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GENETICS OF INHERITED SMALL VESSEL DISEASES

In Search of a Novel Small Vessel Disease and Modifiers of the Clinical Course of CADASIL

Maija Siitonen née Junna

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To my family

ABSTRACT

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GENETICS OF INHERITED SMALL VESSEL DISEASES – IN SEARCH OF A NOVEL SMALL VESSEL DISEASE AND MODIFIERS OF THE CLINICAL COURSE OF CADASIL

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The genetic and environmental risk factors of vascular cognitive impairment are still largely unknown. This thesis aimed to assess the genetic background of two clinically similar familial small vessel diseases (SVD), CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy) and Swedish hMID (hereditary multi-infarct dementia of Swedish type). In the first study, selected genetic modifiers of CADASIL were studied in a homogenous Finnish CADASIL population of 134 patients, all carrying the p.Arg133Cys mutation in *NOTCH3*. Apolipoprotein E (*APOE)* genotypes, angiotensinogen (*AGT)* p.Met268Thr polymorphism and eight *NOTCH3* polymorphisms were studied, but no associations between any particular genetic variant and first-ever stroke or migraine were seen. In the second study, smoking, statin medication and physical activity were suggested to be the most profound environmental differences among the monozygotic twins with CADASIL.

Swedish hMID was for long misdiagnosed as CADASIL. In the third study, the CADASIL diagnosis in the Swedish hMID family was ruled out on the basis of genetic, radiological and pathological findings, and Swedish hMID was suggested to represent a novel SVD. In the fourth study, the gene defect of Swedish hMID was then sought using whole exome sequencing paired with a linkage analysis. The strongest candidate for the pathogenic mutation was a 3'UTR variant in the *COL4A1* gene, but further studies are needed to confirm its functionality.

This study provided new information about the genetic background of two inherited SVDs. Profound knowledge about the pathogenic mutations causing familial SVD is also important for correct diagnosis and treatment options.

Keywords: CADASIL, *NOTCH3*, Swedish hMID, autosomal dominant, familial small vessel disease, vascular cognitive impairment, exome sequencing, linkage analysis, association study

TIIVISTELMÄ

Maija Siitonen (o.s. Junna)

PERINNÖLLISTEN AIVOJEN PIENTEN SUONTEN TAUTIEN GENETIIKKAA – UUDEN TAUDIN GENEETTINEN TAUSTA JA CADASILIN TAUDINKUVAA MUUNTELEVAT TEKIJÄT

Turun yliopisto, Lääketieteellinen tiedekunta, Biolääketieteen laitos, Lääketieteellinen biokemia ja genetiikka, Geriatria, Turun molekyylilääketieteen tohtoriohjelma (TuDMM) ja Helsingin yliopisto, Lääketieteellinen tiedekunta, Patologia

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Verisuoniperäisistä häiriöistä johtuvan kognition heikentymisen syntyyn vaikuttavia geneettisiä ja ympäristötekijöitä tunnetaan toistaiseksi vähän. Tässä työssä tutkittiin kahden kliinisesti samankaltaisen aivojen pienten suonten taudin, CADASILin (**C**erebral **A**utosomal **D**ominant **A**rteriopathy with **S**ubcortical **I**nfarcts and **L**eukoencephalopathy) ja ruotsalaisessa suvussa esiintyvän perinnöllisen multi-infarktidementian (Swedish hMID) genettistä taustaa. Ensimmäisessä osatyössä CADASILin taudinkuvaan vaikuttavia tekijöitä tutkittiin 134:n samaa *NOTCH3*-geenin mutaatiota (p.Arg133Cys) kantavan suomalaisen potilaan aineistossa. Potilaiden apolipoproteiini E (*APOE)* -genotyypit, angiotensinogeeniä koodittavan geenin (*AGT)* p.Met268Thr-polymorfia ja kahdeksan *NOTCH3*-polymorfiaa tutkittiin, mutta minkään niistä ei voitu osoittaa assosioituvan potilaan ensimmäisen aivoinfarktin tai migreenin kanssa. Toisessa osatyössä tupakointi, statiinilääkitys ja aktiivinen liikunta osoittatutuivat merkittävimmiksi eroiksi elintavoissa taudinkuvaltaan toisistaan poikkeavien CADASILia sairastavien identtisten kaksosten välillä.

Kolmannessa osatyössä ruotsalaisen multi-infarktidementiaa sairastavan suvun CADASIL-diagnoosi osoitettiin vääräksi geneettisten, radiologisten ja patologisten tutkimusten perusteella. Neljännessä osatyössä pyrittiin selvittämään tämän taudin taustalla oleva geenivirhe eksomisekvensoinnin ja kytkentäanalyysin avulla. Tutkimusten perusteella lupaavin variantti sijaitsee *COL4A1*-geenin 3'UTR-alueella. Koska kyseessä on UTR-alueen variantti, lisätutkimukset ovat tarpeen variantin patogeenisyyden osoittamiseksi.

Tämä tutkimus valotti kahden perinnöllisen vaskulaarisen taudin geneettistä taustaa. Taudin aiheuttavan geenivirheen ja taudinkuvaa muuntelevien tekijöiden tunteminen helpottaa sairauksien diagnostiikkaa ja uusien hoitomuotojen kehittämistä.

Avainsanat: CADASIL, *NOTCH3*, multi-infarktidementia, autosominen dominantti, Swedish hMID, perinnöllinen vaskulaarinen dementia, eksomisekvensointi, kytkentäanalyysi, assosiaatiotutkimus

TABLE OF CONTENTS

ABBREVIATIONS

AD	Alzheimer's disease
ADAM	ADAM Metallopeptidase Domain
AGT	angiotensinogen
APOE	apolipoprotein E
CADASIL	cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CBF	cerebral blood flow
CELSR1	cadherin, EGF LAG Seven-Pass G-Type Receptor
CI	confidence interval
CoR	corepressor
DHPLC	denaturing high performance liquid chromatography
DLL	delta-like
DNA	deoxyribonucleic acid
DSL	delta/serrate ligand
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor-like
EM	electron microscopy
FFPE	formalin fixed paraffin embedded
GM	grey matter
Gmaf	global minor allele frequency
GOM	granular osmiophilic material
GWAS	genome-wide association study
HAc	histone acetylase
HANAC	hereditary angiopathy with nephropathy, aneurysms, and muscle cramps
HDAc	histone deacetylase
HGP	human genome project
hMID	hereditary multi-infarct dementia
HR	hazard ratio
IHC	immunohistochemical
Jag	jagged
LD	linkage disequilibrium
MAM	mastermind

MCI	mild cognitive impairment
MID	multi-infarct dementia
MRI	magnetic resonance image
N3ECD	NOTCH3 extracellular domain
N3ICD	NOTCH3 intracellular domain
N3TMD	NOTCH3 transmembrane domain
NGS	next generation sequencing
NOTCH3	Notch (drosophila) homolog 3
OMIM	Online Mendelian Inheritance in Man
PAS	periodic acid-Schiff
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDGRF	platelet-derived growth factor receptor
PEST	NOTCH3 transactivation domain
PET	positron emission tomography
PRKCH	protein Kinase C, Eta
RAS	renin-angiotensin system
rs	reference SNP accession number
RT-PCR	reverse transcriptase-PCR
SI	sclerotic index
SNP	single nucleotide polymorphism
SVD	small vessel disease
TACE	tumor necrosis factor –alpha converting enzyme
TIA	transient ischemic attack
VaD	vascular dementia
VCI	vascular cognitive impairment
VSMC	vascular smooth muscle cells
WES	whole-exome sequencing
WGS	whole-genome sequencing
WM	white matter
WMH	white matter hyperintensity
WML	white matter lesions

All gene symbols are italicised in the text: human genes in upper-case and mouse genes only the first letter in upper-case

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals I-IV.

I **Siitonen, M.**, Mykkänen, K., Pescini, F., Rovio, S., Kääriäinen, H., Baumann, M., Pöyhönen, M., Viitanen, M. APOE and AGT in the Finnish p.Arg133Cys CADASIL population. *Acta Neurol Scand*. (2015) Dec;132(6):430-4.

II Mykkänen, K., **Junna, M**., Amberla, K., Bronge, L., Kääriäinen, H., Pöyhönen, M., Kalimo, H., Viitanen, M. Different clinical phenotypes in monozygotic CADASIL twins with a novel NOTCH3 mutation*. Stroke.* 2009 Jun;40(6):2215-8.

III Low, W.C., **Junna, M.**, Börjesson-Hanson, A., Morris, C.M., Moss, T.H., Stevens, D.L,. St Clair, D., Mizuno, T., Zhang, W.W., Mykkänen, K., Wahlstrom, .J, Andersen, O., Kalimo, H., Viitanen, M., Kalaria, R.N. Hereditary multi-infarct dementia of the Swedish type is a novel disorder different from NOTCH3 causing CADASIL. *Brain.* (2007) Feb;130(Pt 2):357-67.

IV **Siitonen, M.,** Börjesson-Hanson, A., Pöyhönen, M., Pasanen, P., Bras, J., Kern, S., Kern, J., Andersen, O., Baumann, M., Singleton, A., Kalaria, R., Kalimo, H., Hardy, J., Myllykangas, L., Viitanen, M. and Guerreiro, R. Seeking the pathogenic mutation in a family with hereditary multi-infarct dementia of Swedish type. *Manuscript*.

1. INTRODUCTION

During the last few decades, the prevalence of cognitive impairment has increased together with life expectancy, and there are at least 93 000 patients suffering from moderate to severe cognitive impairment in Finland (Viramo & Sulkava 2015). In addition, approximately 100 000 people are estimated to be suffering from mild cognitive impairment (MCI) (Viramo & Sulkava 2015). Altogether, dementia affects around 7% of the general population older than 65 years and 30% of people older than 80, and its prevalence is expected to continue rising together with life expectancy (O'Brien et al. 2003).

Vascular dementia (VaD) is the second most common cause of dementia after Alzheimer's disease (AD) in elderly adults. VaD or vascular cognitive impairment (VCI) is a group of syndromes caused by insufficient supply of blood to the brain, typically caused by a series of minor strokes (Craggs et al. 2014). Hence, it has also been called multi-infarct dementia. In addition to multiple strokes, VaD can be caused by small vessel disease (SVD), strategic infarcts, hypoperfusion injury and intracranial haemorrhages as well as combined vascular pathogenetic mechanisms (Wiesmann, Kiliaan & Claassen 2013). The cognitive impairment in VaD differs from that in classical dementia as the symptoms usually occur stepwise due to recurrent infarctions and the executive functions are more severely impaired than memory (Wiesmann, Kiliaan & Claassen 2013, Craggs et al. 2014).

There are no commonly approved neuropathological diagnostic criteria for vascular dementia, and a diagnosis of VCI is hard to establish, since vascular lesions are frequently also seen in cognitively normal patients (Wiesmann, Kiliaan & Claassen 2013). It is also common that the vascular component is involved in mixed type dementia, in which the addition of a cerebrovascular disease to other defined neurodegenerative diseases lowers the threshold for the development of dementia (Craggs et al. 2014). Hence, primary VaD, in which the pathologic cause of cognitive decline is pure cerebrovascular disease, is quite uncommon.

The difficulty in diagnosing VCI clinically or by neuropathological examination highlights the importance of research in the field. Since most sporadic VaDs have a mixed pathogenic background, it is reasonable to use familial disease as a model when studying the molecular mechanisms and the pathogenesis of VCI. In this approach, the underlying genetic background is the same through the entire patient cohort, which is an enormous benefit when studying the phenotypic effect of different genetic factors, demographics and traditional vascular risk factors. The most profoundly studied example of familial disease causing VaD is CADASIL (**C**erebral **A**utosomal **D**ominant **A**rteriopathy with **S**ubcortical **I**nfarcts and **L**eukoencephalopathy). CADASIL is the most common form of VCI causing inherited SVD, and the causative mutations in the *NOTCH3* (Notch homolog 3, *Drosophila*) have been studied for almost twenty years (Joutel et al. 1996).

Despite the fact that vascular lesions are the second most common cause of dementia, the genetic risk factors for sporadic VCI are still largely unknown. Even the genetic background of the

dominantly inherited forms of VCI remains mostly unsolved. With new genome-wide methods, such as genome-wide association study (GWAS) and whole-genome sequencing (WGS), the genetic background of familial and even complex diseases can be revealed. After the gene defect has been identified, research efforts can be focused on the pathogenesis and possible treatment options. A profound knowledge of the pathogenic mutations behind the familial forms of VCI could help to obtain the correct diagnosis, which is important to patients and also for their relatives who face the risk of having the disease. Furthermore, improving our knowledge of VCI, the defective genes behind different forms of VCI and the risk factors affecting the disease are essential means for reducing the economic and societal costs of this disease group.

This thesis project aimed to uncover the genetic background of two forms of inherited SVDs causing VCI: CADASIL and Swedish hMID (hereditary multi-infarct dementia of Swedish type). Specifically, 1) the genetic modifiers of CADASIL were examined in a genetically homogeneous Finnish patient population, and 2) the gene defect of Swedish hMID was sought by taking advantage of the new genome-wide methodologies.

2. REVIEW OF THE LITERATURE

2.1. Vascular cognitive impairment

Vascular cognitive impairment (VCI) is a syndrome characterised by the presence of clinical stroke or vascular brain injury and cognitive impairment affecting more than one cognitive domain (Wiesmann, Kiliaan & Claassen 2013). VCI is a spectrum from mild to severe impairment and vascular dementia (VaD) can also be understood as the most severe form of VCI (Wiesmann, Kiliaan & Claassen 2013). In recent years, the prevalence of dementia has increased and the spectrum of pathogenic mechanisms that cause dementia has changed. In 1965, Fisher presented the lacunar hypothesis, suggesting that lacunes are due to a chronic vasculopathy related to systemic hypertension. Since then many large national and international studies have highlighted the importance of effective treatment of hypertension. Owing to these studies, today hypertension is typically treated early and effectively. Therefore, the burden of VCI caused by hypertensive disease has diminished. This, together with the effective treatment of embolism and new imaging methods in addition to the increased use of advanced magnetic resonance imaging (MRI), has increased the importance of small vessel diseases (SVD) behind the VCI. VCI can be divided into cortical and subcortical VCI according to the region that is most damaged in the brain, but other divisions have also been suggested (Wiesmann, Kiliaan & Claassen 2013). Cortical VaD is typically caused by multiple bilateral infarcts affecting the cerebral cortex, whereas subcortical VaD is usually associated with lacunar infarcts and white matter lesions (WML) in the subcortical area (DeKosky, Kaufer & Lopez 2004).

According to recent studies, subcortical ischemic VaD results from a SVD (Craggs et al. 2014). Although the occurrence of SVD increases as the population ages, there remains a relative lack of knowledge concerning the molecular mechanisms of the pathogenesis of SVD (Craggs et al. 2014). Hence, diagnostic and therapeutic strategies are restricted. The majority of the VCI cases are sporadic, but some inherited forms of VCI have been reported (Table 1). CADASIL (**C**erebral **A**utosomal **D**ominant **A**rteriopathy with **S**ubcortical **I**nfarcts and **L**eukoencephalopathy) is by far the most common form of inherited VCI, but even though the defective gene has been known for almost 20 years, the underlying pathogenesis is still debated. Nonetheless, the situation with CADASIL is far better than with most inherited VaDs. For example, hereditary multi-infarct dementia of Swedish type (Swedish hMID) was first described in 1977 by Sourander and Wålinder, but both the pathogenic gene mutation and the disease pathogenesis are still unknown.

Table 1 *Sporadic and hereditary vascular disorders leading to vascular cognitive impairment.*

Disorder	Full name	Inheritance pattern	Gene	Reference
Large vessel disease				
Multi-infarct dementia		sporadic		
Strategic infarct dementia		sporadic		
Small vessel disease				
Sporadic vascular dementia		sporadic		
Hereditary small vessel diseases				(MINI-symposium, Brain Pathol. 2014. 24:5)
CADASIL (OMIM #125310)	Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy	AD	NOTCH3	(Joutel et al. 1996)
CARASIL (OMIM #600142)	Cerebral Autosomal Resessive Arteriopathy with Subcortical Infarcts and Leukoencephalopathy	AR	HTRA1	(Hara et al. 2009)
Swedish hMID	Hereditary Multi-Infarct Dementia of Swedish type	AD		(Sourander, Wålinder 1977a)
PADMAL	Pontine Autosomal Dominant Mikroangiopathy and Leukoencephalopathy	AD		(Ding et al. 2010)
RVCL (OMIM #192315) *HERNS* *CRV* *HVR*	Retinal Vasculopathy and Cerebral Leukodystrophy Hereditary Endotheliopathy, Retinopathy and Nephropathy Cerebroretinal Vasculopathy Hereditary Vascular Retinopathy	AD	TREX1	(Ophoff et al. 2001, Kolar et al. 2014)
HAS	Hereditary Systemic Angiopathy			(Winkler et al. 2008)
COL4-related disorders (OMIM #192315)		AD	COL4A1, COL4A2	(Aguglia et al. 2004, Plaisier et al. 2007)
SVDB	Hereditary Small Vessel Disease of the Brain	AD		(Verreault et al. 2006)
HDLS (OMIM #221820)	Hereditary Diffuse Leukoencephalopathy with Spheroids	AD	CSF1R	(Rademakers et al. 2011)
CAA (OMIM #605714, 105150, 176500, 117300)	Cerebral amyloid angiopathies	AD	APP, CST3, ITM2B	(Revesz et al. 2009, Yamada 2015)

AD = autosomal dominant, AR = autosomal recessive

2.1.1. Differential diagnosis of vascular cognitive impairment

Most disorders causing VCI are slowly progressive adult onset diseases; therefore, the early diagnosis can be challenging. When pure subcortical VaD progresses in a slow manner, it can clinically resemble Alzheimer's disease (AD), but pathologically the characteristic β-amyloid and

neurofibrillary burden is lacking. Usually, the symptoms of VCI are easiest to detect when they happen soon after a stroke. In a Statement for Healthcare Professionals from the American Heart Association/American Stroke Association (Gorelick et al. 2011), the following three criteria were reported to suggest that mild cognitive impairment (MCI) or dementia is caused by vascular changes:

1. The diagnosis of MCI or dementia is confirmed by neurocognitive testing that provide thorough assessment of specific thinking skills (judgment, planning, problem-solving, reasoning and memory).

2. Imaging evidence of either a recent stroke or other changes in the brain vasculature. The severity and pattern of these changes is consistent with the types of impairment shown in neurocognitive testing.

3. In addition to vascular changes, there is no evidence of other factors contributing to cognitive decline.

However, the vascular changes often coexist with brain changes associated with other types of dementia, which makes the diagnostic criteria less clear-cut.

When similar symptoms and/or findings are also present in relatives, CADASIL should be considered since it is by far the most common form of inherited VaD. The first diagnostic criteria for CADASIL were suggested by Davous (1998). The proposed main criteria were: 1) early onset (<50 years), 2) at least two clinical symptoms (stroke-like episodes, migraine, mood changes, cognitive impairment), 3) lack of traditional vascular risk factors, 4) positive family history and 5) MRI showing leukoencephalopathy with subcortical infarcts in the basal ganglia and white matter (WM). As the phenotype of CADASIL is highly variable, even within the same family, the criteria above (Davous et al. 1998) have not gained wide acceptance. Hence, the clinical diagnosis is usually made based on a combination of otherwise unexplained cerebral ischemic events or cognitive impairment, brain MRI abnormalities and a family history of stroke or dementia (Rutten et al. 2014). Several other diseases, such as multiple sclerosis (Pandey & Abubacker 2006) or Binswanger's disease (van Bogaert 1955) can mimic CADASIL in symptoms and MRI findings, although the pattern of MRI changes in multiple sclerosis is different (Ayrignac et al. 2015) and Binswanger's disease (also known as subcortical-arteriosclerotic encephalopathy or subcortical arteriosclerosis) is not a familial disorder and is frequently associated with hypertension (Pantoni & Garcia 1995, Huisa & Rosenberg 2014). Hence, clinical diagnosis can only raise a suspicion of CADASIL, and the diagnosis needs to be confirmed with genetic testing or by a method that shows the pathological characteristics of the disease.

Rutten et al. (2014) have suggested recommendations for the clinical diagnosis and interpretation of *NOTCH3 (*Notch homolog 3*, Drosophila)* mutations in CADASIL. A genetic test presenting an archetypal CADASIL mutation (Joutel et al. 1997a) in the defective *NOTCH3* gene confirms the diagnosis. If the mutation has been previously found in a relative, the molecular genetic testing can be targeted to this particular mutation. Otherwise, the whole gene, or the exons 2-24 harbouring most of the mutations (Appendix 1), can be sequenced for CADASIL-

causing mutations (Tikka et al. 2009). The correct diagnostic interpretation of variants other than the stereotypical cysteine-altering missense mutations requires expertise in both the clinical features and distinguishing molecular aspects of CADASIL (Rutten et al. 2014). A recent study suggests that a single-particle in vitro aggregation assay might be a reliable tool to evaluate the clinical significance of the non-cysteine variants (Wollenweber et al. 2015). However, the study consisted of only one family, and it is still debated whether the CADASIL diagnosis in this family is conclusive and thus wether the method can be used as diagnostic tool (Rutten, van Duinen & Lesnik Oberstein 2015, Wollenweber, Haffner & Duering 2015).

Another approach to confirm the diagnosis of CADASIL is to analyse the skin biopsy of a clinically suspected CADASIL patient. Electron microscopy can be used to seek CADASIL-specific granular osmiophilic material (GOM) in the walls of small arteries (Ruchoux et al. 1995, Tikka et al. 2009). At present, GOM deposits have not been seen in any other disease, so they confirm the diagnosis with a very high probability. However, it should be recalled that even if GOM is not detected, CADASIL cannot be excluded, since there is the possibility that the sample simply does not have enough informative arteries to show the deposition of GOM. A third method for confirming the diagnosis is to use immunohistochemical (IHC) methods to show the accumulated NOTCH3 extracellular domain (N3ECD) in the walls of dermal arteries (Joutel et al. 2001), although non-specific staining is an inherent caveat of IHC (Lesnik Oberstein et al. 2003) and makes the definite diagnosis problematic in young patients with only small amounts of GOM (Tikka et al. 2009).

If the CADASIL diagnosis cannot be confirmed in a patient with an inherited disorder causing VCI, then other rarer disease entities (Table 1) should be considered. Some of these diseases can be ruled out due to clinical differences, but, for example, Swedish hMID can resemble CADASIL. The first report of Swedish hMID was already published in 1977 (Sourander & Wålinder 1977a & 1977b), and for 30 years it was thought to represent the first published CADASIL family.

