was found on the net (http://stroke.mc.duke.edu/related_links_and_software.htm), later published [4]. This computerized algorithm was developed and validated as part of the Veterans Administration Acute Stroke Study (VASt), for improving the reliability of the TOAST algorithm for assigning ischemic stroke subtype in retrospective data. Thus after 2000 all cases were reevaluated with the new algorithm in conjunction with the "classical"

algorithm manually. The main difference of the two versions is that the VASt algorithm is computerized so it excluded the physician's subjective feelings and taking historical evidence into account in classifying subtype. We did not use the computerized algorithm but the extensive operations manual designed to guide data abstraction containing comprehensive list of other etiology subtype strokes and lacunar syndromes.

The TOAST subtypes include a) Large Artery Atherosclerosis (LAA), b) Cardio-embolism (CE), c) Small Arterial Occlusion (SAO) i.e. lacunar infarction, d) Other aetiologies (OA) and e) Unknown cause. Unknown cause was further divided into; a) unknown cause with complete workup (including investigation of large vessels, heart and auxiliary blood testing), b) unknown cause with incomplete workup (usually due to severe strokes or high age, where investigation was deemed un-ethical since it would not affect treatment), and finally c) multiple possible causes. The subtypes can also be graded as probable or possible based on their degree of certainty. Frequencies of TOAST-subtypes were similar to recently published cohorts [255] with the exception of the SAO subtype which was more uncommon than previously described, probably due to the fact that data extraction was performed retrospectively and lacunar syndromes were harder to define, but possibly also due to a different age distribution in our cohort.

The LAA subtype was defined as >50% stenosis or occlusion of an appropriate precerebral artery or stenosis of a cerebral artery, deemed to be atherosclerotic.

Small arterial occlusions were defined as one of 5 classic lacunar syndromes with or without evidence of small (<1.5 cm) subcortical or brainstem infarctions and excluding LAA and CE etiology. Lacunar syndrome definitions are shown in table 4.

Cardioembolic ICVD is defined as a presence of a cardioembolic cause (table 5) excluding LAA and SAO.

The abstraction manual defined other specific etiologies stringently (please see homepage specified above and table 1) including; arterial dissection, coagulation disorders, migraineous stroke, vasculitis, monogenic disorders among others.

In **Study III** to **V** we a priori decided to analyze patients with ICVD and any evidence of CS compared to patients with no evidence CS by subdividing the ICVD cohort into patients with evidence of carotid artery stenosis of the internal carotid artery (ICA) identified by duplex ultrasonography and defined as greater than 50% or 80% (according to ECST method), irrespective of whether the CS was ipsilateral or contralateral to the stroke, and a non-CS group with no evidence of significant CS. In **Study IV** the stenosis degree for severe CS is reported according to NASCET (<70%) as to be able to compare it to the Icelandic study.

Table 4. Lacunar syndromes and their definitions.

Lacunar syndrome	Definition
Pure motor	New onset weakness of the face, arm and leg. All should be involved on the same side of the body, usually but not always to the same degree. Dysarthria is frequently present. Sensory findings and incoordination inappropriate for muscle weakness should not be present.
Pure Sensory	New onset sensory findings occurring on one side of the body, involving at least two our of three body areas (face, arm, leg). Objective findings of sensory loss are not required – sensory symptoms (e.g. numbness) in the above noted distributions qualify. Motor and cerebellar findings should be absent.
Mixed sensorimotor	Motor and sensory findings simultaneously located in at least 2 out of 3 body areas (face, arm and leg). Tongue deviation and dysarthria may be present.
Ataxic Hemiparesis	Ipsilateral cerebellar ataxia and hemiparesis. Gait ataxia may be present, and the presence or absence of sensory findings on the same side of the body is variable. The face need not be involved, and arm and leg may be weak to varying extents.
Dysarthria-Clumsy hand	Severe dysarthria, clumsy ataxic hand (especially on writing); facial weakness; tongue deviation may or may not be present, as may ipsilateral hyperreflexia and Babinski sign.

Table 5. Cardioembolic Sources.

High Risk Source for Emboli	**Medium Risk Source for Emboli**
Left Ventricular Thrombus	Congestive Heart Failure
Dilated Cardiomyopathy	Left Ventricular Aneurysm
Akinetic Left Ventricular Segment	Mitral Stenosis without Atrial Fibrillation
Left Atrial Thrombus	Mitral Valve Prolapse
Atrial Myxoma	Mitral Annular Calcification
Infective Endocarditis	Atrial Septal Defect
	Patent Foramen Ovale
	Interatrial septal aneurysm
	Nonbacterial Endocarditis

Frequencies of the TOAST subtypes in the whole SSISS as well as the dichotomized ICVD and CS/non-CS are shown in the table below.

Table 6. T.O.A.S.T subtyping [n, (%)] of Ischemic Cerebrovascular Disease (ICVD) patients (a) and ICVD dichotomized according to carotid stenosis (b).

(a)	ICVD	Stroke	TIA	
	n=928	n=710	n=218	
Large Artery Atherosclerosis (LAA)	213 (23)	124 (18)	89 (40)	
Cardio-embolic (CE)	200 (22)	172 (24)	28 (12)	
Small Arterial Occlusion (SAO)	85 (9)	85 (12)	0 (0)	
Other Causes	36 (4)	24 (3)	12 (5)	
Multiple Causes	47 (5)	40 (6)	7 (3)	
Unknown, Complete workup	89 (10)	56 (8)	33 (15)	
Unknown, Incomplete workup	258 (28)	209 (29)	49 (22)	

(b)		Carotid Stenosis		Non Carotid Stenosis
	All	>80%	50-79 %	All
	n=261	n=208	n=53	n=461
Large Artery Atherosclerosis (LAA)	188 (72)	165 (79)	23 (43)	23 (5)
Cardio-embolic (CE)	8 (3)	4 (2)	4 (8)	127 (28)
Small Arterial Occlusion (SAO)	6 (2)	3 (1)	3 (6)	67 (15)
Other Causes	2 (1)	2 (1)	0 (0)	16 (3)
Multiple Causes	39 (15)	24 (12)	15 (28)	6 (1)
Unknown, Complete workup	2 (1)	2 (1)	0 (0)	86 (19)
Unknown, Incomplete workup	16 (6)	8 (4)	8 (15)	136 (30)

Genetic analysis

DNA purification

In **Study I-III** DNA was isolated from peripheral blood mononuclear cells from 10mL of EDTA anticoagulated venous blood by means of the salting out procedure. In **Study IV** and **V** genomic DNA was extracted from peripheral blood mononuclear cells using the salting out procedure and a multitude of other methods over the following years.