2.2. CADASIL

According to the state of current knowledge, the first CADASIL family was first described in 1955 by van Bogaert (van Bogaert 1955, Davous 1998). At that time, the molecular background of CADASIL was still unknown, and CADASIL was not described as an independent disease entity. Hence, the family was reported to have a familial Binswanger's disease (van Bogaert 1955). In that same article, van Bogaert referred to a report of a similar disease described by Mutrux (1951), which suggests that even earlier reports of CADASIL families may be available in the literature. After van Bogaert's publication, several other families with similar disorders were published in Europe: chronic familial vascular encephalopathy (Stevens, Hewlett & Brownell 1977), autosomal dominant syndrome with stroke-like episodes and leukoencephalopathy (Tournier-Lasserve et al. 1991), familial subcortical dementia with arteriopathic leukoencephalopathy (Davous & Fallet-Bianco 1991), a familial disorder with subcortical ischemic strokes, dementia and leukoencephalopathy (Mas, Dilouya & de Recondo 1992) and, lastly, a slowly progressive familial dementia with recurrent strokes (Salvi et al. 1992). In Finland, the first CADASIL family was described in 1987, when Sonninen and Savontaus

reported a large family suffering from multi-infarct dementia (Sonninen & Savontaus 1987). In 1998, after genetic testing had become available, the multi-infarct dementia in that family was confirmed to be CADASIL (Kalimo et al. 1998).

Even though there had been many reports of families with inherited VaD, the detailed genetic background remained unknown until 1993, when the disease locus was mapped by linkage analysis to chromosome 19 (chr19q12) in a large French pedigree (Tournier-Lasserve et al. 1993). In that same paper, Tournier-Lasserve and others also introduced the name "cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy" and its acronym CADASIL for the disease. Three years later, the linkage region was refined to chr19p13.1 (Ducros et al. 1996), and soon after that the mutations in the *NOTCH3* gene were reported to cause the disease (Joutel et al. 1996).

In 1997, Joutel and others described the archetypical nature of the CADASIL-causing *NOTCH3* mutations, which enabled genetic testing and helped to define the clinical characteristics of CADASIL. Subsequently, many of the previously published families were studied in detail, one of them being the Swedish hMID family (Sourander & Wålinder 1977a).

2.2.1. Epidemiology

After 1996, when the gene defect of CADASIL was found, the number of reported CADASIL patients rapidly increased, and many of the previously published families with VCI were confirmed as CADASIL cases. At present, CADASIL is the most common familial VaD, and patients have been reported in all ethnic groups (Tikka et al. 2014). However, detailed studies about the prevalence of CADASIL are few. In 2005, the prevalence of patients with a confirmed diagnosis of CADASIL in West Scotland was reported to be 1.98/100 000 and the prevalence of predicted mutation carriers was 4.14/100 000 (Razvi et al. 2005). In Northeast England, the minimum prevalence of confirmed CADASIL cases in 2012 was 1.32/100 000 (Narayan et al. 2012). The prevalence of CADASIL in Finland is 4/100 000 (Mykkänen et al. 2004), which is similar to other heavily-studied populations (Markus et al. 2002, Razvi et al. 2005). Due to a founder effect, most Finnish patients carry the same dominant mutation, p.Arg133Cys (c.397C>T), which has been dated to the late 1600s or early 1700s (Mykkänen et al. 2004). On the island of Gran Canaria, the prevalence of CADASIL is reported to be 14/100 000 with the dominant mutation p.Arg207Cys (c.619C>T) (Tikka et al. 2014). This exceptionally high prevalence could be due to genetic isolation or ancestral phenomena such as a founder effect. On the other hand, in Canada the prevalence of CADASIL is <1/100 000 in most provinces and the same is true for the United States (Tikka et al. 2014).

In Western countries, stroke is one of the most common causes of death, but strokes at young age (<50 years of age) are fairly rare (annual occurrence 10.8/100 000) (Putaala et al. 2009). In 2003, Dong and co-workers analysed the number of CADASIL patients among 218 patients with lacunar infarcts and white matter hyperintensities (WMH). The reported frequencies were 11% for patients under 50 years and 2% for patients under 65 years of age (Dong et al. 2003). In a Korean study of 151 patients with acute ischemic stroke, 4% were diagnosed as having CADASIL (Choi et al. 2013).

2.2.2. Clinical charcteristics

As the full name of the disease states, CADASIL (Online Mendelian Inheritance of Man, OMIM #125310) is an autosomally dominantly inherited disease affecting the cerebral vasculature, thus leading to subcortical infarcts and leukoencephalopathy. The clinical course of the disease is characterised by four main features: 1) migrainous headache with aura, 2) recurrent cerebral ischemic attacks (transient or stroke), 3) psychiatric symptoms and 4) cognitive symptoms that progress most commonly in a stepwise manner into subcortical VaD. Usually, the first symptoms occur at an early age, but the age at first ever stroke varies greatly from approximately 25 to 70 years (Figure 1).

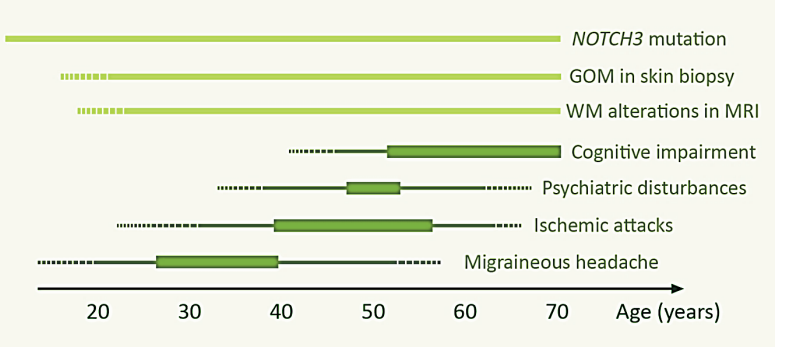

Figure 1 *The manifestation of symptoms and findings throughout the disease course in CADASIL. GOM: granular osmiophilic material; WM: white matter; MRI: magnetic resonance imaging. Figure from Mykkänen (2009), modified from a figure by Chabriat and Bousser (2007). Figure reproduced with the permission of copyright holder.*

2.2.2.1. Typical clinical findings

Migrainous headache is one of the major symptoms of CADASIL, and it occurs in up to 64% of patients. In 22–87% of cases it is associated with aura (Chabriat et al. 1995a, Davous 1998, Markus et al. 2002, Vahedi et al. 2004). Usually migrainous headache is the first symptom of the disease (Figure 1). Since migraine is also frequent as an independent disease, the age of onset of the disease is often reported on the basis of the first ischemic attack rather than migraine. The average age of onset of migraine is in the late twenties, but the attacks may begin even before the age of ten years (range 6–54 years of age) (Dichgans et al. 1998, Vahedi et al. 2004, Granild-Jensen et al. 2009). In most cases (71%), the aura is typical, which means that the symptoms are mainly visual, sensory, aphatic or motor features. In some cases the migrainous headache is associated with atypical aura (basilar or hemiplegic), or it may be prolonged or unusually severe (Dichgans et al. 1998, Vahedi et al. 2004, Granild-Jensen et al. 2009). The frequency of the migrainous attacks varies among patients, and the frequency may change as the disease progresses. It is common for patients to report that the frequency of migrainous headaches has increased before the first ischemic attack and decreased after the first stroke (Dichgans et al. 1998). In female patients, the first migraine attack with aura is often provoked by late pregnancy or puerperium (Roine et al. 2005).

The most common and characteristic manifestation of CADASIL is ischemic strokes, often preceded by transient ischemic attacks (TIA). CADASIL affects mainly small vessels and major

strokes caused by larger infarcts are rare. In the large clinical studies, up to 83% of symptomatic patients had experienced TIAs or strokes (Chabriat et al. 1995a, Dichgans et al. 1998, Peters et al. 2004a), and in almost 50% of the cases an ischemic event was the first manifestation of CADASIL (Dichgans et al. 1998, Desmond et al. 1999). Frequently, the first ischemic events are TIAs, which may be challenging to differentiate from severe migrainous aura. The age at first-ever stroke shows remarkable variability. The youngest confirmed CADASIL patient was only 11 years of age when he had his first-ever stroke (Granild-Jensen et al. 2009), whereas some patients have their first-ever stroke after their 70th birthday (Watanabe et al. 2012). Many of the late-onset cases may remain undiagnosed if no familial history of CADASIL is available, since stroke is not uncommon in the elderly population. The mean ages at first-ever stroke are around 40–50 years (Dichgans et al. 1998, Opherk et al. 2004, Chabriat et al. 2009, Adib-Samii et al. 2010). Most frequently, the strokes are subcortical and present as lacunar syndromes, such as pure motor or sensory symptoms, dysarthria, clumsy hand syndrome, expressive dysphasia, visual field defects or ataxic hemiparesis (Kalimo et al. 2008, Tikka et al. 2014). As the disease progresses and the strokes recur, different physical disturbances such as gait disturbances accumulate. This leads to a stepwise progression towards severe disability and by the age 60 over 50% of the patients are unable to walk and need help in daily life (Dichgans et al. 1998).

As the disease progresses, impaired cerebral blood flow (CBF) and repeated ischemic attacks damage cerebral tissue to an increasing extent, thus causing progressive cognitive symptoms. The cognitive impairment predominantly affects frontal lobe functions, and usually the cognitive symptoms begin with deterioration of executive functions. Working memory, executive and organising functions are often impaired, and the patients have problems in concentrating and slowness in general mental and psychomotor skills (Amberla et al. 2004). The changes in working memory and executive function can be observed in the very early phase of the disease, even before any ischemic attacks have occurred (Taillia et al. 1998, Amberla et al. 2004). In 2004, Amberla and others reported that prestroke and poststroke patients show significant differences in tests for working memory, executive functions and mental speed, and a similar profile was also published by Peters and co-workers (2005). In most cases, the progress of cognitive deterioration is slow and corresponds to the occurrence of TIA and stroke. In 5–15% of the CADASIL cases, VaD seems to develop without any clinical strokes, but MRI normally reveals silent infarcts contributing to the WM changes (Dichgans et al. 1998, Mellies et al. 1998, Kalimo et al. 2008, Chabriat et al. 2009). Episodic memory stays relatively well preserved until later stages of the disease (Chabriat et al. 1995b, Dichgans et al. 1998, Peters et al. 2005, Chabriat et al. 2009). The cognitive decline begins to manifest itself between 40–70 years of age, and by the age of 65 approximately 65% of the patients have developed a serious subcortical VaD (Dichgans et al. 1998).

Approximately 30% of CADASIL patients have psychiatric symptoms, most commonly mood changes (Chabriat et al. 1995b, Dichgans et al. 1998, Valenti et al. 2008). The most prevalent mood disturbance is depression (Desmond et al. 1999, Amberla et al. 2004, Peters et al. 2005, Singhal, Rich & Markus 2005), which is only to be expected in patients who have seen the disease progression in their families and know what to expect from their own disease. In a French CADASIL cohort, apathy was reported in up to 41% of patients and it seemed to associate with

age, severity of cognitive impairment, other neuropsychiatric symptoms and subcortical tissue damage (Reyes et al. 2009). Furthermore, anxiety disorder, schizophrenia, psychotic episodes, paranoia, agitation, aggression and dysthymia have also been reported, but their prevalence is low and their connection to CADASIL is not definitely verified (Verin et al. 1995, Lågas & Juvonen 2001, Chabriat & Bousser 2007, Valenti et al. 2008, Ho & Mondry 2015).

The results of general laboratory tests such as general blood status and cerebrospinal fluid are usually within the normal limits, and the frequencies of traditional vascular risk factors are similar to those reported in the general population (Kalimo et al. 2008). Epileptic seizures are present in about 10% of patients at the later stage of the disease (Chabriat et al. 1995b, Dichgans et al. 1998, Valko et al. 2009, Velizarova et al. 2011, Haddad et al. 2015). Abnormalities in the retinal vessels of CADASIL patients are reported in multiple studies, but they seem to cause only minor functional deficits (Robinson et al. 2001, Cumurciuc et al. 2004, Harju et al. 2004, Roine et al. 2006). Some cases of "CADASIL coma" (reversible acute encephalopathy) have also been reported (Feuerhake et al. 2002, Le Ber et al. 2002, Schon et al. 2003, Ragno et al. 2013).

Many studies have reported mitochondrial mutations in CADASIL patients, and it has been shown that the amount of mutations in mitochondrial DNA is higher in CADASIL patients than in control group (Annunen-Rasila et al. 2006). Although the actual mechanism is unknown, it has been hypothesised that mutations in *NOTCH3* may lead to decreased mitochondrial oxidative phosphorylation and to an increased amount of reactive oxygen species and hence cause secondary mutations in mtDNA (Annunen-Rasila et al. 2006). It has also been suggested that that alteration of proliferation and mitochondrial function in vascular smooth muscle cells (VSMC) might influence cellular functions essential for CADASIL pathology (Viitanen et al. 2013).

In the first phases of the disease CADASIL is a slowly progressive disease and usually the progression happens in a stepwise manner after the recurrent strokes. The mean age at death is reported to be around 65 years of age (Dichgans et al. 1998, Opherk et al. 2004, Chabriat et al. 1995b) and in older studies the disease has been said to lead to death in a mean of 23 years after the manifestation of first symptoms (Dichgans et al. 1998, Opherk et al. 2004). It is likely that the lifespan after the diagnosis has prolonged in recent years, since better awareness of CADASIL has made the diagnosis possible in at an earlier phase of the disease. In Finland, the oldest known CADASIL patient was a 96-year-old female patient who died 28 years after diagnosis (Hannu Kalimo and Matti Viitanen, personal communication, May 2015)

2.2.2.2. Imaging findings

Typically, the first and also most prominent imaging findings in CADASIL are hyperintensities caused by leukoencephalopathy in the brain. These signal abnormalities can be detected in T2-weighted MRI (Figure 2) (Chabriat et al. 1998) in both symptomatic and asymptomatic patients (Coulthard et al. 2000). Hyperintensities are usually found in the subcortical WM regions, whereas lesions in cortical areas and the cerebellum are rare. In the first stages of the disease, the alterations are usually located in temporopolar and periventricular WM and in the capsula externa (Figure 2A); as the disease progresses, the MRI signals become more diffuse (Figure 2B) and symmetrical in the external capsule and anterior temporal lobes (O'Sullivan et al. 2001,

Markus et al. 2002). The degree of the microstructural alterations correlates with the severity of the clinical phenotype, and the lacunar infarct lesion burden is the most significant MRI parameter associated with cognitive impairment in CADASIL (Liem et al. 2007).

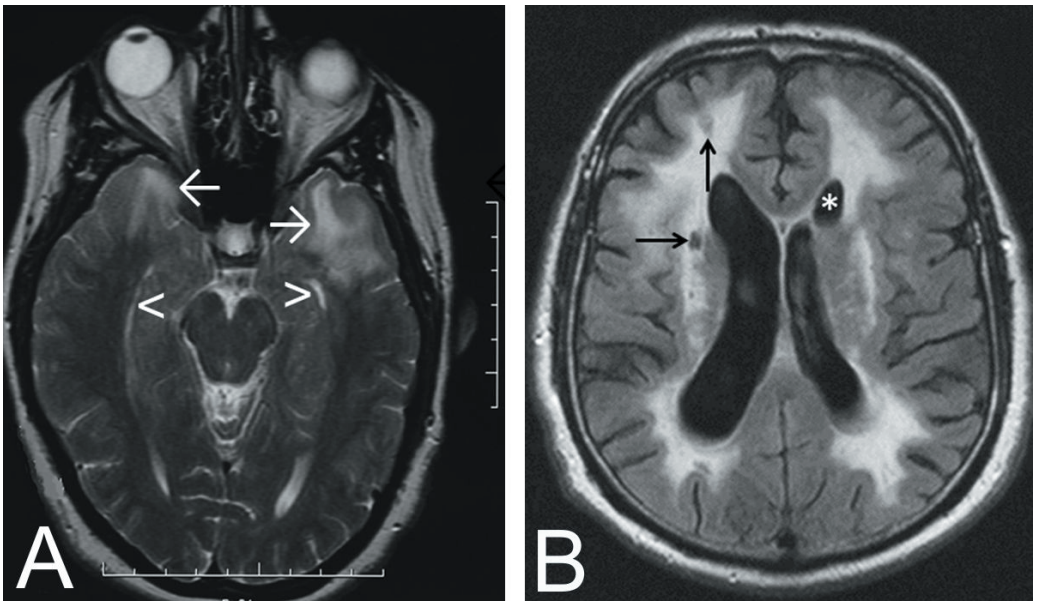

Figure 2 *Typical findings in T2-weighted magnetic resonance imaging (MRI) in CADASIL patients. A) Characteristic MRI in the early stages of the disease (29-year-old female) shows hyperintensities in anterior temporal lobes (arrows) and in capsula externa (arrowheads). B) In the advanced stage of the disease, lacunar infarcts are visible both in the white matter and in basal ganglia (arrows) in fluid-attenuated inversion recovery MRI (57-year-old female). * = frontal horn of the lateral ventricle. Figures from the article by Kalimo et al. (2008), reproduced with the permission of the copyright holder.*

Microbleeds are present in 31–69% of CADASIL patients (Stojanov et al. 2015). These normally occur outside the ischaemic lesions and in most cases have no clinical manifestation (Lesnik Oberstein et al. 2001, Dichgans et al. 2002, Maclean et al. 2005, Choi et al. 2006, Rinnoci et al. 2013, Lee et al. 2015). In China, the occurrence of primary intracerebral haemorrhage reaches 25% (Tan et al. 2014b). It has been suggested that the distribution and burden of cerebral microbleeds could be a clinically useful marker for the risk of symptomatic stroke (Lee et al. 2015). Microbleeds are mainly located within the cortical or deep grey matter (GM), and the amount seems to increase with age as well as with vascular risk factors and anticoagulant treatment (Lesnik Oberstein et al. 2001, Viswanathan et al. 2006, van den Boom et al. 2006, Rinnoci et al. 2013).

The impaired CBF in CADASIL can be measured with functional imaging methods, such as positron emission tomography (PET). Chabriat and others (1995) have reported a 40% decrease in CBF in an asymptomatic patient and a 50% decrease in CBF and oxygen consumption rate in the later stage of the disease. In another PET study, the mean CBF has been shown to be reduced already in early adulthood, and the CBF was shown to decrease significantly with increased severity of the disease (Tuominen et al. 2004). Therefore, it has been suggested that impaired CBF is not secondary to neuronal damage but arises from the arterial dysfunction (Kalimo et al. 2008).

2.2.2.3. Variation in the CADASIL phenotype

There is a wide variability in the age of onset and progression of the disease not only between but also within families (Dichgans et al. 1998). In a study consisting of 18 families with more than one affected patient, Dichgans and others (1998) found considerable intra- and interfamilial variation in the disease phenotype. The mean age at onset of the disease varied from 37–59 years of age between families and a similar variation was also reported within families. The extent of disability was somewhat age-related, but there was clear evidence of intrafamilial variation in the degree of disability (Dichgans et al. 1998). In addition, the disease progression has been shown to vary considerably between patients (Peters et al. 2004a).

Even though this clinical variability has been acknowledged for years, the reasons behind it remain unknown. Some *NOTCH3* mutations have been correlated to certain phenotypes, but many studies have failed to show any association between a given mutation and a phenotype (Dichgans, Herzog & Gasser 2001, Singhal et al. 2004). In 2000, Joutel and others reported a p.Gly114_Pro120del (c.341-2A>G) splice site mutation and suggested that it may contribute to the phenotype with few ischemic strokes, high frequency of migraines and episodes of confusion or CADASIL coma. The p.Arg153Cys (c.457C>T) mutation has been shown to correlate with cerebral microbleeds (Lesnik Oberstein et al. 2001), and the p.Cys455Arg (c.1363T>C) mutation has been described with an early occurrence of strokes (mean age of onset 31 years) (Arboleda-Velasquez et al. 2002). In two German families, a certain mutation was associated with shorter survival: the p.Cys174Tyr (c.521G>A) mutation was associated with a significantly lower median age at first-ever stroke, immobilisation and death, and the p.Cys117Phe (c.350G>T) mutation was associated with a significantly lower median age at death (Opherk et al. 2004).

Not only different *NOTCH3* mutations, but also *NOTCH3* polymorphisms may affect the disease phenotype. A common p.Arg1560Pro (rs78501403 (reference SNP accession number in dpSNP (Sherry et al. 2001))) *NOTCH3* variant has been reported to be protective against ischemic stroke (Ross et al. 2013), and the neutral p.Ala202Ala (rs1043994) polymorphism has been associated with migraine in the general population (Schwaag et al. 2006). In other studies, no association between any one *NOTCH3* mutation and cerebrovascular disease (Ito et al. 2002, Dong et al. 2003) or migraine (Borroni et al. 2006) could be detected.

In addition to *NOTCH3* variants, other genetic factors may also contribute to the variability of the disease. Opherk and co-workers (2006) studied the variation and heritability of the number of ischemic brain lesions and concluded that heritability estimates in CADASIL suggest a strong modifying influence of genetic factors distinct from the causative *NOTCH3* mutation. In a more recent study, they showed that the polygenic risk score is associated with WMH volume, suggesting that multiple common variants with small effect sizes influence the WMH burden in CADASIL (Opherk et al. 2014).

As the main pathogenic feature of CADASIL is ischemic lesions, the genes affecting arterial or brain functions may also be involved in the variable outcome of the clinical course of CADASIL. Apolipoprotein E (ApoE) functions mainly in lipid transportation, but it has also been shown to have an effect in many neurological processes. *APOE* (chromosome 19q13.2) has three different alleles coding for isoforms ε2, ε3, ε4, and the influence may vary according to different protein

isoforms (Shi, Han & Kuniyoshi 2014, Lopez, Krastins & Ning 2014). *APOE* ε4 carriers are shown to be at increased risk of atherosclerosis and stroke (Bangen et al. 2013, Lopez, Krastins & Ning 2014). ApoE ε4 is also a well-known cardiovascular risk factor and associates with Alzheimer's disease and neurodegeneration (Shi, Han & Kuniyoshi 2014, Lopez, Krastins & Ning 2014). Another potential candidate gene is angiotensinogen (*AGT*). *AGT* is a component of the renin-angiotensin system (RAS) that regulates blood pressure, vasoconstriction and vascular muscle tone. *AGT* is located at chromosome 1 (q42-q43) and its p.Met268Thr polymorphism (rs699, previously published as p.Met235Thr) significantly contributes to stroke risk, at least in East Asian populations (Wang et al. 2012, Liang et al. 2013).

In addition to genetic factors, environmental and lifestyle factors can also alter the phenotype, and at least arterial hypertension, hypercholesterolemia, smoking and diabetes mellitus are known to modify the disease phenotype (Dichgans et al. 1998, Kalimo et al. 2002, Singhal et al. 2004, Kalimo et al. 2008, Chabriat et al. 2009, Adib-Samii et al. 2010).