Genetic Markers

Single Nucleotide Polymorphisms (SNPs)

For **Study I** three different SNPs were typed, although the word SNP did not exist at the time being. The SNPs were selected based on previous findings in premature atherosclerosis (the Asn291Ser mutation), evidence of increasing triglyceride (TG) levels (PvuII; C497T) and increasing TG and decreasing HDL levels (HindIII; T+495G).

In the MTHFR gene (**Study II**) we decided to test the C677T polymorphism, where the less common T allele correlates to increased homocysteine (Hcy) levels in its homozygous state in conjunction with the observations of increased Hcy levels in patients with ICVD.

The ACE insertion/deletion polymorphism located in the first intron of the gene was investigated in **Study III**. It has been reported to correlate with plasma levels of the enzyme, hypertension, cardiovascular disease and SAO strokes but not ischemic strokes due to carotid stenosis.

The PDE4D gene was investigated since it is the only gene found by linkage analysis associated to common forms of stroke [256]. When investigating the PDE4D gene in **Study IV** we decided to analyze the most significant markers from the original publication, two SNPs and a microsatellite were genotyped. These three markers were used to describe a protective, at-risk and wildtype haplotype in the original study and as such seemed the most relevant to investigate. The two SNPs (SNP41 and SNP45) are in LD and as such the statistical algorithm used in this study can use them to fill in missing genotypes (see statistics section). The ALOX5AP gene coding the 5-lipooxygnease activating protein (FLAP) was also discovered by linkage analysis in an Icelandic myocardial infarction population and later in common stroke. The four SNPs defining the HapA haplotype were genotyped.

In the final study we investigated 100 suspected susceptibility polymorphisms in 47 different genes. The candidate genes were selected on the basis of being present in biochemical pathways that have been implicated in the development and progression of cardiovascular disease. In addition to the biological relevance of the selected candidate genes, the polymorphisms were further selected based on prior evidence of potential functionality, validated allele frequency and heterozygosity, and sequence-proven allelic variation. The selected genetic polymorphisms focused broadly on the atherosclerotic pathway, including genes involved in lipid metabolism, inflammation, cell adhesion, thrombosis and haemostasis, and platelet function. The polymorphisms genotyped are shown in Supplementary Table 2 in **Study V**.

Microsatellite

In **Study IV** a microsatellite marker (AC008818-1) was genotyped as it was part of the haplotype described above.

Genotyping and allele calling.

In **Study I** and **II**, SNPs were genotyped by restriction fragment length polymorphism (RFLP), state of the art at the time and still used to day. The ACE polymorphism was typed by allele-specific PCR in **Study III**. Alleles were visualized on an agarose gel. Genotyping for **Studies I – III** was done at the Department of Neurology, research lab.

Phosphodiesterase 4D and ALOX5AP SNPs were genotyped with a fluorescent polarization template directed dye-terminator incorporation (SNP-FP-TDI) assay [257] and the AC008818-1 microsatellite with Perkin Elmer/Applied Biosystems 877 Integrated Catalyst Thermocyclers. The PCR products were supplemented with the internal size standard, and the pools were separated and detected on an Applied Biosystems model 377 Sequencer by use of Genescan version 3.0 peak-calling software. Alleles were automatically called with the TrueAllele program (Cybergenetics), and the DecodeGT program (deCODE Genetics) was used both to fractionate according to quality and to edit the called genotypes [258].

Genotyping in **Study V** was performed using multiplex PCR and linear immobilized probe array assays (Strips) (Roche Molecular Systems, Alameda, CA, USA), essentially as described previously [259]. Allele calling was performed both manually (K Kostulas, V Brophy, S Cheng) and automatically with the StripInterpreter_1.3.3.R program (R designating new thresholds for calling alleles). A typical view of the strips laid out for analysis is shown below in figure 1.

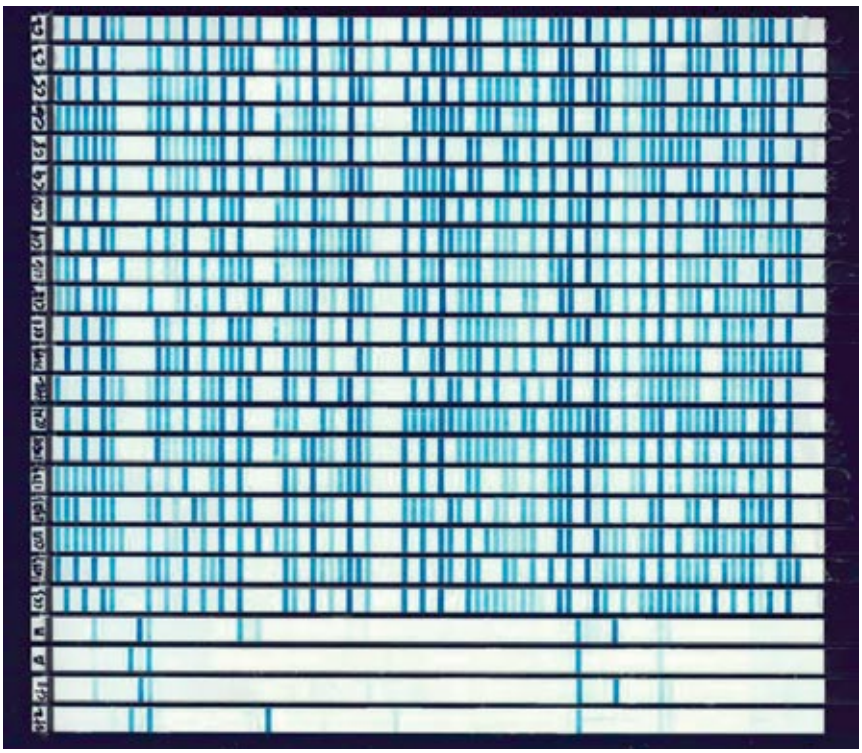

Figure 1. Nylon strips laid out for analysis.

The genotyping of The PDE4D, ALOX5AP and Strips polymorphisms was performed at deCODE Genetic in Reykjavik, Iceland.

Statistical Analysis

Statistical analyses were performed using the GraphPad Instat software, version 2.04a (**Study I** and **II**), SAS 6.12 software (**Study III**) and NEMO [234] (**Study IV** and **V**). For more details see the separate publications/manuscript.