2.2.3. Vascular pathology

In the pathological examination of the brain, lacunar infarcts are common in the cerebral WM, deep GM and brain stem (Figure 3). During the disease course, white matter lesions become widespread, whereas cortical GM is usually relatively well preserved (Figure 3). Nevertheless, cortical infarcts (Paquet et al. 2010, Jouvent et al. 2011) and stenosis of large cerebral arteries (Choi, Choi & Kim 2005) have also been reported.

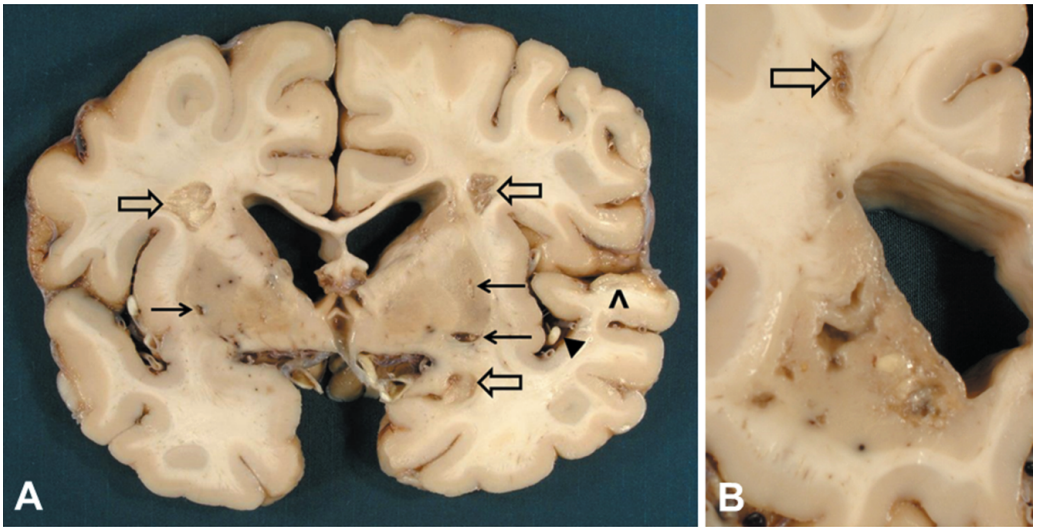

Figure 3 *Brain of a 65-year-old female CADASIL patient with the p.Arg133Cys mutation. A) Open arrows indicate old infarcts in cerebral white matter and narrow arrows point to smaller lacunar infarcts in putamen bilaterally. Cortex is spared except for the ischemic lesion in temporal cortex (arrowhead) due to atherosclerotic occlusion of a middle cerebral artery branch (black triangle); B) The lesions in caudate nucleus are more severe than in the putamen. Open arrow shows small cystic infarct in the white matter of the centrum semiovale. Lateral ventricles in both (A) and (B) are widened because of ischemic tissue loss. Figures from Tikka et al. (2014), reproduced with the permission of the copyright holder.*

It has been said that CADASIL is the most aggressive hereditary SVD in terms of overall vascular pathology and severe arteriolosclerosis affecting mainly frontal WM and basal ganglia regions (Craggs et al. 2013). Even though the symptoms are mainly neurological, CADASIL is a systemic disease and the vascular lesions can be detected in almost all small- and medium-sized arteries (Ruchoux et al. 1995). In the central nervous system, the vascular pathology is characterised by electron-dense deposits of GOM, which has not been reported in any other disease. GOM, thickening of the arterial walls and degeneration of the VSMCs can be detected via electron microscopy (EM) (Figure 4) (Baudrimont et al. 1993, Ruchoux et al. 1994, Tikka et al. 2009). GOM is located in close vicinity to VSMCs (Figure 4). GOM, together with consequent degeneration of the VSMCs and marked fibrosis and stenosis, is most prominent in the cerebral WM arterioles and leads to extensive WMH and multiple lacunar infarcts (Kalimo et al. 2008, Tikka et al. 2014). The detailed structure of GOM is still unknown, but immunohistochemical analyses have shown that N3ECD accumulates on the cytoplasmic membrane of VSMCs (Joutel et al. 2000a), and immunoEM studies suggest that N3ECD is one of the main components of GOM (Ishiko et al. 2006, Yamamoto et al. 2013).

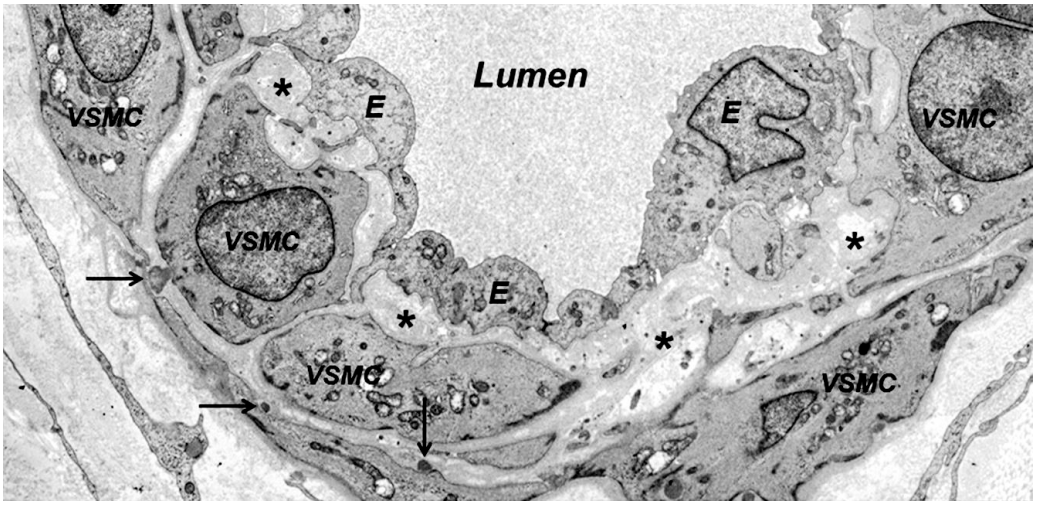

Figure 4 *An electron micrograph of a dermal artery in a 28-year-old CADASIL patient showing the irregular vascular smooth muscle cells (VSMC). Asterisks present the widened endothelial spaces and arrows point to granular osmiophilic material (GOM) deposits in the close vicinity of VSMCs. E= endothelial cells. Figure from Tikka et al. (2014), reproduced with the permission of the copyright holder.*

2.2.4. The genetics of CADASIL

The defective gene in CADASIL was discovered in 1996 when Joutel and co-workers reported that mutations in *NOTCH3* cause CADASIL. This large gene is located at chromosome 19p13.1-13.2, consists of 33 exons and codes for the 2321-amino-acid-long single-pass transmembrane receptor protein NOTCH3 (Figure 5).

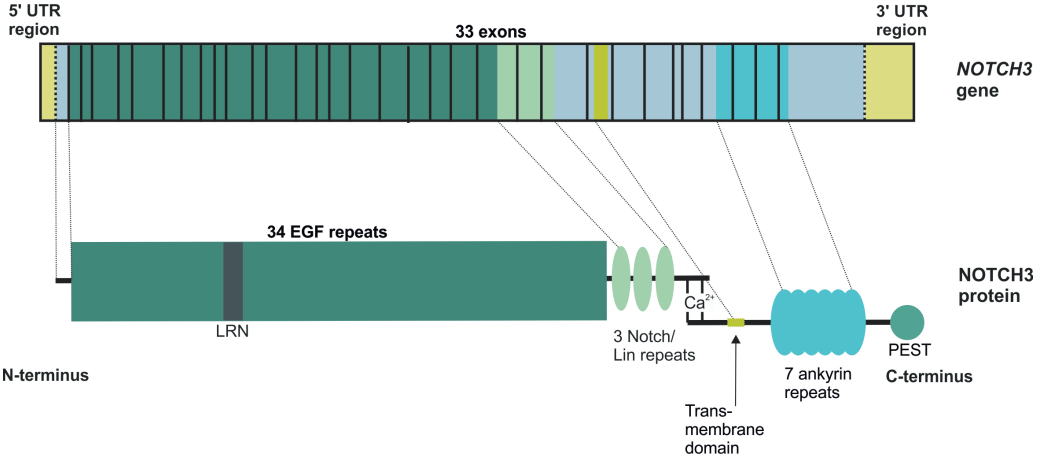

Figure 5 *The schematic presentation of the Notch homolog 3, Drosophila gene (NOTCH3) and the domains of the NOTCH3 receptor protein. LRN: ligand recognition site on the 11th and 12th EGF repeats, EGF: epidermal growth factor -like, PEST: transactivation domain. Modified from Mykkänen (2009).*

2.2.4.1. The NOTCH3 *protein*

The schematic structure of the NOTCH3 receptor protein is illustrated in Figure 5. NOTCH3 is synthesised as a full length protein which is then proteolytically cleaved (S1 cleavage) into two parts by furin (Figure 6). The resulting products are bound together with calcium ions, and this heterodimeric NOTCH3 is then transported to the cell membrane (Blaumueller et al. 1997). The large N-terminal extracellular domain, N3ECD, (210 kDa) is composed of 34 tandemly repeated epidermal growth factor-like domains (EGF) (Wharton et al. 1985). All these EGF-like repeats contain six highly conserved cysteines within a certain distance of each other. These cysteines form disulphide bridges, creating the spatial shape of each EGF domain (Artavanis-Tsakonas, Matsuno &

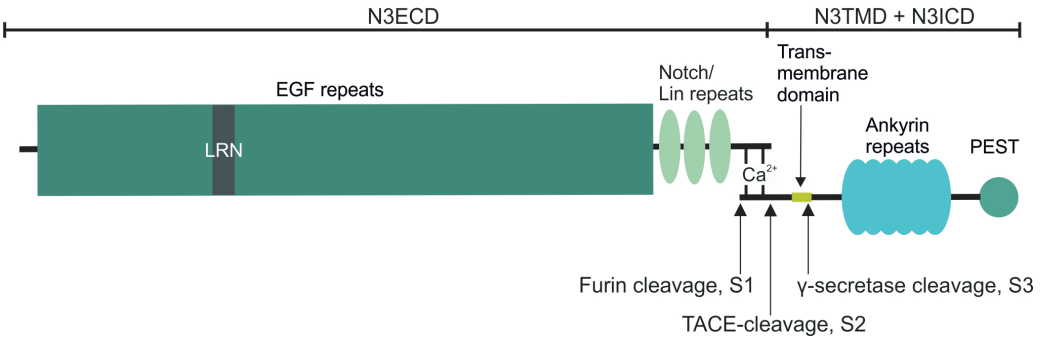

Figure 6 *The cleavage sites of NOTCH3 protein. NOTCH3 is syntethised as a full length protein which is then cleaved into two parts by furin (S1 cleavage). These two parts are bound together with calcium ions (Ca^{2+}), and the resulting heterodimeric NOTCH3 is then transported to the cell membrane. Ligand binding reveals next cleavage site and tumour necrosis factor alpha converting enzyme (TACE) cuts NOTCH3 just outside the plasma membrane. Finally, gamma(γ)-secretase releases NOTCH3 intracellular domain (N3ICD) from NOTCH3 transmembrane domain (N3TMD). N3ECD: NOTCH3 extracellular domain, LRN: ligand recognition site on the 11th and 12th EGF repeats, EGF: epidermal growth factor -like, PEST: transactivation domain.*

Fortini 1995). Hence, the correct number of cysteine residues is important for the correct folding and function of the receptor protein. In man, ligands of NOTCH3 bind to the 11th and 12th EGF-repeats (Joutel et al. 2004), although it has been suggested that other EGF domains may also influence the binding (Lawrence et al. 2000, Xu, Lei & Irvine 2005). The EGF-repeats are followed by three Notch/Lin12-repeats. The smaller (97 kDa) C-terminal part contains a transmembrane domain (N3TMD) and an intracellular domain (N3ICD), which encompasses seven ankyrin repeats and transactivation domain (PEST) (Figure 5). The intracellular part is responsible for transporting the signal from the signal-sending cell to the nucleus of the signal-receiving cell.

2.2.4.2. NOTCH3 *signalling*

The NOTCH3 protein belongs to an evolutionarily conserved Notch receptor family whose members are involved in cell-cell interactions, and the Notch signalling pathways are crucial during the development of most organs. During organogenesis, NOTCH proteins are involved in stem cell renewal, cell proliferation, determination of cell fate and differentiation and apoptosis (Tikka et al. 2014). In adult humans, NOTCH3 is expressed mainly in the VSMCs, but the exact function is still debated (Joutel et al. 2000a, Prakash et al. 2002).

The ligands for NOTCH3 receptors belong to the Delta-like family (DLL1, 3 and 4) and to the Serrate family Jagged (Jag1 and 2). Like the NOTCH3 receptor, they are transmembrane proteins. Both the ligands and NOTCH3 receptors are present on the membrane of VSMCs, but only ligands from the neighbouring cells can activate the signalling pathway (Fleming et al. 2013). Ligand binding causes N3ECD to dissociate from the heterodimeric NOTCH3, and the bound ligand and N3ECD are endocytosed together into the ligand-expressing cell (Figure 7). The endocytosed non-mutated N3ECD is then degraded and the ligand is returned to the cell membrane (Nichols, Miyamoto & Weinmaster 2007). The endocytosis of N3ECD leaves the membrane-bound C-terminal part of NOTCH3 (N3TMD and N3ICD) exposed for cleavage by ADAM metallopeptidase domain 10 (ADAM10) or by ADAM17 (also known as tumour necrosis factor alpha converting enzyme; TACE) (Brou et al. 2000). This S2 cleavage happens just outside the plasma membrane. Finally, gamma(γ)-secretase releases N3ICD from N3TMD (S3 cleavage), and N3ICD enters the nucleus of the signal-receiving cell (Figure 7) (Bozkulak & Weinmaster 2009, Nichols et al. 2007, De Strooper et al. 1999). In the cell nucleus, N3ICD interacts with CBF1/RBP-Jκ and co-activators to activate transcription of its target genes (Fouillade et al. 2008, Fouillade et al. 2012).

2.2.4.3. NOTCH3 mutations in CADASIL

The majority of the pathogenic mutations in CADASIL are located in exons 2-24 of the *NOTCH3* (Appendix 1). Stereotypically, they cause an uneven number of cysteine residues in one of the extracellular EGF-like repeats, thus affecting the disulphur bridges that normally form between the cysteine residues in the protein (Joutel et al. 1997b, Rutten et al. 2014). This stereotypical nature of CADASIL mutations was first described in 1997 when Joutel and others reported 25 different mutations that all altered the number of cysteines in the protein. To date, over 250 different CADASIL mutations have been found. The majority of them are missense point mutations causing a replacement of one cysteine with another amino acid or vice versa

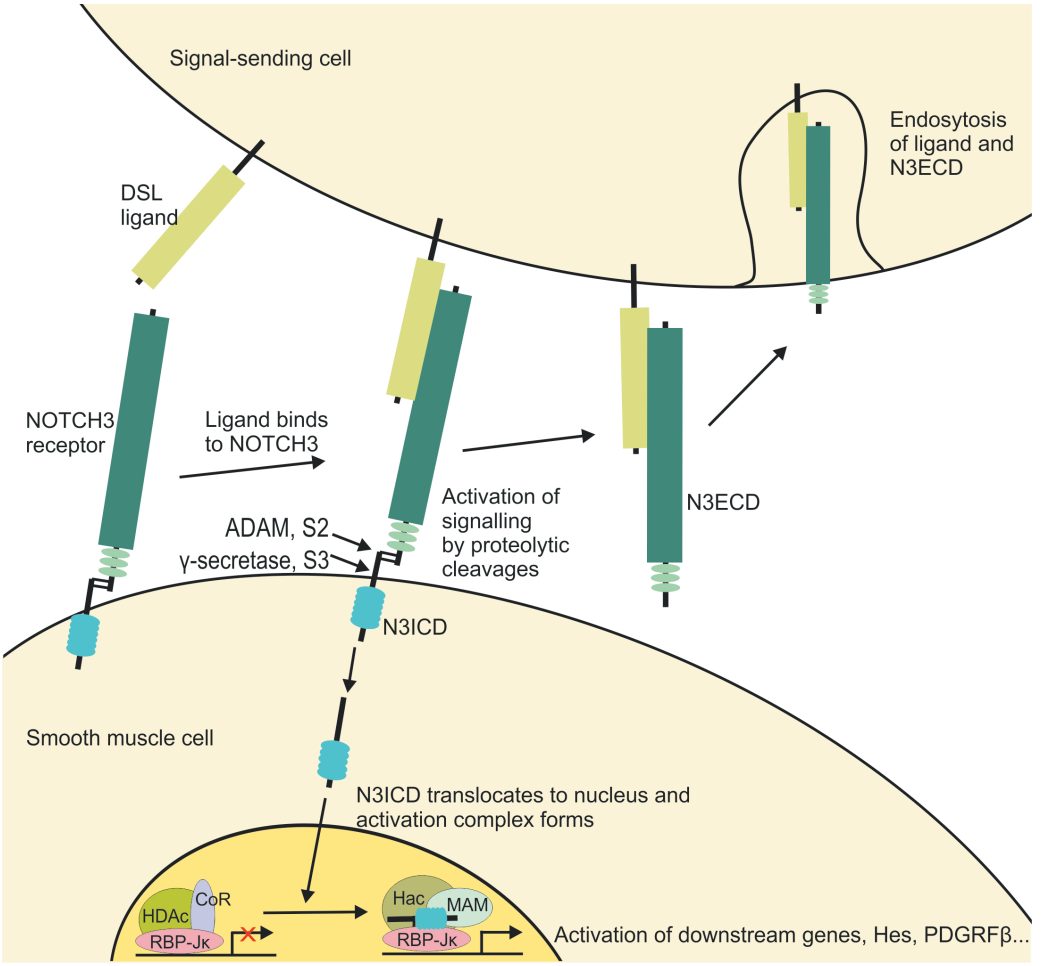

Figure 7 *An overview of NOTCH3 (Notch homolog 3, Drosophila) signalling. Signalling is activated when a Delta/Jagged (DSL) ligand interacts with the extracellular domain of a NOTCH3 receptor on a neighbouring cell. Ligand binding to a receptor causes a structural change in the receptor, exposing S2 and S3 cleavage sites. The third cleavage (S3) by gamma(γ)-secretase releases the intracellular domain (N3ICD), which is then translocated to the nucleus. The extracellular domain (N3ECD) is endocytosed by the ligand-bearing cell. In the nucleus, the N3ICD forms a complex with RBP-Jκ, leading to the transcriptional activation of target genes by displacement of corepressors (CoR) and histone deacetylases (HDAc) and recruitment of Mastermind (MAM) and coactivators such as histone acetylases (Hac).*

(Appendix 1). In addition to these stereotyped mutations, deletions, combined deletion and insertion, duplications and splice site mutations altering the number of cysteine residues have been described (Rutten et al. 2014) (Appendix 1). In addition to cysteine altering mutations, approximately 20 non-cysteine mutations have been reported (Appendix 1). In most reported non-cysteine cases, not all of the exons of *NOTCH3* were sequenced, nor was the diagnosis confirmed with detection of GOM from a skin biopsy (Rutten et al. 2014). However, in six non-cysteine cases CADASIL-specific GOM has been reported to be present in the close vicinity of VSMCs (Mazzei et al. 2004, Kim et al. 2006, Mizuno et al. 2008, Brass et al. 2009, Abramycheva et al. 2015, Wollenweber et al. 2015). At present, GOM has not been reported in any other disease,

which suggests that at least these GOM-causing non-cysteine variants are truly pathogenic. However, in one of these studies GOM was detected in only one patient, although the same p.Asp80Gly variant in *NOTCH3* was also found in three other family members (Wollenweber et al. 2015). In experienced hands, EM has been reported to be highly reliable method for detecting GOM, but it requires technically adequate biopsies and distinction of true GOM from fallacious deposits (Tikka et al. 2009). Hence, the pathogenicity of non-cysteine variants is still debated (Rutten et al. 2014, Wollenweber et al. 2015).

Five patients with homozygous mutations affecting the number of cysteines (p.Arg133Cys, p.Arg578Cys, pGly528Cys, p.Arg544Cys and p.Cys183Ser) have been reported in the literature (Tuominen et al. 2001, Liem et al. 2008, Ragno et al. 2013, Soong et al. 2013, Vinciguerra et al. 2014). CADASIL is a dominantly inherited disease and *de novo* cases are quite rare, but in three cases CADASIL has been confirmed as resulting from a *de novo* mutation (Joutel et al. 2000b, Coto et al. 2006, Stojanov et al. 2014).

2.2.5. Molecular mechanisms of CADASIL pathogenesis

The exact molecular pathogenic mechanisms and events during the pathogenesis of CADASIL are still unknown. The only feature common to all CADASIL mutations is the aggregation of N3ECD on the surface of VSMCs. According to one hypothesis, CADASIL may be caused by abnormal processing and trafficking of mutated NOTCH3. The archetypical CADASIL mutations cause an uneven number of cysteine residues in one of the EGF-like repeats; thus, the normal formation of sulphur bridges between paired cysteine residues is prevented, which is likely to cause misfolding. The change in the 3D-structure of NOTCH3 could lead to impaired S1 cleavage or aberrant dimerisation of NOTCH3 (Arboleda-Velasquez et al. 2005), and hence cause aggregation of N3ECD on the cell surface. Normally, misfolded proteins are directed to proteasomal degradation, and Ihalainen and others (2007) have shown that CADASIL cells suffer from oxidative stress and that their proteasomal degradation is upregulated. However, some of mutated NOTCH3 seem to escape this pathway since it has been shown that at least part of the mutated NOTCH3 molecules are proteolytically cleaved (S1) and transported to the cell membrane in the usual manner (Joutel et al. 2004, Peters et al. 2004b, Low et al. 2006). However, Peters and others (2004b) have shown that cells transfected with mutated human *NOTCH3* display reduced S1 (furin) cleavage. In genetically genuine cultured human CADASIL VSMCs, the S1 cleavage seemed to be normal, and intracellular aggregates were no more common than in control VSMCs (Tikka et al. 2012). Thus, aggregation of N3ECD seems to occur only after the receptor has reached the cell surface; therefore, the accumulation of N3ECD on VSMCs is most likely due to its local aggregation and/or its impaired clearance. To conclude, defective proteolytic processing does not appear to be the key problem in CADASIL.

The effect of a certain mutation varies according to its location in the *NOTCH3* gene. Mutations in the ligand binding site of the NOTCH3 receptor cause a significant decrease of transcriptional activity via the RBP/Jk pathway, while all other mutations tested were shown to activate the signalling cascade normally (Joutel et al. 2000a). Nevertheless, the clinical picture of the disease is similar to those with a mutation not affecting ligand binding (Monet-Lepretre

et al. 2009). Further, several other studies oppose the loss of function of *NOTCH3*: The *Notch3* knock-out mice show no signs of tissue or arteriolar changes such as GOM (Krebs et al. 2003), and another mouse study proved that physiological levels of both the wild-type human *NOTCH3* and mutant p.Arg90Cys *NOTCH3* rescued the arterial deficiencies of *NOTCH3* $^{-/-}$ mice to similar degrees and exhibited normal Notch3/RBP-Jk activity in brain arteries (Monet et al. 2007). Lastly, the similarity of phenotypes of the homozygous and heterozygous CADASIL patients (Tuominen et al. 2001, Vinciguerra et al. 2014) in addition to the rarity of truncating mutations in CADASIL (Peters et al. 2004b) are in favour of another mechanism than loss of function.

Another hypothesis for the pathogenesis of CADASIL is a gain of function caused by the mutation in *NOTCH3*. However, the signalling is not affected due to mutated *NOTCH3* (Joutel et al. 2004, Peters et al. 2004b), which argues against this hypothesis. On the other hand, these two studies were limited to the major NOTCH signalling pathway, so it is possible that the mutations affect other published NOTCH signalling pathways (Martinez Arias, Zecchini & Brennan 2002, Hu et al. 2003, Peters et al. 2004b, Jin et al. 2008). Fouillade and others (2008) have described a cerebral SVD patient with a *NOTCH3* mutation, but no GOM or NOTCH3 aggregation. The mutation destabilises the metal ion bridge in the NOTCH3 protein and hence causes the receptor to be constitutively active in a ligand-independent manner (Fouillade et al. 2008). This evidence suggests that gain of function leads to a cerebral SVD different from CADASIL.

One of the main histological findings in CADASIL is the accumulation of NOTCH3 on the VSMC membrane. The accumulated NOTCH3 is shown to include only the extracellular domain N3ECD (Joutel et al. 2000a). The aggregation of NOTCH3 is believed to be essential for the pathogenesis, but the reason for the accumulation is still unclear. In the normal signalling pathway N3ECD is endocytosed together with the ligand into the ligand-bearing cell (Nichols et al. 2007). It seems that by binding to the N3ECD the ligand physically pulls off the extracellular domain of NOTCH3, which then reveals the cleavage site for S2 cleavage (Wang & Struhl 2005). If the ligand is unable bind to N3ECD, the mechanical force pulling N3ECD could be too weak to reveal the S2 cleavage site, and hence the transendocytosis of N3ECD could be disturbed (Wang & Struhl 2004). However, the amount of aggregated N3ECD on the cell membrane is similar regardless of whether the mutation is affecting ligand binding or is located outside the ligand binding area (Joutel et al. 2004).

One of the main pathologies of CADASIL is arteriopathy, which leads to reduced CBF especially in the resistant arteries (Tuominen et al. 2004). The main effectors in VSMC contraction are myosin and actin, and the smooth muscle cell specific α-actin is targeted in the canonical NOTCH3 signalling pathway (Noseda et al. 2006). The expression of constitutively active NOTCH3 in cultured VSMCs caused changes in the cell shape and increased actin stress fibres and steady-state levels of polymerised actin (Domenga et al. 2004). It has also been shown that genetically genuine human CADASIL VSMCs have an impaired ability to contract, and numerous proteins interacting with the actin cytoskeleton are differentially expressed (Ihalainen et al. 2007). Accordingly, it was shown that actin organisation in these VSMCs is altered (Tikka et al. 2012). Together, these reports posit that NOTCH3 has a part in the regulation of the actin cytoskeleton and may act as a sensor or a signal transmitter permitting VSMCs to react to stretching of the vessel wall. NOTCH signalling has also been linked to platelet-derived growth factor (PDGF) signalling as PDGF-receptor β (PDGFR-β)

is an immediate Notch target gene (Jin et al. 2008). Defects in PDGF-β/PDGFR-β signalling have been shown to cause vascular abnormalities such as microaneurysms, haemorrhage and VSMC hypoplasia (Betsholtz 2004). Furthermore, in authentic human p.Arg133Cys CADASIL cells, NOTCH-ligand stimulation of VSMCs resulted in significantly reduced induction of PDGFR-β expression when compared to control cells (Jin et al. 2008). The significance of PDGFR-β expression by NOTCH is not immediately apparent, although it could explain why mutations in ligand binding and non-ligand-binding sites cause a similar phenotype.

2.3. Hereditary multi-infarct dementia of Swedish type

In 1977, Sourander and Wålinder described a Swedish family suffering from hMID. At the time, the genetics and pathogenesis of SVDs were largely unknown; hence, the disease was considered to be a phenotypic variant of the same inherited entity as CADASIL. When the clinical and pathological picture of CADASIL was described in greater detail, the differences between the Swedish family and CADASIL became more noticeable.

2.3.1. Epidemiology

Since the genetic cause of Swedish hMID has remained unclear, the prevalence of the disease is still unknown. There are some reports of similar disease in the literature (Hagel et al. 2004, Ding et al. 2010), but until further genetic identity of the Swedish hMID is revealed it is impossible to verify whether these families truly have the same type of hMID or not.

2.3.2. Clinical characteristics of Swedish hMID

2.3.2.1. Typical clinical findings

As the name of the disease states, the clinical picture is characterised by multiple infarctions due to disturbances of the CBF. The first symptoms of the disease have occurred when the patients were in their thirties (mean 34 years, range 29-38 years) and included headache, transient vertigo, impaired control of a hand and acute partial hemiparesis (Sourander & Wålinder 1977b, Zhang et al. 1994). As the disease progresses, recurrent ischemic attacks lead to pyramidal, bulbar and cerebellar symptoms such as spasticity, dysphagia, dysarthria and ataxia (Zhang et al. 1994). The disease is also characterised by depressive illness and behavioural symptoms, including mood changes (Zhang et al. 1994). Results of the blood tests and cerebrospinal fluid are reported to be normal, and there are no signs of arterial hypertension (Zhang et al. 1994). The disease course gradually evolves towards severe cognitive impairment (Sourander & Wålinder 1977b). The duration of the disease has reported to be 12-14 years, although one patient died six months after the first symptoms due to an intracerebral haemorrhage (Zhang et al. 1994). The age at death has varied from 29 to 47 years (Zhang et al. 1994).

2.3.2.2. Imaging findings

Most of the previous studies have concentrated on the *post mortem* findings, and the imaging findings are less well reported. In their article, Sourander and Wålinder (1977b) conclude that

in the air encephalogram central and cortical cerebral atrophy was present in all four patients who were studied.

2.3.3. Pathological characteristics

In the macroscopic *post mortem* studies, the general pattern of cerebral changes was reported to be strikingly uniform in all patients. The most prominent changes were multiple small infarcts in the basal ganglia, thalamus, periventricular WM and pons (Sourander & Wålinder 1977b). In addition, cortical and cerebral WM atrophy was present, and there were no alterations in the spinal cord and sciatic nerve (Sourander & Wålinder 1977b).

In the histological studies, widespread occlusive vascular changes and diffuse ischaemic lesions were the most remarkable findings (Sourander & Wålinder 1977b). Vascular changes were reported to be most prominent in the small arteries and arterioles of pia-arachnoidea, basal ganglia, thalamus, mesencephalon, pons and cerebellum. The most common changes included thickening of the vascular wall and narrowing of the lumen, probably caused by subendothelial proliferation or hyaline degeneration of the intima (Sourander & Wålinder 1977b).

The most typical histochemical finding was a strong periodic acid-Schiff (PAS) reaction for carbohydrates in the thickened intima and also in the media (Sourander & Wålinder 1977b). The positive PAS staining is probably caused by acid glycosaminoglycans known to be present in the intima of arteriosclerotic vessels. No amyloid deposits were detected in the cerebral arteries (Sourander & Wålinder 1977b). Staining with Luxol cresyl violet showed regions with WM degeneration and relative sparing of U-fibres (Zhang et al. 1994). In some of the studied arterioles, the smooth muscle cells were lost and replaced by connective tissue (Zhang et al. 1994). Further, the border between media and adventitia was reported to be blurred and some smooth muscle cells seemed to be replaced by connective tissue (Zhang et al. 1994).

EM examination of skin biopsies from an affected Swedish hMID patient showed degenerative irregularly shaped smooth muscle cells with a thickened basement membrane and an increased amount of collagen fibres. Increased type IV collagen immunoreactivity has been shown in the vessels of grey matter of basal ganglia (Craggs et al. 2013, Zhang et al. 1994), and strong immunoreactivity to collagens I, II and III has been reported in the thickened walls of arterial vessels (Zhang et al. 1994). In conclusion, electron microscopy and immunohistochemical studies of Swedish hMID cases show obliterative, non-amyloid angiopathy with degeneration of the media and deposition of the fibrillary collagen types I and III as well as basal lamina components type IV collagen and laminin (Zhang 1997, Craggs et al. 2013). Nonetheless, it is unknown if these deposits of extracellular matrix components cause the disease or if they are merely secondary phenomena to some other primary change.

2.3.4. Genetics

As early as in 1977, when they first described the Swedish hMID, Sourander and Wålinder concluded that the disease had appeared in three generations, affected both genders and had been transmitted from male to male, male to female, female to male and female to female. They

also concluded that none of the affected patients were born of a consanguineous marriage. Hence, it is evident that Swedish hMID is an autosomally dominantly inherited monogenic disease. However, the detailed genetic background remains unsolved almost 40 years after the first report describing this disease entity.

2.3.5. Molecular mechanisms of Swedish hMID pathogenesis

At present, the pathogenesis of Swedish hMID remains largely unknown. Nevertheless, in their article, Zhang and others (1994) presented three different hypotheses for the pathogenesis. According to the first hypothesis the defective gene itself may cause abnormalities in the endothelial or smooth muscle cells of the cerebral arterioles and hence affect the cerebral blood flow. Another theory is that the mutations cause an abnormal protein product that accumulates in the vessel walls or in the near vicinity, thus affecting the normal function of the VSMCs. A third hypothesis is that the disease is caused by an immune-mediated defect acting on the cerebral vessels.

2.4. Identifying disease susceptibility genes in monogenetic disorders

Until recently, mapping the causative gene for monogenetic diseases depended on finding informative families. The families needed to be fairly large, with demonstrable Mendelian inheritance of the disease, preferably across multiple generations. If all these criterias were met, linkage approaches were often succesful for the more common monogenic diseases (Peltonen, McKusick 2001). For the rare genetic diseases, especially when associated with late onset, early lethality or low reproductive fitness, linkage approaches proved to be less succesfull. The Human Genome Project (HGP) set the stage for success in overcoming these challenges.

The HGP launched in 1990 as an international collaborative scientific research programme. The main focus was on the discovery of the whole sequence of human DNA for further biological studies. The first drafts of the human genome were published in 2001 by the International Human Genome Sequencing Consortium (Lander et al. 2001) and by the private company, Celera Genomics (Venter et al. 2001). These reports presented a 90 percent complete sequence of the entire genome's three billion base pairs and the startling finding that the number of human genes appeared to be significantly smaller than previous estimates had suggested. It took another two years to complete the sequence, and, in April 2003, researchers announced that the HGP had completed a high-quality sequence of almost the entire human genome. This new sequence (International Human Genome Sequencing Consortium 2004) closed the gaps from the previously published draft. Since then, the sequence of the human genome has been actively updated by International Hapmap Project Consortium 2010, the 1000 Genomes Project Consortium 2010, The UK10K project and several other studies (Lander 2011). According to current information, the haploid human genome comprises about 20500 protein-coding genes (Clamp et al. 2007). Nonetheless, only a small fraction (approximately 1.5%) of our genome is protein-coding, whereas most of it is associated with regulatory DNA sequences, introns, non-coding RNA and sequences with uncertain functions (Lander 2011).

2.4.1. Genome-wide studies

2.4.1.1. Linkage analysis

For most of the twentieth century, linkage mapping was the standard means of identifying the gene underlying a familial disease. Linkage analysis is based on the tendency of the pathogenic variant and the genetic marker to be inherited together if they are located near each other on the same chromosome. Linkage analysis tests for this co-segregation of a chromosomal region and a trait of interest. The aim of the analysis is to identify the chromosomal loci harbouring the variant predisposing to a certain disease or genetic trait. To do this, the whole genome is studied by genotyping a large number of genetic markers, such as single nucleotide polymorphisms (SNP), microsatellites or restriction fragment length polymorphisms, and the co-segregation of these markers and the disease is then analysed. Markers close to a pathogenic mutation will be inherited together with the pathogenic mutation, unless separated by a meiotic event. The closer the marker to the disease-causing gene, the less likely it will be separated at meiosis. Linkage studies are good for localising areas of disease risk across the genome. However, the linkage areas are often wide and may harbour hundreds of genes. Hence, some other method is needed to narrow down the region of interest and identify the causative gene. The completion of the HGP facilitated the identification of candidate genes within the linkage region and permitted the efficient identification of altered DNA sequences.

At present, linkage analysis is often used to complement the modern next generation sequencing (NGS) methods. Targeted sequencing can be used to study the linkage region in detail or linkage analysis can be paired with whole-exome (WES), or even whole-genome sequencing (WGS). Conducting a linkage analysis is not always possible, since a large number of families with several affected generations are needed. Furthermore, linkage analysis is not suitable for complex traits, where multiple genes are important in disease causation and phenocopies can disturb the analysis. Despite these limitations, mapping monogenic diseases by linkage has been quite successful, even for rare diseases, with well over 1000 monogenic disorders mapped by the turn of the century (Peltonen & McKusick 2001).

2.4.1.2. Next generation sequencing

The high demand for fast and affordable sequencing has driven the development of high-throughput NGS methods. Since 2005 when NGS paltforms became widely available, the causative genes for many monogenic disorders have been identified using these massive parallel sequencing techniques and the rate of their discovery has been exponential (Duncan, Brown & Shore 2014). Even though every NGS platform is unique in how the sequencing is accomplished, the basic methodology is often similar and includes 1) template preparation, 2) sequencing and imaging, and 3) data analysis. When compared to the automated Sanger sequencing, NGS produces an enormous amount of sequence data far more cheaply. In addition to sequencing, NGS can be used, for example, in gene expression studies instead of microarrays. These sequencing-based methods can also be used to identify and quantify rare transcripts and to provide information about alternative splicing and sequence variation in identified genes (Wold & Myers 2008, Metzker

2010). NGS has also revolutionised evolutionary studies, since it is now possible to sequence the whole genome from many related organisms and then compare them (Metzker 2010). However, the widest application area of NGS could be the resequencing of human genomes to improve our knowledge of how genetic variation in our genomes affects health and disease.

WGS provides a vast amount of data, and as a result data analysis can be both expensive and laborious. It is also often hard to predict the pathogenicity of a variant that locates in the non-coding area. For these reasons, the sequencing is often started from the protein-coding regions of the genome, referred to as the exome. The size of an exome is just over 1% of the genome (International Human Genome Sequencing Consortium 2004), and WES is therefore a much more cost effective method to detect pathogenic variants and discover gene targets. In 2009, Ng and others proved that targeted exome sequencing can be used to identify causal variants of rare genetic disorders. Subsequently, WES has become a popular tool for seeking new mutations, but some limitations remain. Even with high sequencing depth (95x-160x), WES successfully captures only 95% of the coding regions with a minimal coverage of 20x (Lelieveld et al. 2015). At present, more and more intronic variants are shown to affect gene expression or cause a disease (Soemedi et al. 2014). Some of the splice sites are covered in the exome, but most often WES cannot find intronic variants that may be important for controlling transcriptional regulation or splicing. In addition, the current understanding of the genome limits the method. Nucleotides in regions of the genome that currently are not recognised as genes will be missed by exome approaches, since they are not targeted. Finally, exomes are not ideal for understanding structural alterations and copy number variations in genomes.

In cases where a certain disease group or condition is suspected, targeted sequencing of specific genes or genomic regions is preferred over WGS and WES. Targeted sequencing yields much higher coverage of regions of interest and reduces sequencing costs and time used (Xuan et al. 2013). A sequencing panel can be targeted only for desired regions of the genome (such as known cancer-causing mutations (Rehm 2013)), eliminating the majority of the genome from analysis. Targeted sequencing helps in the rapid diagnosis of many genetic diseases, and it can also be useful in therapeutic decision-making if the effects of certain treatments are mutation specific (Rehm 2013).

2.4.1.3. Identification of modifier genes in monogenic disorders

The variability in the disease expression can be vast, even when all the patients have the same disease causing mutation. This variability may be due to additional variant(s) in the disease causing gene, epigenetic changes, environmental factors or modifier genes. Studying the modifier genes needs a different approach than seeking the causative gene itself, as the patients do not all carry the modifying variant, and there may be more than one gene affecting the phenotype. The first step is common to all approaches: the clinical phenotype needs to be carefully defined. After that, the classical approach is to focus on specific candidate genes or candidate pathways and resequence chosen genomic areas. The advantage of this approach is that strong modifiers of a certain phenotype can be found even in fairly small cohorts. However, choosing the best candidates can be difficult and time consuming.

Another option for studying modifier genes is the use of genome-wide association studies (GWAS). The first succesful GWAS were published in the second half of the last decade (Klein et al. 2005). GWAS examine the common variants (usually SNPs) in the population in order to discover whether some of the variants are associated with a certain disease or trait. The method has become widely used and a large number of disease-associated loci in the human genome have been identified (Welter et al. 2014). One of the most common approaches used in GWAS is the case-control study, where all participants are genotyped for the majority of common SNPs, and the allele frequencies are then compared between the cases and controls. If the allele frequencies are significantly different between the groups, it can help to identify the disease-causing gene or risk allele. The main limitation of GWAS is the need for large sample sizes, which means that the method is not suitable for rare diseases. Further, common problems affecting GWAS have included the lack of well-defined case and control groups, control for population stratification, and multiple testing.

In the field of neurogenetics, GWAS studies have revealed strong associations with certain genetic variants and stroke. Most associations occur with specific stroke subtypes, for example a variant in the protein kinase C family (*PRKCH*) has been associated with small-vessel stroke (Kubo et al. 2007) and SNPs in *CELSR1* (Cadherin, EGF LAG Seven-Pass G-Type Receptor 1) gene have been identified as risk factors for ischemic stroke (Yamada et al. 2009) in the Japanese population. Even though CADASIL is a monogenic disease, variants associated with stroke could partly explain the vast variability between the patients' clinical pictures. Recent GWAS studies have also yielded four SNPs, located on chromosome 8q22.1, 2q37.1, 12q13.3 and 1p36.32, which are significantly associated with migraine (Tan et al. 2014a). With pooled analysis and international collaboration the genetic modifiers of the clinical picture of CADASIL could be more effectively analysed with GWAS.

2.4.2. Monogenic diseases in the Finnish population

The majority of genes in the Finnish gene pool today are thought to originate from rather small founder populations. The initial founder populations immigrated 4,000 and 2,000 years ago to southern and western Finland. In the 16th–17th centuries an internal migration movement from a small southeastern area to the middle, western, and finally northern and eastern parts of the country populated the entire geographical area of modern Finland (Peltonen, Jalanko & Varilo 1999). Due to the relatively small founder population, geographical isolation, bottlenecks and rapid expansion, genetic drift has affected the population (Peltonen, Jalanko & Varilo 1999) and the frequency of some rare diseases has increased, thus creating the Finnish disease heritage (Norio, Nevanlinna & Perheentupa 1973). In addition to this enrichment of almost 40 diseases, the evidence of a founder effect includes longer regions of linkage disequilibrium (LD), increased kinship coefficients between pairs of randomly chosen individuals, and extended runs of homozygosity (Wang et al. 2014). This homogeneity and the longer LDs make the Finnish population well suited for genetic mapping. Therefore identification of the genes underlying the rare monogenic diseases enriched in Finland has been remarkably successful (Peltonen, Jalanko & Varilo 1999). In GWAS based works, the Finnish founder effect is less likely to provide specific advantage, as common variation is less influenced by population history (Wang et al. 2014). However, with current NGS and chip approaches focused on rare variation, founder populations such as Finland can provide additional power for the analysis (Wang et al. 2014).

3. AIMS

The aim of this study was to clarify the genetics of two familial small vessel diseases, mainly focusing on the genetic variants causing the disease or modifying its phenotype.

The specific aims of the study were:

I To study the possible genetic factors causing the phenotypic variation in CADASIL in a Finnish, mutationally homogenous (p.Arg133Cys) CADASIL population.

II To study the environmental and lifestyle factors affecting the CADASIL phenotype in monozygotic twins, whose disease phenotypes are remarkably different.

III To show that one of the first reported CADASIL families is actually presenting a novel autosomally dominant SVD causing vascular cognitive impairment (Swedish hMID).

IV To search for the specific gene defect behind the Swedish hMID.

4. MATERIALS AND METHODS

4.1. Subjects

4.1.1. The p.Arg133Cys CADASIL population (I)

Altogether, 134 CADASIL patients with the same p.Arg133Cys (c.397C>T) *NOTCH3* mutation were included in the study. All samples were sent to the former Department of Medical Genetics at the University of Turku during the years 1996–2012 as either research or diagnostic samples. In the research samples, the *NOTCH3* mutation was analysed as described in Methods (4.3.2). All samples with a genetically confirmed p.Arg133Cys mutation were included in the study. The *APOE* alleles, *AGT* p.Met268Thr (rs699) polymorphism and neutral intragenic *NOTCH3* p. Ala2902Ala (rs1043994) polymorphism were analysed in all patients. Seven intragenic amino-acid-changing polymorphisms of the *NOTCH3* gene were analysed in a subgroup of 50 patients.

Medical records and extensive structured questionnaires were used to evaluate the patients' health status, health behaviour, and medical history. The main information collected was the occurrence of and age at first-ever TIA and stroke, as well as the incidence of migrainous headache, myocardial infarction and vascular risk factors such as hypertension (>140/90 mmHg or medication), diabetes mellitus (established diagnosis) and high total serum cholesterol (>6.2 mmol/l or statin therapy). The patients' height and weight were used to calculate body mass index (BMI) and >25.0 kg/m^2 was considered overweight. Finally, patients were divided into smokers or non-smokers, and into heavy drinkers (men >280 g of alcohol/week, women >190 g/week), moderate drinkers (men <280 g/week, women <190 g/week) and non-drinkers according to their current smoking and drinking habits (see Table I in the original article I)

4.1.2. The monozygotic twins with CADASIL (II)

A pair of monozygotic twins of Swedish background was included in the study, and their clinical symptoms, imaging findings and genetic background were studied. According to medical records, twin brothers had remarkable differences in their clinical picture. The diagnosis of CADASIL was based on the detection of GOM in the EM examination of a skin biopsy.