Singlepoint analysis

In all studies Hardy-Weinberg calculations were performed to ensure that affected and controls were in H-W-equilibrium. For details see each separate study. In **Study I**, **II** and **III** we compared allele frequencies and genotype frequencies using Chi square-test and Fischer's exact test when appropriate. No corrections for multiple comparisons were done, as these studies were exploratory.

In **Study IV** and **V** we used the in-house ProjectManager program at deCODE Genetics to perform the statistical analyses calculating likelihood ratios using the NEMO algorithm.

Haplotype analysis

No haplotype analysis was performed in **Study I** to **III**. For **Study IV** we used the NEMO-algorithm to calculate likelihood ratios for the PDE4D haplotypes and HapA haplotype. For **Study V** we used the NEMO-algorithm and Haploview v3.2 (2005). No haplotypes were reported in the manuscript.

STAVE 4: THE LAST OF THREE SPIRITS

STROKE OF CHRISTMAS FUTURE

The Phantom slowly, gravely, silently, approached. When it came near me, I *bent down upon* my *knee; for in the very air through which this Spirit moved it seemed to scatter gloom and mystery.* I was hoping to scatter the mystery of the genetics of ischemic stroke but as I delved more deeply into this enigmatic field the gloom descended upon me for my results were dubious at best.

RESULTS AND INTERPRETATION

Study I. Lipoprotein lipase gene polymorphisms in ischaemic stroke and carotid stenosis

Our first approach to study this field was to collect a phenotypically distinct group of stroke patients. We decided to use carotid stenosis as a marker for atherosclerosis and risk factor for stroke and compare this to overall ischemic stroke, which was not sub phenotyped according to TOAST at this time.

We collected a small cohort of patients with ischemic stroke (n=128) and a group of patients with evidence of carotid stenosis with or without previous stroke (n=56). These patients were matched to healthy controls matched for ethnicicity (n=95), meaning the controls were of Scandinavian origin.

Lipoprotein lipase (LPL) has been implicated in the lipid metabolism and hyperlipidemia in early atherosclerosis [260]. LPL is localized on chromosome 8q22, comprising 10 exons spanning about 30 kb and encodes a protein of 475 amino acids. Analysis of the sequence indicates that LPL, hepatic lipase and pancreatic lipase are members of the same gene family. The function of the LPL protein is to hydrolyze lipids in lipoproteins, like those found in chylomicrons and very low density lipoproteins (VLDL), into fatty acids and glycerol, with Apo-CII as a cofactor. It compartmentalized to endothelial cells lining the capillaries. Insulin has been shown to enhance LPL synthesis. LPL deficiency leads to hypertriglyceridemia. Many polymorphisms have been described in LPL over the years. We chose to examine three mutations/polymorphisms in the gene: 1) the Asn291Ser mutation, 2) the PvuII polymorphism and 3) the HindIII polymorphism. When we started **Study I** no prior study of LPL polymorphisms had been published regarding ischemic stroke.
Reymer et al. found a single nucleotide substitution in exon 6 of the LPL gene resulting in an asn291-to-ser substitution (N291S) in a group of patients with familial combined hyperlipidemia. This mutation was shown to have an allele frequency of 0.02 in the general population [261].

The PvuII II polymorphism is the result of a C→T transition in the restriction site of the LPL gene sixth intron, 1.57 kb from the splice acceptor (SA) site. The region containing the PvuII II site resembles a splicing site in its homology to the consensus sequence required for 30-splicing and the formation of the lariat structure, suggesting that the C497T may interfere with correct splicing of mRNA.

The HindIII polymorphism is a T/G base transition at position +495 inside intron 8. As such it should be non-functional.

The Asn291Ser mutation has been reported to be associated with high levels of cholesterol and triglycerides and with lower levels of HDL in patients with premature coronary artery atherosclerosis. Previous reports have linked 1) the Asn291Ser mutation with hypercholesterolemia, reduced HDL and increased LDL, 2) the PvuII polymorphism with increase of triglycerides or absence of such an increase, and 3) the HindIII polymorphism with increase in triglycerides and decrease of LDL. The reason for the various findings may well be due to different sample sizes as well to the possibility that these polymorphism are of lesser importance for lipid metabolism. In addition the different markers are in linkage disequilibrium with each other which makes it difficult to determine which is most closely linked to the functional property of the LPL gene. For more details please see **Study I**.

After our study four stroke populations have been examined for some of the LPL polymorphisms we examined. In a Japanese population of ischemic stroke patients the HindIII polymorphism was significantly more common than in healthy controls (freq 0.234, p=0,031) [187]. A prospective Finnish study of elderly patients (>85 years) with neuropathologically verified infarctions could not find an association to the Asn291Ser mutation [189]. In the ARIC study (Atherosclerosis Risk In Communities) no association was found for the same mutation [191]. Finally no IS association could be found for the HindIII polymorphism in individuals of Yakut ethnicity in the Russian Federation [262]. The recent meta-analysis of IS could not find an association for the Asn291Ser polymorphism with IS (n cases / n controls: 458 / 8879) [132]. In a recent meta-analysis in CHD the Asn291Ser polymorphism was associated with dyslipidemia and CHD [263]. In a larger meta-analysis in CHD no association was found for the PvuII polymorphism [264].

Regarding carotid artery atherosclerotic phenotypes, in the ARIC cohort a gender-specific association with the HindIII polymorphism and carotid artery wall thickness was found [265]. A British study could find no association between cross-sectional carotid plaque area and the Asn291Ser polymorphism [266]. In the Framingham Offspring Study, 3380 patients underwent carotid ultrasound and determined IMT or carotid stenosis ≥25%. Carriers of the Asn291Ser susceptibility variant showed a higher risk of carotid stenosis (OR=2.59, p=0.028) [267].

In our study neither the polymorphic markers nor the Asn291→Ser mutation differed significantly from controls, although the later mutation had significant influence on serum levels of triglycerides in both patients with stroke ($p<0.02$) and carotid stenosis ($p<0.004$). This is in concordance with previously reported studies for patients with myocardial infarction [260, 268], also in ischemic stroke and carotid artery stenosis, the Asn291Ser mutation is associated with hyperlipidemia, but without this being an independent risk factor for the development of disease. These findings may indicate that the level of metabolic disturbance caused by this mutation is not predisposing in any major way to atherosclerosis. The studies previously done in different carotid atherosclerotic phenotypes have the obvious limitation that they all test plaques in

different ways and in the larger study the cut-off for stenosis is only 25% which is a very low degree of stenosis and with low reliability [269, 270].