4.1.3. The Swedish hMID family (III & IV)

21 members of the Swedish hMID family were involved in the study (see pedigree in Figure 1 of original publication IV). Ten of them were affected, another ten were unaffected and one was an unrelated spouse used as a control (Table 2). The clinical data were collected from medical records, and imaging data were also collected when possible. Blood-derived DNA samples were available from 17 individuals, and saliva samples were also collected for DNA for eight of them (Table 2). For four affected patients, only formalin fixed paraffin embedded (FFPE) tissue blocks were available.

Table 2 *Swedish hMID patients and sample types.*

ID in pedigree	Sample type	Affected y/n	Exome sequenced
III:2	Blood-DNA	no (spouse)	
IV:14	Blood-DNA	no	yes
IV:16	Blood-DNA	yes	yes
IV:5	Blood-DNA	no	yes
IV:7	Blood-DNA	yes	yes
V:3	Blood-DNA	yes	yes
V:5	Blood-DNA	yes	
V:7	Blood-DNA	no	
III:1b	Blood-DNA, Saliva-DNA	no	
III:1c	Blood-DNA, Saliva-DNA	yes	yes
III:1d	Blood-DNA, Saliva-DNA	no	
IV:1c	Blood-DNA, Saliva-DNA	no	
IV:1g	Blood-DNA, Saliva-DNA	no	
IV:1i	Blood-DNA, Saliva-DNA	no	
V:a9	Blood-DNA, Saliva-DNA	no	
V:a11	Blood-DNA, Saliva-DNA	no	
III:5	FFPE-DNA	yes	
III:7	FFPE-DNA	yes	
IV:10	FFPE-DNA	yes	
IV:3	FFPE-DNA	yes	
IV:1a	Blood-DNA	yes	

4.2. Ethical aspects

This study was accepted by the ethics committees of the Hospital District of Southwest Finland and Turku University Hospital in Finland and by the Research Ethics Committee at Huddinge University Hospital and the Ethical Committee of Uppsala Academic Hospital in Sweden. The use of Finnish CADASIL DNA samples (I) and the collection of these patients' medical information were approved by the National Authority for Medicolegal Affairs (TEO) and the Ministry of Social Affairs and Health (STM). The Swedish CADASIL samples (monozygotic twins, II) and Swedish hMID samples (III, IV) were collected for research, and all patients and family members gave their informed consent before taking part in this study.

4.3. Methods

The molecular genetic methods used in this study are summarised in Table 3 and described in detail in the next chapters.

Table 3 *Molecular laboratory methods used in this study*

Method	Used in study
DNA extraction	
From leukocytes	I,II,III,IV
From saliva	IV
From FFPE samples	IV
PCR	I,II,III,IV
RT-PCR	III
Sanger sequencing	I,II,III,IV
NOTCH3 mutation analysis	
Amino acid changing *NOTCH3* polymorphisms	
Validation of exome data	
Restriction enzyme analysis	I,II
NOTCH3 mutation testing: p.Arg133Cys, p.Arg182Cys	
Genotyping: *AGT, NOTCH3* p.Ala202Ala	
DHLPC analysis with a Wave™	II
Amino acid changing *NOTCH3* polymorphisms	
Linkage analysis	III,IV
Limited haplotype analysis with microsatellite markers	
Whole genome analysis with microarrays	
Whole exome sequencing	IV

FFPE: formalin fixed paraffin embedded, PCR: polymerase chain reaction, RT-PCR: reverse transcription-PCR, DHPLC: denaturing high performance liquid chromatography

4.3.1. DNA extraction (I, II, III, IV)

The leukocyte DNA was extracted from ethylenediaminetetraacetic acid (EDTA) or lithium-heparin blood samples with the Nucleon BACC3 Genomic DNA Extraction Kit (GEHealthcare, Little Chalfont, Buckinghamshire, UK). Saliva samples were collected with the Oragene DNA Kit (DNA Genotek Inc. Ottawa, Ontario, Canada), and the DNA was extracted according to the manufacturer's protocol. DNA from the FFPE samples was extracted with the NucleoSpin® FFPE DNA kit (Macherey-Nagel, Düren, Germany).

4.3.2. Testing for the p.Arg133Cys and p.Arg182Cys mutations (I, II)

The p.Arg133Cys (c.397C>T) alteration is the most common CADASIL-causing mutation in Finland due to a founder effect (Mykkänen et al. 2004). The presence of this mutation and the p.Arg182Cys (c.544C>T) mutation can be analysed with the same restriction enzyme analysis. Exon 4 of *NOTCH3* was amplified in polymerase chain reaction (PCR) with specific primers (Table 4) and the resulting amplicons were treated with the MspAI1 restriction enzyme (New England Biolabs, Ipswich, Massachuttes, USA). Both p.Arg133Cys and p.Arg182Cys destroy a recognition site of the MspAI1 enzyme; hence, the presence of these mutations can be detected according to the restriction pattern seen on agarose gel electrophoresis.

4.3.3. Sequencing of *NOTCH3* (II, III)

The CADASIL diagnosis of the monozygotic twins was confirmed with an EM analysis showing GOM in a skin biopsy. For the detection of the actual mutation site, exons 2–24 of *NOTCH3* were amplified with specific primer pairs (Table 4). These exons code for the 34 EGF-like repeats in NOTCH3 and cover all published cysteine-altering CADASIL mutations confirmed to date. To rule out a CADASIL-causing mutation in the Swedish hMID patients, the entire coding area of *NOTCH3* was amplified with specific primer pairs (Table 4). The resulting amplicons were then sequenced using an automated sequencing system (Applied Biosystems, Foster City, CA, USA). Sequence Scanner v1.0 or v2.0 (Applied Biosystems) was used to check the quality of the sequences and conduct the preliminary analyses. After the quality check, all sequences were analysed manually against the reference sequence.

Table 4 *NOTCH3 primer sequences, annealing temperatures and polymerase chain reaction (PCR) conditions.*

Exon	Primer sequences	T_{ann} and special PCR conditions
1	5′ – CGG CTC TGG GTG TGT ACT G – 3′ 5′ – GTC CCA TCC GCC AGG T – 3′	61°
2	5′ – GGC ACC TGG CTG ATC CT – 3′ 5′ – CCG CCC CCA CAC ACA – 3′	63°, 1x GC-RICH Solution
3	5′ – TGT GCT GCC CAA CCA AGC CA – 3′ 5′ – TCC AGA CTC TTC CCC TCT CA – 3′	65°C
4	5′ – TAG TCG GGG GTG TGG TCA GT – 3′ 5′ – CCT CTG ACT CTC CTG AGT AG – 3′	65°C
5 & 6	5′ – TGA GTG AGC CCT ACT CAG GA – 3′ 5′ – GCC CTC ACT AAA AAC CAT CC – 3′	58°C
7 & 8	5′ – TGG GCA GAG CAG GAA GAT – 3′ 5′ – GCC CCC TGC CTC AGG AC – 3′	63°
9 & 10	5′ – ACC CCG TTC ACA CCA TAG G – 3′ 5′ – CCG CCT CCT GAT TCT TGT C – 3′	62°
11 & 12	5′ – AAG TGG GCG GAG CCT GAC – 3′ 5′ – TCG ATC TAA GGA CCC CCT CT – 3′	65°C
13 & 14	5′ – CTG GTT GTC CCT GCT GAC TT – 3′ 5′ – AGA AGG CCC ATG GTG TTG – 3′	62°C
15	5′ – GGG AGT CCC TCA AGG CTA TC – 3′ 5′ – GCA GAG GAG ATG GAG AGG AG – 3′	61°C
16	5′ – CCC TGC TCT GTA CCC TGT AA – 3′ 5′ – TGT TCC CCA GAG CAG CAC – 3′	61°C
17	5′ – CTA ATG GGG GCA AGG TAG GT – 3′ 5′ – AAG CCA GAG TCC CTG CTC TC – 3′	61°C
18 & 19	5′ – GAT CCT CCC TCC CAC TCC T – 3′ 5′ – CTT CCC AAG GCC CCA CAC – 3′	65°C, 1x GC-RICH Solution
20	5′ – TGG GGT TAC CTC TGT TCC TG – 3′ 5′ – CCC ACC TCC TCT TCC CTC T – 3′	61°C
21 & 22	5′ – GGT CTG TGT CCC ACT AAG CTG – 3′ 5′ – CAG CCA CAA TGG GGG AAT – 3′	62°C
23	5′ – CTG TCA TTC CCC CAT TGT G – 3′ 5′ – GCC CCT ACT CCT CCT CCA – 3′	61°C

Exon	Primer sequences	T_{ann} and special PCR conditions
24	5′ – CCC CAC CCT CAT TTT TAT CC – 3′ 5′ – AAA CAG ACT GGG ATG GAT GC – 3′	61°C, 1x GC-RICH Solution
25	5′ – TGT CTC CTC TGA CCC CTG AC – 3′ 5′ – CCT CTC CCC AGC CAC CAC – 3′	65°C
26 & 27	5′ – CAA TTT TTG AGC CCT CTG GT – 3′ 5′ – GCA GGG TTC TCA CTT CAT GC – 3′	61°C
28	5′ – CCT CTA GTG TCC CCC TCA CA – 3′ 5′ – CTG AGG GGA GGG GTC AGA – 3′	62°C
29	5′ – CCA GCA CCA AAG GGT GAG – 3′ 5′ – CAC AGG TAG GAT GGG TGA GG – 3′	61°C
30	5′ – GGC CCT GTG TTT ACC TTC CT – 3′ 5′ – CTA ATG CCT GCC CCA GCT CT – 3′	64°C
31	5′ – CTG CCA TGA CCC CTC CTG – 3′ 5′ – TGA CAC CAA CCC AGC TTA GA – 3′	63°C
32	5′ – AAT TTC TGC CTC CCT GAC AT – 3′ 5′ – CAC TGT GCC ACT GCT GAC A – 3′	61°C
33	5′ – TGC TAC TGT TAG CTG GGG TTT – 3′ 5′ – GGC TGA GTA CAC ATC CTC CAG – 3′	60°C
	5′ – GCT GCC ACT GCC ACT GC – 3′ 5′ – GAA GAG GAT GAA AAA GAC TAA AAG GA – 3′	63°C
	5′ – TTC TTA GAT CTT GGG GGC CTA – 3′ 5′ – AGT GGG TGC GCC CAA GG – 3′	65°C
	5′ – TGT ACC TAG TAC ACA GGC ATG A – 3′ 5′ – GGG GAG ATA GAA GTC CCA CTG – 3′	60°C

T_{ann} = annealing temperature, GC-RICH Solution (Roche, Basel, Switzerland)

4.3.4. The genotyping of *APOE* and *AGT* (I, II)

Two candidate genes, apolipoprotein E *(APOE)* and angiotensinogen *(AGT),* were studied as possible modifiers of the clinical picture of CADASIL. Both candidate genes were chosen due to their association with stroke. The *APOE* ε4 carriers have been reported to be at increased risk of atherosclerosis and stroke (Bangen et al. 2013, Lopez, Krastins & Ning 2014) and *AGT* p.Met268Thr has been shown to increase the stroke risk at least in East Asian populations (Wang et al. 2012, Liang et al. 2013). All 134 CADASIL patients with the same p.Arg133Cys mutation were genotyped for the three most common allelic isoforms of *APOE* (ε2, ε3 and ε4), and the *AGT* p.Met268Thr (c.803C>T, rs699) polymorphism. The *APOE* isoforms were genotyped by first amplifying the DNA region containing the codons coding for amino acid positions 112 and 158 with specific primers (Table 5) in a standard PCR reaction. The amplicons were then sequenced using the automated ABI3100 system. *AGT* exon 2 was amplified in a PCR reaction in which one of the primers contains a mismatch nucleotide (Table 5). Together with the c.803C>T transition, the mismatch produces a restriction site for the Tth111I enzyme (New Engand Biolabs). Genotypes were then inferred on the basis of the restriction pattern after agarose gel electrophoresis.

Table 5 *Primers used for the detection of APOE alleles and AGT genotypes.*

Gene	Primer sequences	T_{ann} and specific PCR conditions
AGT exon 2	5'-CCG TTT GTG CAG GGC CTG GCT CTC T-3' 5'-CAG GGT GCT GTC CAC ACT GGA CCC C-3'	69°C, final extension 10 min, mismatch in sequence underlined
APOE exon 4	5'-GCG GAT GGC GCT GAG G-3' 5'-GGC ACG GCT GTC CAA G-3'	59°C, 10% Dimetyl sulfoxide (DMSO)

4.3.5. The analysis of the *NOTCH3* intragenic polymorphisms (I)

Altogether, seven amino-acid changing polymorphisms of *NOTCH3* (Table 6) were analysed in a subgroup of 50 CADASIL patients. These polymorphisms were chosen from the first report of *NOTCH3* polymorphisms (Joutel et al. 1997b). For 20 patients, the analysis was done using a heteroduplex analysis in an automated denaturing high performance liquid chromatography (DHPLC) system (Wave™, Transgenomics, Omaha, Nebraska, USA), and for 30 patients using direct sequencing with an automated ABI3100 system. The synonymous *NOTCH3* p.Ala202Ala (rs1043994) polymorphism was analysed in all 134 patients by restriction analysis of the *NOTCH3* exon 4 PCR-amplicons. The analysis was done using the HhaI restriction enzyme (New England Biolabs), which cuts the G allele of the polymorphism. The neutral polymorphism was included in this study due to its reported association with migraine in the normal population (Schwaag et al. 2006).

Table 6 *NOTCH3 polymorphisms included in the study.*

Reported as (Joutel et al 1997b)	Amino acid change	Rs number	SISu* MAF ‡	Notes
c.587A>G, exon 4	p.His170Arg	rs147373451	$8.17261e^{-05}$	
c.606A>G, exon 4	p.Ala202Ala	rs1043994	0.870019	Synonymous
c.941G>C, exon 6	p.Gly288Ala			c.941G>C, NM_000435.2
c.1565C>T, exon 9	p.Pro496Leu	rs11670799	0.0037141	
c.1568C>T, exon 9	p.Ser497Leu	rs114207045	0.0116298	
c.3477C>A, exon 21	p.His1133Gln	rs112197217	0.0147107	
c.3625G>A, exon 22	p.Val1183Met	rs10408676	0.00263979	
c.5632G>A, exon 30	p.Ala1852Thr			c.5632G>A, NM_000435.2

MAF: minor allele frequency,

**The Sequencing Initiative Suomi (The SISu project) is an international collaboration between research groups aiming to build tools for genomic medicine. These groups are generating whole genome and whole exome sequence data from Finnish samples and provide data resources for the research community. Key groups of the project are from Universities of Eastern Finland, Oulu and Helsinki and The Institute for Health and Welfare, Finland, Lund University, The Wellcome Trust Sanger Institute, University of Oxford, The Broad Institute of Harvard and MIT, University of Michigan, Washington University in St. Louis, and University of California, Los Angeles (UCLA). The project is coordinated in the Institute for Molecular Medicine Finland at the University of Helsinki. www.sisuproject.fi*

‡ Paavo Häppölä, personal communication, August 2015

4.3.6. Associations between the *APOE, AGT* and *NOTCH3* polymorphisms and stroke (I)

The possible associations between *APOE* genotypes or the *AGT* p.Met268Thr polymorphism and first-ever stroke or TIA were analysed using Cox proportional hazards regression. The analyses were conducted first unadjusted and then adjusted for age, sex, and known vascular risk factors, such as hypertension, hypercholesterolemia, myocardial infarction, smoking and alcohol consumption. The association between the neutral *NOTCH3* p.Ala202Ala polymorphism and migraine was analysed using logistic regression with a sibling as a random effect. All statistical analyses were conducted using SPSS, version 19.0 (IBM Inc., Armonk, New York, USA) or SAS version 9.1 (SAS Institute, Cary, North Carolina, USA).

4.3.7. Exome sequencing (IV)

A total of 6 individuals were chosen for whole-exome sequencing; four were affected (III:1c, IV:7, IV:16 and V:3) and 2 unaffected (IV:5 and IV:14) (Table 2). At first, only three patients and two controls were available to be sequenced at the Institute of Molecular Medicine in Finland (FIMM, Helsinki, Finland) (IV:5, IV:7, IV:14, IV:16 and V:3). Additionally, two of them (IV:16, V:3) were simultaneously sequenced at the National Institutes of Health (NIH, Bethesda, Maryland, USA). Later, when new samples were available, one more patient (III:1c) was sequenced at University College London (UCL, London, UK). In all cases the target enrichment was done with SeqCap EZ Human Exome Library version 2.0 (Roche Nimblegen Inc., Basel, Switzerland), and the actual sequencing run was performed with HiSeq 2000 (Illumina, San Diego, CA, USA). The raw data from all sequence runs were combined and all the samples were re-called from the fastQ files. The analysis pipeline followed the GATK best practices (Genome analysis toolkit, https://www.broadinstitute.org/gatk/). The alignment of the reads was done against GRCh37/hg19 and *in silico* analysis of the functional effects of coding non-synonymous variants was performed with SIFT (Kumar, Henikoff & Ng 2009) and PolyPhen (Adzhubei et al. 2010). The mean target coverages varied between 22 and 38. Data analysis was based on the autosomal dominant inheritance trait and the hypothesis that none of the neurologically healthy family members carried the variant. Variants not found in all patients in the family and variants that were non-autosomal, homozygous, synonymous or common in the general population were excluded. The validation of variants found with exome sequencing was done using Sanger sequencing with the BigDye Terminator chemistry version 3.1 (Applied Biosystems).

4.3.8. Linkage analysis (III & IV)

A limited haplotype analysis with three microsatellite markers (D19S253, D19S923 and D19S841, see Figure 2 in original publication III), was performed to rule out the association between the Swedish hMID family and the CADASIL causing *NOTCH3* gene. The method is described in detail in original publication III.

To study the chromosomal areas shared by all the affected Swedish hMID family members (IV), a whole genome microarray was performed for all available blood- or saliva-derived DNA samples. The FFPE samples were not included due to fragmentation of DNA. The microarray analysis was performed with the HumanOmniExpress Bead chip (Illumina) and whole genome

parametric linkage analysis was done with Allegro (Gudbjartsson et al. 2000) with a fully penetrant autosomal dominant model. Chromosomal regions achieving LOD scores >2 were included in further analysis.

4.3.9. Imaging and histological methods

The imaging and histological methods used in the study are summarised below in Table 7 and described in detail in the original publications as indicated by their assigned Roman numerals.

Table 7 *Imaging methods used in this study*

Method	Used in study
Gross morphological evaluation of brain	III
Histopathological and immunohistochemical stainings: Haemotoxylin & Eosin Luxol Fast Blue Periodic Acid Schiff Anti-COL4	III & IV
MRI	II & III
Electron microscopy	II & III

5. RESULTS AND DISCUSSION

5.1. Modifiers of the CADASIL phenotype (I, II)

The clinical picture of CADASIL varies greatly both in and between families. Hence, it is suggested that in addition to the actual *NOTCH3* mutation other genetic or environmental factors may also modify the phenotype of CADASIL. In this study, we investigated the possible modifiers of the clinical picture by analysing genetic factors in a mutationally homozygous population of 134 CADASIL patients and also lifestyle factors in a monozygotic twin pair.

5.1.1. Genetic factors modifying the CADASIL phenotype (I)

Our study population consisted of 134 patients (68 females and 66 males), and the mean age in this cohort was 57 (range 20-94) years. Diagnosis of CADASIL was confirmed with genetic testing, and all the patients included in this study had the same p.Arg133Cys mutation. The clinical features and demographical data of this population are described in Table 1 of original article I.

Our study population is unique among other large CADASIL populations (Dotti et al. 2005, Joutel et al. 1997b) since all the patients have the same p.Arg133Cys mutation due to a founder effect (Mykkänen et al. 2004). In addition, the size of the Finnish population is rather small (approximately 5.48 million), and the population has originated from a small founder population (Peltonen, Jalanko & Varilo 1999). This, together with isolation, has enhanced the homogeneity of our patient cohort. The frequencies of stroke, migrainous headache and vascular risk factors in our population (Table 1 in the original article I) were similar to those found in other large CADASIL cohorts (Singhal et al. 2004). Another advantage in our study was the vast amount of clinical data collected using structured questionnaires and medical records. Hence, this population of 134 CADASIL patients is highly suitable for studying associations between different genetic variants and first-ever stroke.

The *APOE* allele frequencies in our study population were ε2 5.4%, ε3 77.5% and ε4 17.1% and the genotype frequencies were ε2/ε2 0,8%, ε2/ε3 9,0%, ε3/ε3 58,6%, ε3/ε4 30,8% and ε4/ε4 0,8%. The allele frequencies in this study are consistent with the frequencies reported in the general Finnish population (Schiele et al. 2000). Cox proportional hazard rgression showed no association between the *APOE* genotypes and first-ever stroke, first-ever TIA or first-ever TIA/stroke (Table 2 in original article I). The results did not change after adjusting for the traditional vascular risk factors. Our results confirm the previous studies by Singhal and co-workers (2004) and van den Boom and others (2006), suggesting that *APOE* ε4 is not a major contributor in the phenotypic variation in CADASIL. Interestingly, a recent study has reported that APOE ε2 is associated with WMH volume in CADASIL (Gesierich et al. 2015). Unfortunately, in our cohort, the frequency of APOE ε2 is too low for statistical analysis.

The *AGT* genotype frequencies in our study population were Met/Met 23.2%, Met/Thr 56.6% and Thr/Thr 20.2%. There were no associations between different *AGT* genotypes and first-ever ischemic events.

Seven amino acid-changing polymorphisms and one neutral polymorphism (Table 6) were studied as potential modifiers of the disease phenotype. None of the amino acid-changing polymorphisms was found in a subgroup of 50 patients. Hence, the analysis was not widened to the entire patient cohort. In this Finnish population, the frequency of these polymorphisms is too low for any statistical approach. The synonymous p.Ala202Ala polymorphism was analysed in all 134 patients, but no association between this SNP and first-ever stroke or migraine could be detected: AA vs. GG, OR 0.71 (95% CI 0.01-40.59); AG vs. GG, OR 1.15 (95% CI 0.27-4.86).

5.1.2. Environmental and lifestyle factors modifying the CADASIL phenotype (II)

As monozygotic twins share an identical genome, they are optimal for evaluating the phenotypic variation that arises from environmental and lifestyle factors. In this study, we assessed the clinical variation between identical twin brothers (twin A and twin B) suffering from CADASIL.