Regarding the investigated markers in **Study I** one must conclude that the Asn291Ser mutation is not a good candidate marker since it is very uncommon in the general population and the other polymorphisms are in linkage disequilibrium with each other (see Table 4, **Study I**), as previously described [271], which makes it difficult to determine which is most closely linked to the functional property of the LPL gene. In our final study (**Study V**) we re-analyzed one of the markers from this study, the Asn291Ser mutation; and found no association to ICVD in the large cohort approximating one thousand cases. This would indicate that if there is an effect of this mutation it is of a small degree.

In conclusion there is evidence that the examined polymorphisms in the LPL gene are of less importance by themselves for ICVD. The recent meta-analysis could not find an association for the Asn291Ser polymorphism with IS, opposite to the effect in CHD. However the meta-analysis in IS contained less than 500 cases compared to the CHD meta-analysis with approximately 20 000 cases.

Study II. A methylenetetrahydrofolate reductase gene polymorphism in ischemic stroke and in carotid artery stenosis

For the second study we increased the number of patients and controls to increase power and to be able to match for age and gender. In total 126 patients with ICVD were collected both consecutively (n=80) and retro- and prospectively (n=46). As the initial analysis showed an increased risk in patients with ICVD and relevant carotid stenosis (22%) we added a consecutively collected group of 70 patients with carotid stenosis. We matched for age from a panel of 350 controls. Different controls were used in the matching of ICVD and CS patients.

The methylenetetrahydrofolate reductase (MTHFR) gene is localized on chromosome 1p36.3 and contains 11 exons and the gene itself does not contain a TATA box but CpG islands and binding sites for several transcription factors. MTHFR is an enzyme that exists in the cytoplasm of cells and catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyl-tetrahydrofolate, a cosubstrate for homocysteine remethylation to methionine by the enzyme methionine synthase.
In 1995 Frosst et al. identified a C-to-T substitution at nucleotide 677 leading to a conversion of an alanine to a valine residue in the catalytic domain of the enzyme and responsible for a thermolabile form of MTHFR. This polymorphism has been implicated in vascular disease, thrombosis, cancer, neural tube defects, cleft lip/palate, preeclampsia, Down's syndrome, depression, schizophrenia and migraine with aura. For details please see OMIM 607093. Another thermolabile form of the enzyme was found in 1998, due to a 1298A-C mutation resulting in a glu429-to-ala substitution [272, 273] localized in the regulatory domain of the gene. We started our study in 1997 prior to the knowledge of the more recently found polymorphism and studied the C677T polymorphism by means of RFLP.

Several observations have suggested the importance of homocysteine (Hcy) in ICVD. These include increased levels of plasma homocysteine in ICVD patients and decreased folate levels in individuals that later develop ICVD. In addition, both Hcy and folate levels have been shown to correlate with risk for occlusive carotid artery disease. For more details regarding the importance of Hcy, folate and B12 in ICVD and atherosclerosis please read **Study II**.

Prior to our study this polymorphism had only been studied in one IS cohort. The first study by Markus et al could not find any association between the suspected susceptibility genotype (TT) and all ICVD, its subtypes or carotid stenosis. However they found an association to Hcy levels and that folate by itself was independently related to Hcy [200]. The same group confirmed that MTHFR acts in SAO through endothelial dysfunction [274].

In the next study no association was found but like our study it lacked in numbers (n=72) [275]. Since then the polymorphism has been confirmed in Japanese [276], in children [277], African Brazilians [278], Chinese (with epistatic effect with the ACE ID genotype) [279] and no association found in other child cohorts [280-282], in an Austrian cohort (but showing effects on Hcy with low folate) [283], Brazilian Caucasians [278], Chinese [284, 285], Italians [286] and Japanese (but significant association in females) [201]. After all these studies with preciously few patients in each study and basically no subgrouping Kelly et al performed the first meta-analysis (excluding some of the mentioned studies) and found no association between the TT genotype and IS (OR 1.23, 95% CI 0.96-1,58, n (IS/Control)=2 788 / 3 962) but an association between mild-to-moderate hyper-Hcy and IS [287]. This did not deter further studies now in larger cohorts with positive association in Chinese (n=1832) [288] and small with no association [289, 290]. Pezzini et al found that having many risk variants in different genes increased risk but the MTHFR also had effect alone [291]. Genotype risk factor interactions were found with smoking and hypertension in a subsequent study [292]. In one study the TT genotype was associated to severe CS in men (n=615) [293]. A second meta-analysis in approximately 3000 cases found a positive association to MTHFR TT genotype [132] and a third meta-analysis with 14870 subjects found that the T allele and TT genotype increased risk in overall ICVD and imaging proven IS (approx OR range from 1,17-1,48) [294]. Researchers are still investigating this polymorphism in new IS populations with different results [295-299]. Other studies have evaluated the effect of MTHFR on Hcy levels, where Hcy levels have been associated with IS [300-304]. Bathum et al who studied Danish twins found that Hcy levels had a high heritability and that the MTHFR locus stood for almost all variation due to genetic factors especially in young (18-39 years) where it 53% of phenotypic variation [300].

Regarding CS and IMT, the MTHFR C677T polymorphism has been examined in numerous studies with varying disease groups or healthy subject with various results [203, 305-315].

In our study we found no association between the T allele, TT genotypes and ICVD or CS. We could also not find any association between MTHFR genotypes and Hcy

levels, even when correcting for elevated creatinine. This wasn't changed when low folate or B12 was taken into account.

Compared to **Study I** we increased the number of carotid stenosis patients but also added new healthy controls and thus we were able to match both according to age and sex. Of course our study did like so many other studies lack in numbers, and as we calculated in this case, we would have a power of 80% to detect an OR of 1.6 at the 0.05 level given the allele frequency in the study, thus the effect of MTHFR on ICVD should be lower, as the above mentioned meta-analyses confirm.
Many of the above-mentioned studies don't report subgroups according to TOAST and therefore it is hard to say whether MTHFR T allele would increase risk for CS and LAA stroke, but at least one study found this association regarding LAA [316] and CS [293]. The analysis of MTHFR in **Study V** did not find the T allele to be associated to overall ICVD (p=0.18, T freq. 0.29) or severe CS (p=0.08, T freq. 0.26). The frequency for the T allele was similar to that in **Study II** (0.30).

In conclusion the MTHFR C677T variant seems to affect the risk of IS, but no definite treatment has yet been found to decrease risk when treating the appropriate biomarker, homocysteine.