The monozygosity of the twins was confirmed with a DNA test comprising ten genetic markers (99.9% likelihood, National Public Health Institute, Finland). The CADASIL diagnosis was established after twin B had his second stroke-like attack at the age of 48 by an EM analysis showing GOM in the near vicinity of VSMCs in his skin arterioles. Sequencing of the *NOTCH3* gene showed a previously unpublished mutation, p.Cys251Tyr (c.752G>A), in the 6th EGF-repeat of the NOTCH3 receptor. Although the mutation is novel, three other mutations (p. Cys251Arg, p.Cys251Ser, p.Cys251Gly) have previously been reported in that same codon (Markus et al. 2002, Lesnik Oberstein et al. 2003, Vikelis, Papatriantafyllou & Karageorgiou 2007), suggesting that variations at that site are truly pathogenic. As the p.Cys251Tyr mutation affects the number of cysteine residues in one of the EGF-repeats of *NOTCH3*, it follows the stereotyped nature of most CADASIL-causing mutations. Naturally, the same mutation could also be found in twin A. Both of the twins were also genotyped for *APOE,* and, as expected, they shared the same genotype, ε3/ε4.

Even though these monozygotic twins share an identical genetic background, there were clear differences in the disease progression. Twin B had his first ischemic attack at the age of 39, with dysarthria as the main symptom. After that, it took 14 years before twin A suffered from his first-ever ischemic attack at the age of 53 years. The clinical findings and differences in the disease progression are summarized in Table 8.

Table 8 *Differences in the clinical picture and the disease course in monozygotic twins with CADASIL. Modified from Mykkänen, 2009.*

Symptom/Finding	Twin A			Twin B		
Ischemic attacks	2 strokes (53y, 55y) TIA (54y)			4 stroke-like attacks (39y, 48y, 49y, 54y) TIA (48y)		
Symptoms	Dysarthria, dysphasia, loss of consciousness, nausea, abdominal pain, dizziness, spell of confusion			Dysarthria, visual symptoms, loss of consciousness, nausea, chills, cold sweat		
Imaging						
MRI: Scheltens scores*	**At the age of** Periventricular Deep WM Basal ganglia Infratentorial	**47y** Σ 6 Σ 16 Σ 7 Σ 7	**53y** Σ 6 Σ 16 Σ 10 Σ 7	**At the age of** Periventricular Deep WM Basal ganglia Infratentorial	**47y** Σ 6 Σ 22 Σ 15 Σ 11	**53y** Σ 6 Σ 22 Σ 15 Σ 12
Number of lacunar infarcts			11			16
PET (at the age of 50)	CBF decreased 2 SD: Pons			CBF decreased over 2 SDs: cerebral WM, cortical areas (except for the visual cortex)		
Neuropsychological tests						
At the age of 48 years	Below normal in verbal abstraction, verbal fluency, complex visuospatial tasks and delayed memory			Below normal in verbal abstraction, verbal fluency, complex visuospatial tasks with high demand on executive functions, verbal learning and retrieval tasks.		
At the age of 55 years	Deterioration in verbal episodic memory, complex visuospatial abilities and visual memory			Further deterioration in verbal fluency, visuospatial abilities, verbal delayed memory, visual memory. Recovery in verbal learning and memory retrieval tasks.		

*Abbreviations: TIA, transient ischemic attack; MRI, magnetic resonance imaging; WM, white matter; PET, positron emission tomography; CBF, cerebral blood flow; SD, standard deviation. *(Scheltens et al. 1993)*

As seen in Table 8, by the time twin A had his first ever ischemic attack at the age of 53 years, twin B had already suffered from at least four ischemic events. There were also clear differences in the MRI and PET results, twin B presenting more severe changes in both brain structures and CBF. In the neuropsychological examination, the twins showed similar profiles, although differences in the level of performance and progression could be observed. The localisation of the infarcts might have an effect on the clinical picture of the disease, but the similarity in twins' neuropsychological profiles suggests that CADASIL is the only major contributor for the phenotype.

As there are no differences in the genomes of monozygotic twins, the variation in the disease progression and symptoms arises mostly from environmental factors and lifestyle choices. The gestation and birth of the twins were described as normal, although we cannot exclude intrauterine placement or asymmetric placental blood flow that might affect the similarity of cognitive functions (Rose 2005). However, the difference in birth weights was minor, which suggests that the intrauterine environment had been similar for both foetuses. Early childhood was also similar for both twins, but later in life some differences in lifestyle could be seen (Table 9).

Table 9 *Differences in the environmental and lifestyle factors between the twins.*

	Twin A	Twin B
Size at birth	2340 g 46,5 cm	2150 g 46,5 cm
Education	Compulsory education and vocational schools, 11 years in total	Compulsory education and upper secondary school, 12 years in total
Alcohol consumption	Moderate	Light
Smoking	Never	Daily from the age of 14 to the age of 38.
Physical activity	Physically active since youth.	Light exercise regularly from early adulthood to the age of 54 when he had to stop due to physical exhaustion
Hypercholesterolemia	Treated with Simvastatin from the age of 43	Fasting plasma lipid levels were within recommendations while treated with Atorvastatin (from the age of 47)
Medication	Simvastatin Aspirin, low dosage Antidepressants Folic acid B vitamins Dipyridamol	Atorvastatin Aspirin, low dosage Folic acid B vitamins

Even though identical twins are not expected to have genetic differences at birth, later in life epigenetic changes do occur. Different environmental factors can have direct effects on the tissue level and cause changes in DNA methylation and histone modifications during the lifetime (Fraga et al. 2005). Hence, environmental factors such as nutrition, physical activity, smoking and alcohol consumption can cause permanent modifications on the DNA level and alter the phenotype. Smoking is known to increase the stroke risk both in the general population (Shinton & Beevers 1989) and in the CADASIL population (Singhal et al. 2004). The effect of smoking was also seen as a trend in this study (I), although it did not reach statistical significance. Twin B had already started smoking in his youth (14 years of age) and continued until he was 38 years of age. Twin A had never smoked. In the light of the previous studies (Adib-Samii et al. 2010), this difference could be the most probable environmental factor contributing to the phenotypic variation in this twin pair. Furthermore, previous studies have also shown that statin treatment (Di Mascio, Marchioli & Tognoni 2000, Asahi et al. 2005) and regular exercise (Gillum, Mussolino & Ingram 1996, Hu et al. 2000) may reduce stroke risk. Since twin A was diagnosed with hypercholesterolemia, he started statin treatment at the age of 43, five years earlier than twin B. Even though statin treatment in the general population has been proven to lower the risk of stroke, Peters and others (2007) could not show a similar effect in a CADASIL population treated with atorvastatin. However, the treatment period in that study was short (8 weeks), and the study population consisted of only 24 CADASIL patients. Hence, it is possible that the longer use of statins by twin A may have contributed to his later disease onset and milder phenotype. Furthermore, twin A exercised more and at a higher intensity than twin B, and this physical activity could also be one of the environmental factors that have protected him from a more severe disease phenotype.

5.1.3. Studying the phenotypic variability in CADASIL (I, II)

As CADASIL is a late onset disease with no known curative treatment, identifying the possible genetic and environmental factors affecting the phenotype is important for the patients and their relatives. The mood changes and depression seen in CADASIL may be partly caused by the fact that the patients feel that there is nothing that can be done for their disease. Hence, knowledge about lifestyle choices that could enhance their health could be also psychologically beneficial. The study of monozygotic twins with CADASIL showed that smoking, physical activity and statin treatment could be potential candidates as environmental factors modifying the phenotype of CADASIL. However, as these observations are based on one pair of monozygotic twins only, further studies with larger sample sizes are needed to confirm the true effect of these candidates.

The limited sample size was an inevitable limitation not only in our twin study (II) but also in the study focusing on the genetic modifiers of CADASIL (I). As CADASIL is a fairly rare disease, the size of the patient cohort is restricted, especially when including only one mutation. Because of the limited patient cohort, a hypothesis driven candidate gene approach was preferred to GWAS. The small population size leads to compromised statistical power, which is true for our study as well. We were able see statistical trends in our cohort, suggesting that with a larger study cohort, some associations could be detected. Opherk and co-workers (2014) have also stated that the polygenic risk score suggests that multiple variants with small effect sizes influence the WMH burden in CADASIL. Hence, GWAS with internationally pooled, relatively large patient cohorts could be an effective method for studying modifiers of the CADASIL phenotype.

In addition to studying the genetic modifiers of CADASIL in our mutationally homogenous CADASIL cohort, we also analysed the effect of certain environmental factors such as smoking, statin medication and alcohol consumption. Even though previous studies have shown an association between smoking and earlier age of onset at stroke (Adib-Samii et al. 2010), our results remained statistically insignificant possibly due to the limited size of the cohort.

One of the problems in studying the variability of the CADASIL phenotype arises from the difficulty in diagnosing certain events. Defining the clinical phenotype representing the variability between affected individuals is perhaps the most important step when studying modifier genes. In our study (I), we could see a statistically insignificant trend between *APOE* ε4 and earlier first-ever stroke, but when TIA together with stroke was used as the end-point, this trend disappeared. Our hypothesis is that this could be due to the difficulty of distinguishing TIA from other neurological deficits, such as severe migrainous aura. The same is true for other neurological symptoms as well: even severe TIA and minor stroke may be hard to separate by clinical symptoms only. Hence, imaging findings such as a WM lesion load might serve as a more reliable outcome. However, the imaging methods are expensive and it is not easy to collect a large patient cohort with comparable imaging results available. To conclude, identifying modifier genes is challenging. The contribution of a single variant may be small, and its effect may vary according to other factors, such as environment or ethnicity. Moreover, multiple modifiers may contribute to the phenotype in a cumulative way.

5.2. Swedish hMID

5.2.1. Excluding the CADASIL diagnosis in the Swedish hMID family (III)

Multiple radiologic, molecular and neuropathological methods were used to assess whether Swedish hMID is a phenotypic variant of CADASIL or a new disease entity with a different genetic background.

5.2.1.1. Imaging findings

In T2-weighted MRIs, the most typical findings were mild cortical and central cerebral atrophy with white matter hyperintensities in subcortical regions. Multiple small cystic infarcts in the central GM and WM and pons were common, and several lesions in the basal ganglia and thalamus were often observed (Figure 8). The most profound difference between the MRI findings in Swedish hMID and the typical MRI changes in CADASIL was the absence of white matter hyperintensities in the anterior temporal pole. In addition, apparent changes in the external capsule or corpus callosum were absent in the MRIs of the Swedish hMID patients (Figure 8).

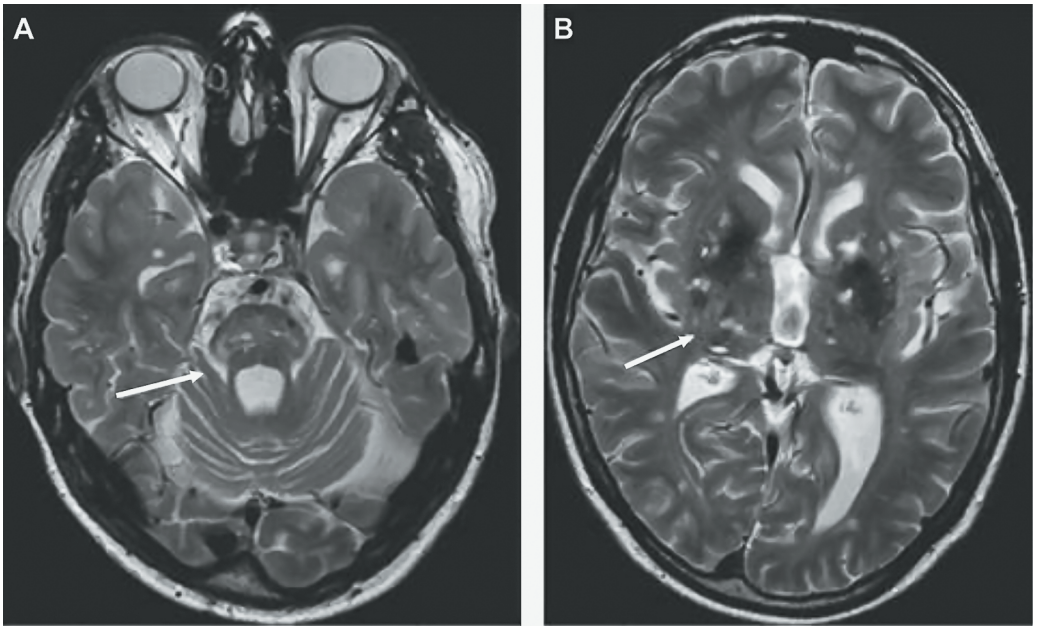

Figure 8 *T2-weighted magnetic resonance images of a 42-year-old male with hereditary multi-infarct dementia of Swedish type. A) Arrow points indicate lesions in the pons, whereas the temporal poles show no signs of lesions. B) Multiple lesions are present in the basal ganglia and thalamus but the capsula externa is intact. (Cf. Figure 2A)*

5.2.1.2. Pathological findings

Pathological examination of brains affected by Swedish hMID showed necrotic lesions in the subcortical structures and the pons. The amount of WM was reduced, and the loss of arterial

smooth muscle cells was prominent in the WM compared to GM. In addition, secondary dilatation of the ventricles was reported. In the close vicinity of infarct sites, many vessels were completely stenosed, and fragmentation of the elastic lamina and perivascular polymorphonuclear leukocytes and macrophages were common. The degree of arterial microvessel sclerosis in the WM of the Swedish hMID patients (sclerotic index SI=0.62) was similar to that in CADASIL (SI=0.64, p=0.243), while in the GM the SI of the Swedish hMID patients (SI=0.54) was significantly less than in CADASIL (SI=0.60, p=0.008).

In the EM examination of a skin biopsy (hMID patient IV:3), smooth muscle cells showed degenerative changes. The contours of the smooth muscle cells were irregular, basement membranes were thickened and the amount of collagen fibres was increased. However, there were no signs of GOM or other granular deposits. GOM has been shown to be present with all stereotypical CADASIL mutations; hence, this finding clearly differentiates Swedish hMID from CADASIL.

The microvascular pathologies of Swedish hMID and CADASIL are compared in Figure 5 of original article III. Immunostaining with α-actin showed no differences between CADASIL and Swedish hMID, and the immunoreactivity to medin, an arterial muscle cell marker, demonstrated disruption of smooth muscle cells in both disorders. Immunostaining for collagens I, II and VI revealed no qualitative differences between these two disease entities; however, immunoreactivity to collagen IV was significantly increased in the frontal GM of CADASIL patients and not in the Swedish hMID patients. The cerebral endothelium was stained with a GLUT1 antiserum, and the results showed abnormalities in the neocortex of the Swedish hMID cases.

As N3ECD accumulation is also reported to be characteristic of CADASIL, an immunohistochemical staining with an N3ECD antibody was carried out. All the tested CADASIL patients showed accumulation of N3ECD, whereas in the Swedish hMID patients no accumulation could be detected (Figure 6 in original article III). To conclude, the microvascular pathologies in these two diseases are somewhat different, and, more importantly, the Swedish hMID cases show no evidence of accumulation of N3ECD on the VSMCs.

To conclude, the pathological findings in Swedish hMID and CADASIL are similar, but not identical. This pathological data suggests that the disease is caused by a microvascular disease affecting arterioles by resulting in thickening their walls, deposition of collagens and obliteration of the lumen. This may lead to compromised CBF and hence cause ischemic lesions in the brain.

5.2.1.3. Genetic screening

The most reliable way to confirm a CADASIL diagnosis is a genetic test showing the actual *NOTCH3* mutation. We therefore sequenced the entire coding region (8091 base pairs) of *NOTCH3* in four affected individuals. The sequencing revealed no CADASIL-causing mutations. Some common *NOTCH3* polymorphisms were found, but they did not segregate with the disease, and a limited reverse transcription -PCR (RT-PCR) analysis showed no differences in the RNA expression. As a result, it is not likely that these common polymorphisms would affect the regulation of *NOTCH3* in this family.

To rule out the possibility of intronic variants or structural variations of *NOTCH3*, we conducted a limited haplotype analysis with three microsatellite markers (Figure 2 in original study III). The haplotype analysis showed that no distinctive haplotype could be attributed to the affected family members only. Further, the D19S923 microsatellite marker located in the *NOTCH3* gene (Figure 1A in original article III) showed allelic differences in the affected family members. As theD19S923 marker located within the *NOTCH3* locus itself, it is highly unlikely that there would have been recombination event that did not affect *NOTCH3*. Hence, these genetic analyses conclude that the Swedish hMID family does not have CADASIL, and their disease is not linked to the same chromosomal region as *NOTCH3*.

5.2.1.4. Swedish hMID is a novel SVD

Even though Swedish hMID clinically resembles CADASIL, there are some differences in the imaging and pathological findings. More importantly, genetic tests proved that Swedish hMID is not associated with the same chromosomal region as CADASIL, and no CADASIL-causing *NOTCH3* mutation could be detected. Therefore, Swedish hMID family suffers from a novel SVD. As the disease resembles CADASIL, NOTCH3 ligands jagged and delta are interesting candidates for the causative gene. However, all reported mutations in jagged cause Alagille syndrome (Gridley 2003), which is clinically different from Swedish hMID.

The precise pathogenesis of Swedish hMID remains unknown. In 1977, Sourander and Wålinder suggested that the pathogenesis of Swedish hMID could be attributed to an autoimmune process or metabolic disturbances. In this study, we showed that the Swedish hMID patients had fewer perivascular cells, which could suggest an immunopathogenic mechanism.

Our study highlights the importance of genetic testing in the field of inherited SVDs. It is not unusual that two disease entities are clinically similar, and even the radiological and pathological examinations may show similar manifestations. SVDs similar to Swedish hMID have been reported in the literature (Tomimoto et al. 2006, Hagel et al. 2004, Santa et al. 2003). As long as the genetic background of these families is unknown, it is impossible to assess whether they present different familial SVDs or the same disease. If the pathogenic gene or mutation is known, genetic testing can easily confirm the diagnosis. The phenotypic variation can also be vast inside a certain disease (I, II), which emphasises the need for genetic testing. Correct and accurate diagnosis is important not only for treatment and genetic counselling but also for the psychological well-being of the patient.

5.2.2. Seeking the pathogenic mutation in the Swedish hMID family

To attempt to locate a pathogenic mutation in the Swedish hMID family, WES was carried out in 6 family members (4 patients and 2 controls). The first exome sequencings (patients IV:5, IV:7, IV:14, IV:16 and V:3) revealed no obvious variants that could cause Swedish hMID. As we had contacted new family members and collected new samples, we decided to widen our search with one new WES (III:1c) and use linkage analysis to narrow down the regions of interest. Previously linkage analysis was not feasible, since we had too few samples for the analysis. As a result of the

parametric multipoint linkage analysis, we could identify four chromosomal regions achieving LOD score >2 (See Table 1, in original article IV). Although the mean age at onset is reported to be 29–38, we cannot definitely exclude the possibility of a later onset, hence all unaffected family members were considered as unknown in the linkage analysis. When the linkage areas were compared to the variants found in the exomes of all patients, but not in the controls, we could identify three variants from the areas covered by linkage peaks (Table 1, in original article IV).

Of the variants found, *SPOCK2* c.*11G>A and *COL4A1* c.4470C>T have such high global minor allele frequencies (Gmaf) that they do not fit with the autosomal dominant inheritance model (Table 1. in original article IV). The third finding was a 3'UTR variant (c*32G>A) in the collagen 4A1 (*COL4A1*) gene located on 13q34 (Emanuel et al. 1986). This 3'UTR variant c.*32G>A in the COL4A1 gene is currently (August 2015) not reported in the public databases 1000 Genomes (1000 Genomes Project Consortium et al. 2012), ExAC (http://exac.broadinstitute.org/) and dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) nor in the 6503 exomes provided by the NHLBI Exome Sequencing Project (ESP, http://evs.gs.washington.edu/EVS/). Further, the COL4 immunoreactivity had previously been shown to be increased in the vessels of Swedish hMID patients (Craggs et al. 2013), which enhanced our interest in this variant.

The presence of *COL4A1* c*32G>A was validated with Sanger sequencing of all available family members. The results showed that *COL4A1* c*32G>A segregates with the disease: All patients carried the variant, and none of the older unaffected cases (age > 40 years) had it. However, a 33-year-old currently unaffected male had the variant. In the Swedish hMID family, the reported mean age at the onset of the disease is 34 years (range 29–38 years of age) (Sourander & Wålinder 1977b), suggesting that this currently unaffected male may still be in the asymptomatic stage of the disease. Furthermore, this young male has suffered from depressive illness and psychosis, which could represent early manifestations of the disease.

Since our finding is in the non-coding area of the genome, its functionality is hard to predict. We used multiple *in silico* programs to study the possible effects of this 3'UTR variant, and both MutationTaster (mutationtaster.org) and Human splicing finder v.2.4.1 (http://www.umd.be/HSF/#) predict the variant to be pathogenic through alterations on the splice site. According to MutationTaster, a new splicing donor site is gained. Due to this new donor site, non-coding material may be present at the transcript, which could lead to a nonfunctional protein. Wild-type collagen IV is an important molecule in the basement membranes of vascular endothelia, VSMCs and several other tissues. Unlike other collagens, collagen IV is a nonfibrillary molecule forming meshworks rather than structured fibres (Poschl et al. 2004). This network is important for cell adhesion, migration and differentiation (Khoshnoodi, Pedchenko & Hudson 2008).

COL4A1 encodes the proα1(IV) chain (Mayne et al. 1984) of functional collagen IV. Two of these proα1(IV) chains pair with a proα2(IV) chain to form a heterotrimeric helix, collagen IV (Mayne et al. 1984). The proα2(IV) chain is coded by another gene, collagen 4A2 (*COL4A2)* (Mayne et al. 1984). The two genes, *COL4A1* and *COL42*, are located on opposite DNA strands, separated by as few as 42 base pairs (Poschl, Pollner & Kuhn 1988, Soininen et al. 1988). The connecting sequence between these genes is suggested to contain a bidirectional promoter that

is common to them both (Poschl, Pollner & Kuhn 1988). As the genes code for the subunits of the same functional molecule and share a promoter, it is understandable that mutations in either gene can result in similar phenotypes.

The first disease that could be associated with mutations in the *COL4A1* gene was autosomal dominant porencephaly (Gould et al. 2005). COL4A1 is an important molecule for the basement membrane stability, and in porencephaly this stability seems to be disrupted (Weng et al. 2012), causing cystic cavities in the brain. Recently, it has been shown that mutations in *COL4A1* cause a systemic disease, but the symptoms differ according to the exact pathogenic mutation (Lemmens et al. 2013). During the last ten years, mutations in *COL4A1* have been reported in a wide variety of diseases including porencephaly, small-vessel disease and haemorrhagic stroke, leukoencephalopathy, hereditary angiopathy with nephropathy, aneurysms and muscle cramp (HANAC) syndrome, and Walker-Warburg syndrome (Meuwissen et al. 2015). Clinically, Swedish hMID is a small vessel disease characterised by multiple small infarcts in the brain. Cerebral hemorrhages have also been described in the family, as one of the patients died of cerebral haemorrhage only six months after the disease onset (Sourander & Wålinder 1977b). Hence, phenotypically Swedish hMID blends into the expanding group of *COL4A1*-related disorders.