Study III. An angiotensin converting enzyme gene polymorphism indicates a genetic distinction between ischemic stroke and carotid stenosis

A common insertion/deletion polymorphism in the angiotensin converting enzyme (ACE) gene has been shown to be of importance in cardiovascular disease and might be of importance to the development of ischemic stroke or carotid stenosis. In our third study we collected 131 patients with ischemic stroke and 68 patients with more than 50% stenosis of the internal carotid artery were investigated and compared with age- and sex-matched healthy controls.

The ACE gene is localized to chromosome 17q23 encoding two isozymes, one somatic expressed in many different tissues, including vascular endothelium (4.3-kilobase mRNA species), epithelial cells and neuronal tissues and a germinal expressed in sperm only. Its two primary functions are the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor and aldosterone-stimulating peptide and involvement in the inactivation of bradykinin, a potent vasodilator. Angiotensin II, the main product of ACE, is a pleiotropic hormone, serving as a vasoconstrictor, neurotransmitter, growth factor, angiogenesis factor, pro-thrombotic agent, and cytokine.

The ACE gene has been suggested as a candidate gene for hypertension and studied in a multitude of different cardiovascular phenotypes (please se OMIM entry MIM +106180 and MIM #145500). It has been shown that variability in ACE concentrations in plasma are determined by a major gene effect [317, 318] which was shown to be associated with an insertion (I)/deletion (D) polymorphism involving about 250 bp situated in intron 16 of the ACE gene, the so-called ACE/ID polymorphism [319]. For more details regarding the ACE/ID polymorphism, see OMIM (entry MIM +106180).

Prior to our study this polymorphism had been studied in some stroke cohorts and other stroke related phenotypes as stated in the **Genetics of multifactorial ischemic cerebrovascular disease** part of **Background** and after our initial submission of the manuscript, Sharma published a meta-analysis on the ACE/ID polymorphism showing the D allele to be a weak risk factor for ischemic stroke under a recessive model [131]. The much larger meta-analysis (including **Study III**) by Casas et al with 2 990 cases and 11 305 controls also found a minor risk for the D/D genotype (OR 1,21, CI 1,08-1,35) [132].

In Study III we could not find an association of the presumed susceptibility allele (D) with IS but there was a significant difference (p=0.038, OR=1.71) in the distribution of ACE alleles, homozygosity for the presumed susceptibility allele being more common in patients with CS than in healthy controls. There was also a significant difference ($p<0.05$) in patients with CS in comparison with matched ICVD patients without CS.
We then concluded that the ACE gene polymorphism is a risk factor for the development of CS. At the time we saw the observed difference in ACE allele distribution as evidence for a genetic distinction between ICVD and CS, two clinically related conditions, which further supported the hypothesis that genetic factors are of importance for this group of diseases.

New for **Study III** the increased awareness of the reviewers that matching for confounding factors should be performed. Since we did not possess that data on our controls we hypothesized about using a restricted group, where we limited the analysis to non-smoking patients free of ischemic heart disease, diabetes and hypertension to confirm the negative result from the overall analysis. Analysis between the subgroups of ICVD (CS and non CS) was done before and after logistic regression. Of interest in this study was that our results went in the opposite direction of the previous study by Markus et al where there was no association between the D allele and carotid atheroma, but the mean stenosis degree in that study was only 35% in 46 cases [121]. In a later study by Pfohl et al the D allele was found to be associated with extracranial stenosis [320]. Our own study mirrors this study as we find the ACE D-allele to be associated to ICVD with severe CS. The analysis of the ACE I/D polymorphism in **Study V** did not find the D allele to be associated to overall ICVD (p=0.35, D freq. 0.52) or severe CS (p=0.31, D freq. 0.53). The frequency for the D allele was similar to that in **Study III** (0.54).

Of course fine tuning the phenotype, as Hassan and colleagues did to leukoaraiosis and lacunar syndromes supported the hypothesis that the DD genotype influences small vessel disease [321]. We could not find this in the SAO subgroup in Study V (p=0.35, D freq. 0.54).

In conclusion the ACE D-allele seems to affect IS. The question remains which subphenotype of IS/ICVD it has the largest effect upon.

Study IV. PDE4D and ALOX5AP genetic variants and risk for Ischemic Cerebrovascular Disease in Sweden

A few years passed by as we continued to collect ICVD cases to increase power. Meanwhile the first and second successful attempts to use linkage analysis to find common stroke loci were performed on Iceland, namely the phosphodiesterase 4D (PDE4D) and Arachnoidate 5-lipoxygenase-activating protein (ALOX5AP) genes.
The PDE4D gene is localized on chromosome 5q22 and contains at least 22 exons and spans about 1.5 Mb, having at least 9 different isoforms that encode functional proteins. The protein degrades the second messenger cAMP, a signal transduction molecule. In the original study by Gretarsdottir et al the at-risk haplotype had significantly lower expression of the PDE4D7 and PDE4D9 isoforms [234]. Information regarding other isoforms and their function can be found in OMIM (entry MIM *600129).
The arachnoidate 5-lipoxygenase-activating protein is located on chromosome 13q12 and contains 5 exons spanning approximately 31 kb. The protein 5 lipoxyganese-activating protein (FLAP), encoded by the ALOX5AP gene, is involved in the leukotriene pathway as a regulator and inititiator, through 5-lipoxygenase (5-LO) and has been implicated in the development of atherosclerosis in a mouse-model as well as in human studies [322-325]. A recent study has shown elevated expression of 5-LO in human symptomatic carotid plaques [326, 327].

PDE4D and FLAP have been studied extensively in a short time period, but the studies either lacked in numbers or did not aim to reproduce the most significant markers and haplotypes from the original publication as stated previously in **Genetics of Ischemic Cerebrovascular Disease**.

We started our collaboration with deCODE Genetics before their publication of the STRK1 locus. At first we tried to map the PDE4D gene with approximately 70 markers across the gene. These markers were chosen on the basis of what was significant in the initial linkage analysis [256]. The markers analyzed are shown in table 7.

Table 7. Markers analyzed in PDE4D. For details see Gretarsdottir et al. [210, 231].

D5S623	AC016604-3	D17-C	D5S2018	AC008498-1	D5S2072
D5S1968	AC016635-2	D17-B	AC027322-3	AC016636-1	D5S647
D5S664	AC016604-5	D5S2500	AC027322-5	AC016636-2	D5S2121
D5S1966	**D5S2028**	D17-D	D5S2071	D5S1990	D5S2048
D5S633	**D5S2107**	AC008804-2	AC008879-2	AC016645-3	D5S2036
D5S461	AC008833-2	D17-H	AC008818-1	AC016645-1	D5S2046
D5S2102	AC008833-3	D17-G	AC008879-3	AC026434-5	D5S2019
D5S1715	D5S468	D5S2080	AC020733-1	D5S1474	D5S629
AC008786-7	AC022125-3	AC027322-12	AC016591-2	D5S427	D5S1999
AC008786-6	AC008833-6	AC027322-10	AC008498-5	D5S1956	
AC008786-5	**D5S2000**	AC027322-9	AC008498-4	D5S2858	
D5S398	D5S2091	AC027322-8	D5S624	D5S2089	

The most significant single markers were D5S664 and D5S2000 localized outside the gene (D5S664) and close to the D8 exon (D5S2000) with the most significant haplotype consisting of the markers D5S2028 (0) and D5S2107 (10) (p=0.000046). We did separate analyses in subgroups according to TOAST and based on risk factors to explore the material, however after correction for multiple testing by permutation no association was significant (unpublished results).

As the initial analyses were performed more SNPs were discovered by deCODE and we collected more patients and controls to increase power and in 2005 we limited the analysis to typing the most significant markers namely SNP45, SNP41 and the microsatellite AC008818-1. At the same time we typed the sevens SNPs defining the HapA and HapB (unpublished) haplotypes in the ALOX5AP gene. In total we had 685 cases and 751 controls typed with varying amount for each marker (due to loss of DNA mainly) as shown in **Study IV**.

Our findings seemed to partly confirm the original report, meaning we found a much weaker borderline association in the two main groups implicated in the original report, namely LAA and CE, but no association in the over all ICVD group when analyzing the SNPs but not the microsatellite. Microsatellites can be expected to be more divergent in the two populations (Icelandic and Swedish) as microsatellites have one order higher magnitude of change compared to SNPs. When analyzing the at-risk haplotype GA0 and the protective haplotype AG-8 a weak association was found for the at-risk haplotype GA (excluding the microsatellite). Since the SNPs are in strong LD, this haplotype corresponds to the complement of the protective haplotype in the Icelandic study.

We did not confirm the association between ALOX5AP HapA haplotype and ICVD, but a non-significant risk was observed in the LAA subtype. No association was significant after correction by permutation, based in the number of haplotypes and subgroups analyzed.

In conclusion there are ample, but heterogeneous associations between PDE4D and IS or ICVD. A forthcoming meta-analysis of the original at-risk and protective haplotypes and future functional studies might help elucidate this gene's effect in ICVD. There is similar heterogeneous association data for ALOX5AP. This together with other genetic variants implying effect in the leukotriene pathway points to relevance in atherosclerosis, but the exact importance of ALOX5AP in stroke remains to be determined.

Study V. Genetic Profile of Ischemic Cerebrovascular Disease and Carotid Stenosis

One of our primary aims was to study multiple susceptibility markers to be able to find gene-gene and gene-environment interactions and to be able to define new subgroups of ICVD based on genetic background.

Through our collaboration with deCODE Genetics we came in contact with Roche Diagnostic Systems in Alameda, CA who hade devised a set of nylon filaments

("Strips") where PCR products from a multiplex PCR could be hybridized. We tested 100 polymorphisms in 47 genes incorporated into 3 different strips in a large cohort of ICVD (928) patients and controls (602). The markers were chosen by Roche and the three strips were aimed at cardiovascular risk factors and thus had more lipid polymorphisms than hypertensive and coagulation polymorphisms. Inflammatory markers were mainly assigned another set of strips (made to investigate inflammatory diseases). All the patients had been subtyped according to TOAST using the original publication and the computerized algorithm [3, 4] and extensive data on investigations and risk factor as well as laboratory data had been collected.

Our primary hypothesis regarding ICVD is that it is a very heterogeneous disorder and since we have a tradition of examining most patients at our clinic with duplex ultrasound of the carotid arteries, which is a sound and reliable method, we decided to use carotid stenosis (CS) to dichotomize the patients. In this way we were able to regress for confounding factors as well.

We mean that ICVD with presence of carotid stenosis would be a more atherosclerotic phenotype than over all ICVD and also easily replicated in other cohorts. This approach, to use an intermediate phenotype, has been used by other investigators with different risk factors such as hypertensive stroke patients and patients with evidence of leukoaraiosis and stroke.

Our a-priori decision was to analyze the over all group of ICVD, IS and TIA separately (mainly to see that the TIAs did not change the overall association), gender specific, etiologically defined TOAST subgroups, early onset of ICVD (defined as ≤65 years) and patients with severe symptomatic CS (defined as ≥80 % stenosis, ECST). It is important to understand that the LAA TOAST subgroup will not be the same as the severe symptomatic CS subgroup, the difference being LAA includes less severe CS stenoses (>50 %), vertebral and intracranial stenoses.

Regarding the a priori decision to analyze patients with ICVD and any evidence of CS, irrespective of whether it was symptomatic or not, compared to patients with no evidence of CS, this group also differs from LAA. Table 8 shows the differences.

Table 8. Difference between subphenotypes with large artery atherosclerosis.

	Stenosis	Vessels	Symp/Asymp	Examination
Subgroup				
LAA	≥50%	All major	Yes/No	US, CTA, MRA, angio
Severe CS	≥80%	ICA	Yes/No	US, CTA, MRA, angio
ICVD with CS	≥50%	ICA	Yes/Yes	US

LAA= large artery atherosclerosis, CS = carotid stenosis, Symp = symptomatic, Asymp= asymptomatic, US = ultrasound, CTA = CT angiography, MRA = MR angiograph, angio = conventional angiography

We found three polymorphisms to be associated to ICVD after correction for age and gender: the lipoprotein lipase (LPL) Ser447Term polymorphism, the angiotensinogen (AGT) A(-20)C promoter polymorphism and guanine nucleotide-binding protein beta-3 (GNB3) 825 C/T polymorphism. Two polymorphisms in MMP9 and MMP12 were borderline significant. The GNB3 polymorphism was however in Hardy-Weinberg disequilibrium in patients. This can be due to genotyping error or an actual different distribution of the susceptibility allele in the subpopulation that develops ICVD.

The LPL Ser447Term polymorphism is well evaluated in IS and previous studies are discussed in **Study I**.

The AGT -20A→C polymorphism is less evaluated in ICVD with only two studies testing it and has been shown to be associated with increased basal promoter activity of the AGT gene. Gormley et al found the marker not to be associated with SAO but an interaction between the susceptibility allele and hypertension [328]. In the Austrian Stroke Prevention Study, there was an association between microangiopathy-related cerebral damage and a haplotype comprised of the -20C allele among others [329]. This could not be shown by Gormley et al.