The majority of the *COL4A1* mutations cause a substitution of glycine in the evolutionarily conserved triple helical domain of collagen IV (Meuwissen et al. 2015). This domain consists of a repetitive sequence of three amino acids (Khoshnoodi, Pedchenko & Hudson 2008). The first amino acid is always glycine, but the other two can vary (Meuwissen et al. 2015). Usually, the repeats are interrupted with cysteine residues that offer binding sites for other molecules and enhance the flexibility of the collagen IV meshwork (Meuwissen et al. 2015). *In vitro* cellular expression assays have shown that mutations in this conserved domain cause a significant reduction in the ratio of extracellular to intracellular COL4A1, suggesting intracellular accumulation of COL4A1 and/or decreased amount of extracellular COL4A1 (Weng et al. 2012). This results in structural disruptions in basement membranes and may cause weakening of the vessels. Based on these results, the autosomal dominant inheritance model and lack of a phenotype in mice heterozygous for the null alleles of *Col4a1* and *Col4a2* (Poschl et al. 2004), a dominant-negative effect has been suggested. In addition, haploinsufficiency has been recently shown with frameshift and splice site mutations of *COL4A1* (Lemmens et al. 2013). The variant found in this study is located in the 3'UTR and an *in silico* analysis predicts that it causes splice site alterations. It might therefore be possible that *COL4A1* c.*32G>A could cause the disease through haploinsufficiency in the Swedish hMID family. It has been hypothesised that the *COL4A1*-related symptoms may be more severe in patients with a missense mutation resulting in a dominant-negative effect than in patients with splice site mutations leading to haploinsufficiency (Lemmens et al. 2013). This could partially explain why hMID of Swedish type manifests only in adulthood, whereas many other COL4-related disorders manifest early, even prenatally (Meuwissen et al. 2015). Nevertheless, more studies are needed to confirm this hypothesis, especially since Lemmens and others described two families with *COL4A1* haploinsufficiency as having a similar phenotype to patients with missense mutations causing a dominant-negative effect (Lemmens et al. 2013).

To conclude, the *COL4A1* c.*32G>A variant segregates with the disease in this large family suffering from Swedish hMID, and the clinical picture of the disease is similar to other reported collagen-related disorders. Hence, according to linkage analysis combined with WES the *COL4A1* c.*32G>A variant is the best plausible candidate for the pathogenic mutation causing Swedish hMID. That said, there are some limitations in our study. First, the LOD-scores in our study did not reach 3.0. This could be due to phenocopies or incomplete penetrance of the disease. According to the pedigree, incomplete penetrance seems unlikely, but phenocopies can not be ruled out. In addition, we had to treat all the unaffecteds as 'unknown' in the linkage analysis, as some of them were on their thirties and the disease can develop later.

Since we performed WES instead of WGS, we cannot exclude another disease-causing variant in the non-coding areas of genome. If all the samples had been available since the beginning of this project, it would have been sensible to do the linkage analysis first and then plan a targeted resequencing for all the areas of interest. In that way, we could have obtained more information about the non-coding areas, and yet the amount of data would still have been reasonable. In addition, targeted sequencing yields a much higher read depth and coverage than WES or WGS. Exome sequencing can also miss structural alterations and copy number variants, but this was not likely in our study, as the whole genome genotyping showed no signs of large structural variants. Another limitation in our study is the lack of functional studies. The expression of *COL4A1* in blood cells is low, and, as a result, new samples from different tissues are needed to study the expression of *COL4A1* on the RNA level. To conclude, this study revealed an interesting candidate gene in the Swedish hMID family, but further studies are needed to confirm the functional effects of *COL4A1* c.*32G>A.

6. CONCLUSIONS AND FUTURE PROSPECTS

I. The variability in the CADASIL phenotype is wide even in a homogeneous cohort of individuals carrying a single predisposing mutation. Different genetic factors, such as the *APOE* or *AGT* genotype or the intragenic *NOTCH3* polymorhisms can alter the phenotype, but the effects are relatively small and hence difficult to detect. A whole-genome association study could be an effective way to study the genetic factors affecting the phenotype if large enough patient cohorts are available. International studies with pooled patient populations are therefore needed to further clarify the effect of different genetic factors on the CADASIL phenotype.

II. The variability in the CADASIL phenotype is present even in monozygotic twins. In our study, twin B had his first stroke 14 years earlier than twin A. This, and other remarkable differences in the phenotypes, suggests that environmental and life-style factors do alter the clinical course of CADASIL. The most prominent differences in the twins life-style were twin B's smoking and twin A's more intense physical activity and longer use of statins. Hence, these three factors should be studied further in large CADASIL cohorts. Knowledge about the environmental factors affecting the phenotype could help to delay the disease onset. As there is no curative treatment for CADASIL, this would be highly important for the patients.

III. For a long time the Swedish hMID family was thought to have CADASIL. In this study, a CADASIL diagnosis was ruled out based on the sequence analysis of *NOTCH3*, a limited haplotype analysis and an EM study showing no signs of GOM. These results suggest that this disease is a new autosomal dominant SVD with an as yet indefinitely identified pathogenic mutation. Our study highlights the fact that many inherited diseases can mimic each other, and a correct diagnosis can be hard to achieve based solely upon the clinical symptoms.

IV. The pathogenic mutation in Swedish hMID was sought using exome sequencing and a parametric multipoint linkage study, and a potential candidate, *COL4A1* c.*32A>G was found. Swedish hMID fits clinically into the expanding group of *COL4A1*-related disorders, but further studies at the RNA level are needed to assess the functionality of this 3'UTR variant. Our study clearly shows the limitations of exome sequencing. At present, increasing numbers of diseases are reported to be caused by a variant in the non-coding areas of genome, and these variants cannot be detected with exome sequencing. Hence, for further studies regarding autosomal dominant diseases we would suggest linkage analysis paired with targeted re-sequencing or whole genome sequencing

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APPENDIX

Appendix 1: *NOTCH3 gene mutations reported in the literature in association with CADASIL. Modified from Tikka et al. 2009.*

	DNA reported*	Coding sequence†	Exon/ intron	Mutations per exon (%)	Amino acid change	EGF repeat	Reference	Notes
1	205T>G	c.127T>G	**exon 2**	0,51	p.Cys43Gly	1	(1, 2)	
2	G206T	c.128G>T			p.Cys43Phe	1	(3)	
3	TGT>GGT	c.145T>G			p.Cys49Gly	1	(4)	
4	c.145T>C	c.145T>C			p.Cys49Arg	1	(5)	
5	224TGT>TAT	c.146G>A			p.Cys49Tyr	1	(6)	
6	224G>T	c.146G>T			p.Cys49Phe	1	(1)	
7	c.157G>T	c.157G>T			p.Gly53Cys	1	(5)	
8	238C>T	c.160C>T			p.Arg54Cys	1	(7)	
9	257C>G	c.179C>G			p.Ser60Cys	1	(1)	
10	-	c.181C>T			p.Arg61Trp	1	(8)	non-cysteine
11	-	c.193T>G			p.Cys65Gly	1	(9)	
12	272G>C	c.194G>C			p.Cys65Ser	1	(1)	
13	TGC-TAC	c.194G>A			p.Cys65Tyr	1	(10)	
14	c.199T>A	c.199T>A	**exon 3**	10,55	p.Cys67Ser	1	(11)	
15	G>A	c.200G>A			p.Cys67Tyr	1	(12)	
16	TGG>TGT	c.213G>T			p.Trp71Cys	1	(13)	
17	224G>C	c.224G>C			p.Arg75Pro	1	(14)	non-cysteine
18	Tgt 304 Cgt	c.226T>C			p.Cys76Arg	1	(15)	
19	c.226_234del	c.226_234del			p.Cys76_Leu78del	1	(5)	
20	306T>G	c.228T>G			p.Cys76Trp	1	(1)	
21	309_326del	c.231_248del			p.Gln77_Cys82del	1 + 2	(1)	deletion
22	-	c.239A>G			p.Asp80Gly	2	(16)	non-cysteine
23	317-331 del	c.239_253del			p.Asp80_Ser84del	2	(16)	deletion
24	-	c.244T>C			p.Cys82Arg	2	(17)	
25	337T>C	c.259T>C			p.Cys87Arg	2	(1)	
26	338G>A	c.260G>A			p.Cys87Tyr	2	(1)	
27		c. 265G>T			p.Gly89Cys	2	(18)	
28	341del12	c.263_274del			p.Ala88_Gly 91del	2	(19)	non-cysteine, deletion
29	346CGT>TGT	c.268C>T			p.Arg90Cys	2	(6)	
30	c.357insTGC	c.277_279dup			p.Cys93dup	2	(20)	duplication
31	tGc 356 tAc	c.278G>A			p.Cys93Tyr	2	(15)	
32	356TGC>TTC	c.278G>T			p.Cys93Phe	2	(16)	
33	p.Arg103X	c.307C>T			p.Arg103Term	2	(21)	
34		c.316T>C			p.Cys106Arg	2	(22)	
35	396C>G	c.318C>G			p.Cys106Trp	2	(1)	
36	322C>T	c.322T>C			p.Cys108Arg	2	(23)	
37		c.322T>A			p.Cys108Ser	2	(24)	
38	401G>A	c.323G>A			p.Cys108Tyr	2	(1)	
39	TGC>TGG	c.324C>G			p.Cys108Trp	2	(25)	
40	406CGT>TGT	c.328C>T			p.Arg110Cys	2	(6)	
41	A>G in 3′ splice site of exon 4	c.341-2A>G	**intron 3**	0,78	p.Gly114_Pro120del	2	(26)	splice site mutation, deletion
42	c.341-26_24delAAC	c.341-26_24delAAC				2	(27)	splice site mutation, 25 aa insertion including cys
43	c.349T>C	c.349T>C	**exon 4**	31,25	p.Cys117Arg	2	(6)	

	DNA reported*	Coding sequence†	Exon/ intron	Mutations per exon (%)	Amino acid change	EGF repeat	Reference	Notes
44	-	c.350G>T			p.Cys117Phe	2	(28)	
45	G428A	c.350G>A			p.Cys117Tyr	2	(29)	
46		c.350G>C			p.Cys117Ser	2	(30)	
47	TCC>TGC	c.353C>G			p.Ser118Cys	3	(31)	
48	446TGC>TTC	c.368G>T			p.Cys123Phe	3	(16)	
49	446G>A	c.368G>A			p.Cys123Tyr	3	(7)	
50	del TTGTG	c.381_385del			p.Cys128Pro fsX32	2	(32)	frame shift
51	TGT>GGT	c.382T>G			p.Cys128Gly	3	(33)	*de novo*
52	tGt 461 tAt	c.383G>A			p.Cys128Tyr	3	(15)	
53	c.383G>T	c.383G>T			p.Cys128Phe	3	(34)	
54	cGGT>TGT	c.391G>T			p.Gly131Cys	3	(35)	
55	475CGC>TGC	c.397C>T			p.Arg133Cys	3	(6, 36)	homozygous
56		c.401G>A			p.Cys134Tyr	3	(24)	
57	-	c.402C>G			p.Cys134Trp	3	(37)	
58	499CGC>TGC	c.421C>T			p.Arg141Cys	3	(6)	
59	tTc 503 tGc	c.425T>G			p.Phe142Cys	3	(15)	
60	509TGC>TCC	c.431G>C			p.Cys144Ser	3	(16)	
61	509TGC>TAC	c.431G>A			p.Cys144Tyr	3	(16)	
62	g509G>T	c.431G>T			p.Cys144Phe	3	(38)	
63	512C>G	c.434C>G			p.Ser145Cys	3	(1)	
64	514TGC>CGC	c.436T>C			p.Cys146Arg	3	(6)	
65	G437A	c.437G>A			p.Cys146Tyr	3	(39)	
66	523G>T	c.445G>T			p.Gly149Cys	3	(1)	
67	-	c.446G>T			p.Gly149Val	3	(40)	non-cysteine
68	527TAC>TGC	c.449A>G			p.Tyr150Cys	3	(16)	
69	C529G	c.451C>G			p.Gln151Glu	3	(29)	non-cysteine
70	535CGC>TGC	c.457C>T			p.Arg153Cys	3	(6)	
71	537-545	c.459_467del			p.Arg153_Cys155del	3	(16)	deletion
72		c.463T>A			p.Cys155Ser	3	(29)	
73	542G>C	c.464G>C			p.Cys155Ser	3	(1)	
74	c.464G>A	c.464G>A			p.Cys155Tyr	3	(34)	
75	562T>A	c.484T>A			p.Cys162Ser	4	(7)	
76	484T>C	c.484T>C			p.Cys162Arg	4	(41)	
77	-	c.486C>G			p.Cys162Trp	4	[30]	
78	G571T	c.493G>T			p.Gly165Cys	4	(29)	
79	CGC>TGC	c.505C>T			p.Arg169Cys	4	(13)	
80	c.509A>G	c.509A>G			p.His170Cys	4	(23)	
81	A587T	c.509A>T			p.His170Arg	4	(29)	non-cysteine
82	589GGT>TGT	c.511G>T			p.Gly171Cys	4	(6)	
83	C.512del611	c.512_611del					(42)	deletion
84	-	c.520T>C			p.Cys174Arg	4	(43)	
85	c.520T>A	c.520T>A			p.Cys174Ser	4	(11)	
86	599G>T	c.521G>T			p.Cys174Phe	4	(44)	
87	-	c.521G>A			p.Cys174Tyr	4	(28)	
88	617C>G	c.539C>G			p.Ser180Cys	4	(7)	
89	-	c.542T>G			p.Phe181Cys	4	(45)	
90	CGC>TGC	c.544C>T			p.Arg182Cys	4	(13, 46)	*de novo*
91	-	c.547T>C			p.Cys183Arg	4	(28)	
92	625TGC>AGC	c.547T>A			p.Cys183Ser	4	(16)	
93	626G>T	c.548G>T			p.Cys183Phe	4	(1)	
94	631TGT>CGT	c.553T>C			p.Cys185Arg	4	(6)	

	DNA reported*	Coding sequence†	Exon/ intron	Mutations per exon (%)	Amino acid change	EGF repeat	Reference	Notes
95		c.553T>A			p.Cys185Ser	4	(47)	
96	-	c.553T>G			p.Cys185Gly	4	(37)	
97	-	c.554G>A			p.Cys185Tyr	4	(48)	
98	A644G	c.566A>G			p.Tyr189Cys	4	(3)	
99	24 bp del ex4 co-dons 192-200	c.574_597del			p. Pro192_Val200del	4	(49)	deletion
100	Tgt 658 Cgt	c.580T>C			p.Cys194Arg	4	(15)	
101	T658A	c.580T>A			p.Cys194Ser	4	(50)	
102	C194G	c.580T>G			p.Cys194Gly	4	(51)	
103		c.581G>C			p.Cys194Ser	4	(52)	
104	tGt 659 tTt	c.581G>T			p.Cys194Phe	4	(16)	
105	659G>A	c.581G>A			p.Cys194Tyr	4	(7)	
106	c.T601C	c.601T>C			p.Cys201Arg	5	(53)	
107	680G>A	c.602G>A			p.Cys201Tyr	5	(1)	
108	590C>T	c.590C>T			p.Ala202Val	5	(54)	non-cysteine
109	-	c.616T>C			p.Cys206Arg	5	(55)	
110	695G>A	c.617G>A			p.Cys206Tyr	5	(7)	
111	697CGT>TGT	c.619C>T			p.Arg207Cys	5	(56)	
112	712TGC>AGC	c.634T>A			p.Cys212Ser	5	(6)	
113	(c713G>A)	c.635G>A			p.Cys212Tyr	5	(57)	
114		c.636C>G			p.Cys212Trp	5	(30)	
115	716G>A	c.638G>A			p.Arg213Lys	5	(58)	non-cysteine
116	TAC>TGC	c.659A>G			p.Tyr220Cys	5	(25)	
117	742TGT>GGT	c.664T>G			p.Cys222Gly	5	(6)	
118	c.664T>A	c.664T>A			p.Cys222Ser	5	(59)	
119	c.665G>C	c.665G>C			p.Cys222Ser	5	(5)	
120	tGt 743 tAt	c.665G>A			p.Cys222Tyr	5	(60)	
121	749TGT>TAT	c.671G>A			p.Cys224Tyr	5	(6)	
122	-	c.697T>A	**exon 5**	6,64	p.Cys233Ser	5	(37)	
123	T697C	c.697T>C			p.Cys233Arg	5	(52)	
124	776G>A	c.698G>A			p.Cys233Tyr	5	(1)	
125	G777T	c.699T>G			p.Cys233Trp	5	(3)	
126	-	c.709G>A			p.Val237Met	6	(61)	non-cysteine
127	del 45bp	c.714_758del			p.Asp239_Asp253del	6	(62)	deletion
128	797G>C	c.719G>C			p.Cys240Ser	6	(1)	
129	Tgt 811 Cgt	c.733T>C			p.Cys245Arg	6	(1)	
130	-	c.733T>A			p.Cys245Ser	6	(63)	
131	Tgc 829 Cgc	c.751T>C			p.Cys251Arg	6	(15)	
132	T829A	c.751T>A			p.Cys251Ser	6	(3)	
133	TGC>GGC	c.751T>G			p.Cys251Gly	6	(64)	
134	c.752G>A	c.752G>A			p.Cys251Tyr	6	(65)	
135	-	c.745G>A			p.Val252Met	6	(51)	non-cysteine
136	851TAT>TGT	c.773A>G			p.Tyr258Cys	6	(6)	
137	856T>G				p.Cys260Gly	6	(66)	
138	857G>A	c.779G>A			p.Cys260Tyr	6	(1)	
139	TGC>TTC	c.812G>C	**exon 6**	4,30	p.Cys271Phe	6	(67)	
140		c.886G>T			p.Gly296Cys	7	(68)	
141	973A>T	c.895A>T			p.Ser299Cys	7	(69)	
142	1033_1034GC>TG	c.955_956delinsTG			p.Ala319Cys	8	(1)	
143	-	c.946G>A			p.Val322Met	8	(51)	non-cysteine
144	CGC>TGC	c.994C>T			p.Arg332Cys	8	(70)	

	DNA reported*	Coding sequence†	Exon/ intron	Mutations per exon (%)	Amino acid change	EGF repeat	Reference	Notes
145	1082C>G	c.1004C>G			p.Ser335Cys	8	(1)	
146	A1088G	c.1010A>G			p.Tyr337Cys	8	(3)	
147	T1090C	c.1012T>C			p.Cys338Arg	8	(71)	
148	-	c.1013G>T			p.Cys338Phe	8	(51)	
149	c.1019G>A	c.1019G>A			p.Cys340Tyr	8	(72)	
150	c.1057_1071dup	c.1057_1071dup	**exon 7**	4,30	p.Asp353_Ser357	9	(73)	duplication, includes Cys
151	-	c.1078T>C			p.Cys360Arg	9	(74)	
152		c.1096T>C			p.Cys366Arg	9	(75)	
153	TGT->TGG	c.1098T>G			p.Cys366Trp	9	(76)	
154		c.1135T>C			p.Cys379Arg	9	(77)	
155	c.1135T/G	c.1135 T>G			p.Cys379Gly	9	(78)	
156	1214G>C	c.1136G>C			p.Cys379Ser	9	(1)	
157	G1222T	c.1144G>T			p.Gly382Cys	9	(3)	
158	1241TGT>TAT	c.1163G>A			p.Cys388Tyr	9	(79)	
159	1261T>C	c.1183T>C			p.Cys395Arg	10	(1)	
160		c.1187C>G			p.Ser396Cys	10	(24)	
161	p.S414C	c.1241C>G	**exon 8**	7,81	p.Ser414Cys	10	(74)	
162	-	c.1255T>C			p.Cys419Arg	10	(49)	
163	-	c.1258G>T			p.Gly420Cys	10	(37)	
164	1339C>T	c.1261C>T			p.Arg421Cys	10	(1)	
165	C1279T	c.1279C>T			p.Arg427Cys	10	(52)	
166	T1360C	c.1282T>C			p.Cys428Arg	10	(71)	
167	-	c.1283G>C			p.Cys428Ser	10	(37)	
168	1361G>A	c.1283G>A			p.Cys428Tyr	10	(1)	
169	c.1300_1308dup	c.1300_1308dup			p.Glu434_Leu-436dup	11	(11)	duplication
170	T1381C	c.1303T>C			p.Cys435Arg	11	(3)	
171	Tgc 1396 Ggc	c.1318T>G			p.Cys440Gly	11	(15)	
172	1396T>C	c.1318T>C			p.Cys440Arg	11	(1)	
173	T1396A	c.1318T>A			p.Cys440Ser	11	(80)	
174	-	c.1337G>T			p.Cys446Phe	11	(81)	
175	1415G>C	c.1337G>C			p.Cys446Ser	11	(1)	
176	Cgc 1423 Tgc	c.1345C>T			p.Arg449Cys	11	(82)	
177	T1441C	c.1363T>C			p.Cys455Arg	11	(83)	
178	-	c.1364G>A			p.Cys455Tyr	11	(74)	
179	G1370T	c.1370G>T			p.Cys457Ser	11	(52)	
180	A1473G	c.1394A>T	**exon 9**	2,73	p.Tyr465Cys	11	(81)	
181	1397G>A	c.1397G>A			p.Cys466Tyr	11	(84)	
182	-	c.1433G>A			p.Cys478Tyr	12	(85)	
183	c.1450T>G	c.1450T>G			p.Cys484Gly	12	(24)	
184	1529G>A	c.1451G>A			p.Cys484Tyr	12	(1)	
185	1529G>T	c.1451G>T			p.Cys484Phe	12	(2)	
186	1562G>A	c.1484G>A			p.Cys495Tyr	12	(1)	
187	-	c.1510T>C	**exon 10**	2,34	p.Cys504Arg	12	(47)	
188	1609T>C	c.1531T>C			p.Cys511Arg	13	(1)	
189	TGC>TAC	c.1532G>A			p.Cys511Tyr	13	(86)	
190	c.1532G/T	c.1532TG>T			p.Cys511Phe	13	(78)	In article p.Cys511Leu
191	G1660T	c.1582G>T			p.Gly528Cys	13	(71)	
192	TGC>TCC	c.1592G>C			p.Cys531Ser	13	(87)	
193	cCGC-TGC	c.1594C>T			p.Arg532Cys	13	(88)	