The GNB3 825 T-allele has been shown to increase risk for hypertension in the original study by Siffert et al. [146], but no overall association to IS has been shown in the only study performed so far [330], which did not lack in power. The polymorphism is located in exon 10 of the GNB3 gene and is associated with alternative splicing of the gene and formation of a truncated but functionally active beta3 subunit. T-allele carriers respond with a stronger decrease in blood pressure to therapy with a thiazide diuretic and with clonidine.

All the significant markers showed varying frequencies in different subphenotypes of ICVD as shown in **Study V**, which in most cases could be interpreted positively when taking the gene function into account.

In our comparison of dichotomizing ICVD according to presence of CS we found Factor VII (F7), apolipoprotein E and two renin polymorphisms to be more frequent in patients with evidence of CS compared to non-CS patients. How should this be interpreted? Our hypothesis was that these markers could be of greater importance to develop stroke when having concomitant large vessel atherosclerosis. With F7 this is quite straight forward as the susceptibility allele has been shown to be prothrombotic.

The APOE Arg158Cys polymorphism which denotes APOE2 and has been shown to lead to type III hyperlipoproteinemia which is typically associated with homozygosity for a change in apolipoprotein E2 from arg158 to cys [331]. Thus the T-allele (Cys) should increase risk, opposite to what we have found. Sullivan et al. found that the cys version of the APOE protein is sufficient to cause type III hyperlipoproteinemia and spontaneous atherosclerosis in mice. Mice expressing human APOE2 (2/2) had elevated plasma cholesterol and triglyceride levels compared to normolipidemic mice that expressed human APOE3 (3/3) and spontaneously developed atherosclerotic plaques [332]. However it has late been shown that APOA5 can be an important cofactor to develop type III hyperlipidemia [333]. Regarding carotid atherosclerosis

and IMT, specifically E2 allele carriers, have been shown to have increased risk of carotid hypertrophy after logistic regression despite a favorable lipid profile [334]. APOE has been studied extensively in stroke (both ischemic and hemorrhagic) but previous publications go in different directions and vary depending on population [226, 335-337]. The meta-analysis by Casas et al could find no increased risk for APOE E4 allele and stroke (approx 1800 cases) in Caucasians with IS [132].

The same problem could be said to affect the renin polymorphisms where the susceptibility allele of C2464T has been shown to be associated with hypertension in some populations [338]. The T-allele was less frequent in ICVD patients with CS compared to ICVD patients without CS in our cohort. The other REN polymorphism G(-1111)A was identified by SNP discovery at Roche Molecular Systems and is in LD with the Bgl I SNP. The susceptibility A allele has been shown to be associated with hypertension [339]. In our study the A allele is less frequent in ICVD with CS compared to ICVD without CS.

A haplotype consisting of BglI(+)/MboI(-) and BglI(+)/MboI(+) was found to be highly associated with stroke [340]. When using the A allele corresponding to Bgl I+ allele and constructing haplotypes, no association can be found in our material (AT vs. GC), but only 13 patients were carriers of the AT haplotype in the ICVD CS group.

How can these discrepancies be explained? Are they discrepancies at all? It is of importance to remember that this is an analysis of differences between patient groups and that such an association is relevant in the perspective of mechanisms and not for the overall risk of ICVD. Of the four polymorphisms, Factor VII G(-402)A and APOE were significant when comparing ICVD with CS to controls as was the LPL Ser447Term polymorphism. The reason that the LPL polymorphism did not show an association in the CS analysis is possibly that the frequency of the susceptibility allele was much lower in non-CS patients compared to controls. The differences in frequencies of the alleles in thee genes could be random chance or there could be a difference in risk factor frequencies that might be of importance even though we performed a logistic regression to take them into account, besides hypertension was equally present in CS and non-CS patients. Hyperlipidemia was however more frequent (38% vs. 25 %, $p<0.001$) in CS vs. non-CS which could dilute the effect of APOE E2 perhaps.

In all significant genes haplotype analysis was performed using Haploview and NEMO with other polymorphisms in the same gene but no significant haplotypes were found after permutation testing. In the end these results need to be confirmed in independent cohorts but show that new associations can be found but hard to interpret.

In conclusion the Ser447Term polymorphism of the LPL gene seems to be a relevant candidate marker in ICVD and in this cohort it is protective. The ICVD with CS phenotype seems to be useful in finding differences in frequencies for known candidate polymorphisms and is similar to the TOAST LAA subtype, but easier to phenotype.

GENERAL DISCUSSION

Association studies

The current trend in genetics of complex diseases is to use LD-mapping/association analysis to find common variants. The common disease common variant hypothesis states that common genetic variants should be present in the affected population and healthy controls in a significant proportion of the population to be seen as disease causing. Opposite to this are variants which are less frequent in the population but in certain cases may lead to disease. In reality both variants should be taken into account, but in reality it is much easier to study common variants compared to rare ones, if you don't happen to have a very specific phenotype which is more prone to get disease from a less common variant.

When I started my studies in genetics, one either performed linkage analysis of "Mendelian" versions of common disease in extended pedigrees or selected candidate genes, often being candidates based on findings in other disease related to stroke (coagulation, hypertension, diabetes) or diseases with the same risk factors as stroke (ischemic heart disease). Typically one gene – one polymorphism was studied at a time and strong associations were often found in the first paper declining with time and effort [252]. Today, state of the art is genome wide association studies, typing hundreds of thousands of markers at the same time [85].

Size is Power

Another difference from then and now is the sizes of the study cohorts, to begin with small or very small ranging from less than a hundred affected to a few hundred. At the time there was a belief that genetic risk factors should have a risk increase close to the same as other common risk factors; in the range of OR 2-5. This fact in combination with the initial strong findings led to the use of small cohorts. As recent meta-analyses of common polymorphisms have shown, the true effect of these variant is in the range of OR 1.15-1.4 and cohorts of about a few thousand affected were needed to find these risks [132, 294]. When using a 100 case cohort, trying for a p-value of less than 0.05 with a power of 80% and a minor allele frequency of 0.3, you would be able to detect an OR of 2.2. When using a 1000 case cohort you would be able to detect an OR of 1.3.

Phenotypes, intermediate or interlocking

As mentioned in the Methods part we tried to use a more specific phenotype to detect genetic susceptibility variants, namely presence of carotid stenosis in an ICVD patient. Carotid stenosis is considered a risk factor and causative agent of ICVD and also an expression of atherosclerosis which is reliably found with duplex ultrasound. A recent systematic review of family history (FHx) in ischemic stroke found that FHx of ischemic heart disease and FHx of hypertension were associated to stroke and particular to large artery atherosclerosis (LAA) and it was concluded that the heritable effect in ischemic stroke is in part due to heritability of hypertension and IHD [341]. There was no association to FHx of diabetes mellitus. Thus hypertension and atherosclerosis can be confounding intermediate phenotypes to IS. The authors mean that these phenotypes should have a stronger genetic component and adjustment for these phenotypes in controls is needed. We have used CS as our intermediate phenotype but have been

unable to adjust for this in our controls and thus tried to compare the cases with the intermediate phenotype to cases without it to be able to find novel genetic associations.

Controls are random

The recommendation in genetic association studies has been to use so called hypercontrols, i.e. controls well described regarding confounding factors and excluding the studied phenotype. The controls should be ethnically matched to avoid population stratification. In the recent genome-wide association study this has been overturned [85]. The authors argue against hypercontrols and mean that population stratification is less of a problem than previously thought, at least in larger cohorts. The authors conclude that blood donors (which were one part of the controls) did not show any significant differences to epidemiological controls and should be used in the future as they are a much cheaper alternative when numbers need to be large.

Multiple genes, Multiple testing

As mentioned above, previously only one gene and one polymorphism at a time was studied in most genetic studies of ischemic stroke. This has changed over the years as faster typing methods have become available peaking with the possibility to type the whole genome with micro array chips. Of course this instead has put the focus on how to correct for multiple testing, whether one should think one marker- one hypothesis. This would be the most stringent way of doing it as done by the Bonferroni correction, but this might be too stringent from a genetic point of view as markers are not independent of each other. Therefore other methods using permutation testing and simulation of data or the much easier "divide the cohort in two" approach have been used or no correction as studies might be exploratory. Not all studies are of course exploratory and therefore correction is needed. In **Study IV** we used permutation but reported both uncorrected and corrected p-values. The problem appeared in **Study V** where we analyzed 100 SNPs in 47 different genes. Some of the markers had been implicated in IS before and were extensively studied including meta-analyses performed cementing the associations. Other SNPs were associated to intermediate phenotypes or risk factors of ICVD. How should one correct in this scenario? We decided not to correct at all and only report our findings.

The best way to correct would be to replicate the finding in a second independent cohort, like people from our research group has done in multiple sclerosis and the interleukin-7 receptor gene [342].

Haplotypes and tagging

A haplotype is a combination of alleles at two or more loci that are linked to each other. Haplotype analysis can be used to detect stronger associations than can be found by single markers. Since a haplotype consists of several markers spanning a region they can be used in mapping parts of a genome or gene that could be of interest.

In early studies we have mainly used the one gene- one polymorphism approach and have not been able to calculate haplotypes as such, but in **Study IV** we tried to replicate a haplotype found in the Icelandic cohort. In **Study V** we performed haplotype analysis in the significantly associated markers but could not find any haplotypes that change the overall strength or effect of any association.

A new way of mapping regions in genes is by using tagSNPs. By taking advantage of SNPs with high LD (calculated by r^2) one can use one tagSNP to infer the alleles of other linked SNPs and thus type fewer markers in the gene. This approach is possible since we have LD data from the HapMap project. As our markers in **Study V** were selected prior to HapMap being completed, this was not part of the selection criteria.

Functional data

Adding functional data as levels of proteins, lipids and mRNA increases the truth of the association. Recommendations from he literature state that functional data demonstrating a biological effect of the at-risk allele enhance the significance of the finding [343]. Novel mRNA and protein arrays will make this feasible on a large scale in the not to far future.

STAVE 5: THE END OF IT

CONCLUSION AND FUTURE

YES! and the bedpost was my *own. The bed was* my *own, the room was* my *own. Best and happiest of all, the Time before* me *was* my *own, to make amends in!*
*"*I *will live in the Past, the Present, and the Future!"* I *repeated, as* I *scrambled out of bed. "The* Strokes *of all Three shall strive within me. Oh* Gregor Mendel*! Heaven and the Christmas Time be praised for this!* I *say it on my knees, old* Gregor*; on my knees!"*

I was so fltstered and so glowing with his good intentions that I *decided to finish this of…*

In this thesis I have aimed to dissect the complex entity of ischemic cerebrovascular disease to increase the knowledge of the pathogenesis by using genetic factors. This was done by investigating candidate genes using the association approach.

In the first three studies I used the outdated one gene- one polymorphism approach based on the common disease –common variant hypothesis. I was not able to find any association, but retrospectively I can say that the markers were well chosen as two of them have been shown to be associated to ischemic stroke in meta-analyses, MTHFR and ACE.

In the forth study I tried to replicate the first finding from a linkage analysis in complex stroke. I found part of the protective haplotype to be associated in the same subgroup of ICVD, which had the strongest initial association in the original study.

In the final study I tried to find association in a group of previously published susceptibility and resistance polymorphisms to ischemic stroke and its intermediate phenotypes. I found weak associations to a few genes; one being the same gene as in the first study, and I also described the different frequencies of these polymorphisms in different subphenotypes of ICVD. By using my own chosen subphenotype, carotid stenosis, I found additional differences in frequency between an atherosclerotic phenotype of ICVD, previously not reported which can be used in future studies.

In the foreseeable future, the field of complex disease genetics will accelerate as genotyping becomes less expensive and faster. The rate limiting step will be collection of well phenotyped large groups of affected, relevant statistical methods to analyze the data as well as collecting mRNA and protein to correlate the genotype with its functional outcome. There are already today expensive ways of measuring genes, expression and protein at the same time.

With the advent of large well described cohorts, testing for gene-gene and gene-environment interactions will be possible and this might facilitate risk in already found associations.

I think the knowledge from studying susceptibility markers will be limiting by itself. The effects will be far too small without a gene-gene or gene-environment interaction

analysis. It may be used in dissecting disease pathology to be able to potentate targeting for drug design. I think an easier approach, and perhaps more fruitful, would be to test risk variants effects in conjunction with specific pharmacological treatments. In this way one might be able to choose relevant individual treatment from the current arsenal of drugs. With the use of pharmacogenetics the correct dose can be chosen.

As this little booklet has been under the auspice of Charles Dickens' "A Christmas Carol", for my own oblique reasons, I would like to make an end of it by this paraphrase.

He had no further intercourse with Strokes, but lived upon the Total Abstinence Principle, ever afterwards…

But I must say I rather suspect this will not come true…

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