	DNA reported*	Coding sequence†	Exon/ intron	Mutations per exon (%)	Amino acid change	EGF repeat	Reference	Notes
194	TGT>TAT	c.1625G>A	**exon 11**	6,25	p.Cys542Tyr	13	(13)	
195	1708CGC>TGC	c.1630C>T			p.Arg544Cys	14	(56)	
196	T1723C	c.1645T>C			p.Cys549Arg	14	(3)	
197	1724G>A	c.1646G>A			p.Cys549Tyr	14	(1)	
198	CGC>TGC	c.1672C>T			p.Arg558Cys	14	(13)	
199	TGT>TAT	c.1703G>A			p.Cys568Tyr	14	(89, 90)	
200	TAC>TGC	c.1721A>G			p.Tyr574Cys	14	(87)	
201	ACA>GCA	c.1729A>G			p.Thr577Ala	14	(90)	non-cysteine
202	CGC>TGC	c.1732C>T			p.Arg578Cys	14	(13)	
203	1735T>C	c.1735T>C			p.Cys579Arg	14	(54)	
204	1759C>T	c.1759C>T			p.Arg587Cys	15	(91)	
205	c.1771T>C	c.1771T>C			p.Cys591Arg	15	(24)	
206	C1774T	c.1774C>T			p.Arg592Cys	15	(52)	
207	c.1790G>C	c.1790G>C			p.Cys597Ser	15	(92)	
208		c.1816T>C			p.Cys606Arg	15	(24)	
209	1897C>T	c.1819C>T			p.Arg607Cys	15	(7)	
210	c.1918C>T	c.1918C>T	**exon 12**	0,39	p.Arg640Cys	16	(34)	
211	c.1999G>T	c.1999G>T	**exon 13**	1,50	p.Gly667Cys	17	(34)	
212	CGC>TGC	c.2038C>T			p.Arg680Cys	17	(93)	
213	2031C/A	c.2053C>A			p.Arg685Thr	17	(94)	non-cysteine
214	c.2129A>G	c.2129A>G			p.Tyr710Cys	17	(95)	
215			**exon 14**	0,78	p.Arg717Cys	18	dbSNP entry	rs144163298
216	2260CGC>TGC	c.2182C>T			p.Arg728Cys	18	(6)	
217	2402G>C	c.2324G>C	**exon 15**	0,78	p.Cys775Ser	20	(2)	
218	-	c.2353C>T			p.Arg785Cys	20	(72)	
219	1279G>T	c.2411-1G>T	**intron 15**	0,39	p.Pro805_Asn856del	20–22	(96)	splice site mutation
220		c.2815T>C	**exon 18**	3,91	p.Cys939Arg	24	(24)	
221	Ggc 2935 Tgc	c.2857G>T			p.Gly953Cys	24	(15)	
222		c.2923G>T			p.Gly975Cys	25	(97)	
223	cTGC>AGC	c.2929T>A			p.Cys977Ser	25	(31)	
224	cAGC-CGC	c.2932A>C			p.Ser978Arg	25	(89)	non-cysteine
225	G2935T	c.2935G>T			p.Ala979Ser	25	(98)	non-cysteine
226	3029T>G	c.2951T>G			p.Phe984Cys	25	(7)	
227	3031CGC>TGC	c.2953C>T			p.Arg985Cys	25	(6)	
228	2963G>A	c.2963G>A			p.Cys988Tyr	25	(91)	
229	gTGC>GGC	c.2989T>G			p.Cys997Gly	25	(35)	
230	-	c.3011A>C	**exon 19**	3,91	p.Cys1004Tyr	26	(99)	
231	3094CGC>GCT	c.3016C>T			p.Arg1006Cys	26	(6)	
232	-	c.3022C>T			p.Pro1008Ser	26	(100)	non-cysteine
233		c.3037G>T			p.Gly1013Cys	26	(24)	
234	3121TGC>CGC	c.3043T>C			p.Cys1015Arg	26	(56)	
235	c.3058G>C	c.3058G>C			p.Ala1020Pro	26	(101)	non-cysteine
236	tAt 3141 tGt	c.3062A>T			p.Tyr1021Cys	26	(60)	
237	c.3065G>T	c.3065G>T			p.Cys1022Phe	26	(102)	
238	c.3084G>T	c.3084G>T			p.Trp1028Cys	26	(103)	
239	3169CGC>TGC	c.3091C>T			p.Arg1031Cys	26	(6)	
240	Ggt 3250 Tgt	c.3172G>T	**exon 20**	2,34	p.Gly1058Cys	27	(15, 37)	
241		c.3182G>A			p.Cys1061Tyr	27	(104)	
242	c.3206A>G	c.3206A>G			p.Tyr1069Cys	27	(11)	
243	-	c.3226C>T			p.Arg1076Cys	27	(81)	
244	c.3292A>T	c.3292A>T			p.Thr1098Ser	28	(5)	non-cysteine

	DNA reported*	Coding sequence†	Exon/ intron	Mutations per exon (%)	Amino acid change	EGF repeat	Reference	Notes
245	TGC>TAC	c.3296G>A			p.Cys1099Tyr	28	(89)	
246	c.3471C>G	c.3471C>G	**exon 21**	0,78	p.Cys1131Trp	29	(105)	
247					p.Arg1143Cys	29	dbSNP entry	rs60373464
248	3769CGT>TGT	c.3691C>T	**exon 22**	0,39	p.Arg1231Cys	31	(6)	
249		c.3750C>G	**exon 23**	1,17	p.Cys1250Trp	32	(106)	
250	TGC>CGC	c.3781T>C			p.Cys1261Arg	32	(13)	
251	3860G>A	c.3782G>A			p.Cys1261Tyr	32	(1)	
252		c.3893C>T	**exon 24**	0,78	p.Cys1298Phe	33	(107)	
253	c.3944G>A	c.3944G>A			p.Cys1315Tyr	33	(108)	
254	c.4544T>C	c.4544T>C	**exon 25**	0,39	p.Leu1515Pro	-	(109)	activating, not CADASIL-related
255	GCT>ACT	c.4822G>A	**exon 26**	0,39	p.Ala1608Thr	-	(13)	non-cysteine
256	c.5284G>A	c.5284G>A	**exon 29**	0,39	p.Val1762Met	-	(110)	rare polymorhisms?

The mutations in NOTCH3 gene that are reported to associate with CADASIL are listed in the table. Mutations not involving cysteine are marked in the "notes" column.

** Description of mutation at the DNA level as reported in original publication (see reference).*

† Mutation description at the DNA level in coding sequence of NOTCH3. Nucleotide number starting from the A of the AGT translation initiating methionine (111).

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ORIGINAL PUBLICATIONS

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I

Clinical Commentary

APOE and *AGT* in the Finnish p.Arg133Cys CADASIL population

Siitonen M, Mykkänen K, Pescini F, Rovio S, Kääriäinen H, Baumann M, Pöyhönen M, Viitanen M. *APOE* and *AGT* in the Finnish p.Arg133Cys CADASIL population.
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Background – CADASIL is an inherited systemic small vessel disease, the affected status of brain vessels leading to subcortical vascular dementia. The defective gene is *NOTCH3* in which over 230 different pathogenic mutations have been identified. The clinical course of CADASIL is highly variable even within families. Previous studies have shown that additional genetic factors modify the phenotype. *Aims and methods* – Altogether, 134 Finnish CADASIL patients with p.Arg133Cys mutation were analysed for possible associations between the apolipoprotein E (*APOE*) genotype, angiotensinogen (*AGT*) p.Met268Thr polymorphism or neutral p.Ala202Ala *NOTCH3* polymorphism and earlier first-ever stroke or migraine. *Results* – We found no association between the *APOE* genotypes, *AGT* polymorphism, *NOTCH3* polymorphism and earlier first-ever stroke or migraine. *Conclusions* – The *APOE*, *AGT* and *NOTCH3* polymorphism did not modify the onset of strokes or migraine in our CADASIL sample, which is one of the largest mutationally homogenous CADASIL populations published to date. International collaboration, pooled analyses and genomewide approaches are warranted to identify the genetic factors that modify the highly variable CADASIL phenotype.

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Key words: genotype–phenotype correlation; founder effect; association study; CADASIL; *NOTCH3*; *APOE*; *AGT*

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Introduction

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is an inherited systemic small vessel disease with characteristic, although variable, clinical features [e.g. transient ischaemic attacks (TIA), strokes, migrainous headache, progressive cognitive decline leading finally to dementia of subcortical vascular type and psychiatric symptoms]. In magnetic resonance imaging (MRI), typical white matter alterations are common (1). The vascular pathology in the central nervous system is characterized by – CADASIL-specific – electron-dense deposits of granular osmiophilic material (GOM) in close vicinity of vascular smooth muscle cells (VSMCs) with subsequent degeneration of the VSMCs and marked fibrosis and stenosis most prominent in the cerebral white matter (WM) arterioles, which together give rise to extensive leukoaraiosis and multiple lacunar infarcts (1). CADASIL is diagnosed by demonstration of GOM in a skin biopsy (2) or by genetic verification of a pathogenic mutation in the *NOTCH3* gene, located on 19p13.1 (1). A vast majority of over 230 mutations responsible

for CADASIL are missense point mutations causing an uneven number of cysteine residues in one of the epidermal growth factor-like repeats in the extracellular domain of the NOTCH3 receptor molecule (2–4).

The age of onset and progression of the disease are highly variable even within the same family. Some genotype–phenotype correlations have been reported (5, 6), and an association between two different mutations and shorter survival was detected in two German families, (7) but many studies have failed to show any association (8, 9). To date, at least 30 *NOTCH3* polymorphisms, 19 neutral and 11 amino acid changing, have been found (4), but only a common p.Arg1560Pro *NOTCH3* variant has been reported to be protective against ischaemic stroke (10), and an association between the neutral p.Ala202Ala (rs1043994) polymorphism and migraine has been reported in the general population (11).

However, heritability estimates in CADASIL suggest a strong modifying influence of genetic factors distinct from the causative *NOTCH3* mutation on the number of ischaemic brain lesions (12). It has also been shown that the polygenic risk score is associated with white matter hyperintensity (WMH) volume, which suggests that multiple common variants with small effect sizes modify the WMH burden in CADASIL (13). As the main pathogenic feature of CADASIL is ischaemic lesions, the genes associated with cardiovascular diseases and stroke such as apolipoprotein E (*APOE)* and angiotensinogen (AGT) are obvious candidates to be involved in the variable outcome of the clinical course of CADASIL. *APOE* ε4 carriers are shown to be at increased risk for atherosclerosis and stroke (14, 15), and *AGT* p.Met268Thr (rs699, previously published as p.Met235Thr) significantly contributes to stroke risk at least in East Asian populations (16, 17). It has also been shown that polymorphisms in the *NOTCH3* gene itself could alter the phenotype of the disease (10, 11). Altogether, over 200 patients with CADASIL have been molecular genetically diagnosed in Finland, and the majority carry the same p.Arg133Cys mutation due to the founder effect (18). Despite the similar mutational background, the patients' clinical pictures are highly variable. Therefore, using this unique material of 134 CADASIL patients with the same p.Arg133Cys mutation, we investigated whether *APOE, AGT*, seven amino acid changing and one neutral p.Ala202Ala *NOTCH3* polymorphisms have an effect on the earlier occurrence of first-ever stroke or migraine.

Patients and methods

Clinical data

Altogether, 134 patients from 24 Finnish families with the same p.Arg133Cys mutation were included in the study. The clinical data were collected retrospectively using medical records and structured questionnaires. The incidence of, and age at incidence of, first-ever TIA and stroke and the occurrence of migrainous headache, myocardial infarction and vascular risk factors such as hypertension (>140/90 mmHg or medication), diabetes mellitus (established diagnosis) and high total serum cholesterol (>6.2 mmol/l or statin therapy) were acquired. Body mass index (BMI) was calculated and >25.0 kg/m^2 was considered overweight. Patients were divided into smokers or non-smokers, and into heavy drinkers (men >280 g of alcohol/week, women >190 g/week), moderate drinkers (men <280 g/week, women <190 g/week) and non-drinkers. The Ethical Committee of the Intermunicipal Hospital District of Southwest Finland approved the study, and informed consent was received from all participants.

Genetic analysis

For the *APOE* genotypes, the genomic DNA was amplified with primers apoE-L: 5′-GGCACGGC TGTCCAAG-3′ and apoE-R: 5′-GCGGATGGC GCTGAGG-3′. After amplification, the genotypes were analysed by direct sequencing of the purified PCR products. The *AGT* p.Met268Thr substitution was analysed as described by Russ et al. (19).

The seven amino acid-changing polymorphisms analysed in this study were as follows: p.His170Arg (rs147373451), p.Gly288Ala (c.941G>C, NM_000 435.2), p.Pro496Leu (rs11670799), p.Ser497Leu (rs114207045), p.His1133Gln (rs112197217), p.Val1183Met (rs10408676) and p.Ala1852Thr (c.5632G>A, NM_000435.2). The polymorphisms were analysed by DHLPC analysis with a Wave™ apparatus (20) for 20 patients and by sequencing for 30 patients. Neutral p.Ala202Ala *NOTCH3* polymorphism was analysed from all 134 patients as described by Schwaag et al. (11).

Statistical analysis

Cox proportional hazards regression was used to analyse the associations between *APOE* genotypes or *AGT* p.Met268Thr polymorphism and the first-ever stroke or TIA. The analyses were

conducted both unadjusted and after adjusting for age, sex, hypertension, hypercholesterolaemia, myocardial infarction, smoking and alcohol drinking. The association between the neutral *NOTCH3* p.Ala202Ala polymorphism and migraine was analysed using logistic regression with sibling as a random effect. All statistical analyses were conducted with SPSS version 19.0 (IBM Corp. Armonk, NY, USA) or SAS version 9.1 (SAS Institute Inc., Cary, NC, USA.)

Results

There were 68 females and 66 males in our cohort, and their mean age was 57 (range 20–94 years), while their mean age at first-ever stroke or TIA was 45. The demographic and clinical features of our CADASIL population are presented in Table 1, and detailed genotypes are given in Table S1.

The *APOE* allele frequencies were ε2 5.4%, ε3 77.5% and ε4 17.1%, which is congruent with the normal population in Finland. We could not show any association between the *APOE* genotypes and first-ever stroke (Table 2), and the results did not change after adjusting for the traditional vascular risk factors listed in Table 1.

The frequencies of the *AGT* p.Met268Thr genotypes were as follows: MM 23.2%, MT 56.6% and TT 20.2%. No association was found between the *AGT* genotypes and first-ever stroke (TT genotype and stroke, HR 0.76 and 95% CI 0.40–1.46).

All of the 50 patients analysed for the seven amino acid changing *NOTCH3* polymorphisms were homozygous for the common allele. Hence, the sequencing was not widened to the rest of the patient population for it was obvious that the frequency of these polymorphisms was too low for any statistical analysis. Interestingly, there was no association between the neutral p.Ala202Ala *NOTCH3* polymorphism and migraine in our CADASIL population (AA vs GG, OR 0.71 95% CI 0.01–40.59; AG vs GG, OR 1.15 95% CI 0.27–4.86).

Table 1 Demographics and clinical features of the Finnish CADASIL cohort

	Patients with the feature/with information
Male sex	66/134 (49.3%)
Stroke	70/116 (60.3%)
TIA	53/102 (52.0%)
Migrainous headache	58/104 (55.8%)
Hypertension	24/120 (20.0%)
Hypercholesterolaemia	47/115 (40.9%)
Myocardial infarction	9/117 (7.7%)
Diabetes mellitus	10/119 (8.4%)
Overweight (BMI>25)	51/91 (56.4%)
Current smokers	30/102 (29.4%)
Alcohol drinking	
Non-drinkers	31/87 (35.6%)
Moderate drinkers	56/87 (64.4%)
Heavy drinkers	6/87 (6.9%)

Table 2 Association between *APOE* ε4 allele and earlier first-ever stroke, TIA or stroke/TIA

	HR	95% CI	*n*
APOE ε4 vs stroke	1.50	0.75–2.99	111
APOE ε4 vs stroke/TIA	1.07	0.50–2.28	111
APOE ε4 vs TIA	0.89	0.49–1.61	94

Values are hazard ratios (HR) with 95% confidence intervals (95% CI) for APOE ε4 carriers from Cox proportional hazards regression analyses.

Discussion

The great advantage of our study is the large number (134) of patients with the same p.Arg133Cys mutation. This homogeneity makes our population exceptional among other large CADASIL populations (3, 4). In addition to the same mutation, our Finnish patients can also be expected to have other similarities in their genetic background due to the founder effect (18) and the relatively small size of the Finnish population (approximately 5.4 million). When compared to other published founder populations (21, 22), our patient cohort is the largest and most profoundly characterized. Together, these facts make our population highly suitable for studying associations between genetic risk factors and their effects among patients with CADASIL. Furthermore, the frequencies of stroke, migrainous headache and vascular risk factors in our population are similar to those found in a large prospective series of patients with CADASIL (8), which increases the credibility of our study.

In our CADASIL population, we could not show any association between the analysed genetic factors and first-ever stroke. This is in line with two previous CADASIL studies in which *APOE* genotype had no modifying effect on the clinical course (8, 23). When TIA together with stroke as the end point were considered, the results of the statistical analyses confirmed this still less. This could be due to the difficulty in distinguishing TIA from other neurological deficits. Hence, we recommend that this difficulty should be considered when using TIA as an outcome in the analyses.

Only few studies have considered *NOTCH3* polymorphisms in the context of cerebrovascular diseases or migraine. One study has reported an association between a *NOTCH3* polymorphism

(p.Ala202Ala) and migraine (11), whereas three other studies have failed to show any association (24–26). Interestingly, the neutral p.Ala202Ala polymorphism does not associate with migraine in our mutation-homogeneous CADASIL population.

CADASIL has a very restricted overall incidence, and the patient cohorts are usually fairly small, especially when focusing on one mutation. This leads to compromised statistical power, which is an inevitable limitation that also applies to our study, as reflected in the large confidence intervals. However, we could see some statistical trends in our cohort, and previous studies have shown that multiple variants with small effects influence the WMH burden in CADASIL (13). Together, these facts suggest that with a reasonably large population size these genetic variants could be found with genomewide approaches. Additional studies in collaboration with other large CADASIL populations are warranted to further clarify the genetic factors modifying the clinical course of CADASIL.

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Conflict of interest and sources of funding

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1. Detailed clinical information about the patients and their *APOE, AGT* p.Met268Thr (rs 699) and *NOTCH3* p.Ala202Ala (rs1043994) genotypes.

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Different Clinical Phenotypes in Monozygotic CADASIL Twins With a Novel *NOTCH3* Mutation

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Background and Purpose—CADASIL is a hereditary arteriopathy causing recurrent strokes and cognitive decline. Because monozygotic twins have identical genetic background, differences in their environment and lifestyle could reveal factors that may influence CADASIL patients' clinical course, which is highly variable even within the same family.

Methods—We describe differences in clinical and imaging findings in a pair of monozygotic CADASIL twins.

Results—Twin B experienced his first-ever stroke 14 years earlier than twin A, and his symptoms, signs, and imaging findings were more severe. Distinguishing factors were twin B's smoking as well as twin A's physical activity and earlier statin treatment. Causative *NOTCH3* mutation was a novel c.752G>A -substitution (p.Cys251Tyr).

Conclusions—The phenotypic differences in these monozygotic twins suggest influence of environmental and lifestyle factors on the clinical course of CADASIL. **(*Stroke*. 2009;40:2215-2218.)**

Key Words: CADASIL ■ *NOTCH3* monozygotic twins

II

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a hereditary, adult-onset, systemic arteriopathy caused by a mutation in *NOTCH3* gene,[1] which codes for NOTCH3 receptor molecule expressed in vascular smooth muscle cells (VSMCs). *NOTCH3* mutations cause degeneration of VSMCs in small arteries with consequent fibrosis and stenosis of penetrating arteries in the cerebral white matter (WM). This results in recurrent subcortical lacunar infarcts and WM degeneration, detectable with MRI, which lead to progressive cognitive decline and finally to vascular dementia. Although 1 of the more than 170 different *NOTCH3* mutations have been associated with earlier onset of CADASIL,[2,3] the reasons for the wide variation—even between patients with the same mutation—in the age of onset, severity of symptoms, rate of progression, and duration of the disease[4] are largely unknown.

Reports on discordance or phenotypic differences between monozygotic twins in Mendelian conditions are rare.[5–7] CADASIL has not been previously reported in monozygotic twins. We describe here the considerable variation in clinical symptoms and imaging findings in a pair of monozygotic CADASIL twins. Genetic analysis of the twins revealed a novel *NOTCH3* mutation.

Case Reports

Family History

Since the age of 70, the twins' mother had episodes of dysarthria and difficulties in concentration and developed memory problems. Diagnosis of vascular dementia was given on the basis of declined cognitive functions and WM hyperintensities as well as multiple small infarcts at the MRI. She died of cancer at the age of 72 years. Her CADASIL diagnosis was afterward verified based on the presence of characteristic histopathology in kidney arteries. The twins' father died at the age of 81. His postmortem neuropathological examination confirmed Alzheimer disease and also excluded CADASIL.

Pregnancy and delivery of the twin brothers were uneventful, but the nature of their placentation is unknown. The birth weights were 2340 g (twin A) and 2150 g (twin B), and height 46.5 cm for both.

Twin A

Twin A was normotensive, with normal blood glucose and homocysteine levels and modest alcohol consumption. He has been on simvastatin for hypercholesterolemia since the age of 43. Twin A has participated actively in sports since youth and

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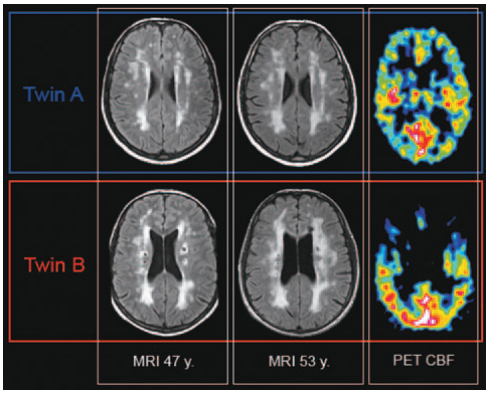

Figure. MRIs and PET scans of cerebral blood flow.

has never smoked. Twin A was asymptomatic at the age of 47, although MRI showed mild changes in WM (Figure and Table). Low-dose aspirin treatment was prescribed. After the age of 49, he experienced episodes of losing consciousness, followed by nausea, abdominal pain, and dizziness. At the age of 50, positron emission tomography (PET) with ^{15}O-labeled water revealed significantly decreased (by 2 SD compared to healthy controls) cerebral blood flow (CBF) only in the pons (Figure). PET with 2-[18F]-fluoro-2-deoxy-D-glucose (FDG) showed regional cerebral metabolic rate of glucose ($rCMR_{gluc}$) within normal variation. At the age of 53, he experienced his first stroke (dysarthria, dysphasia). Antidepressant was prescribed. At this age, MRI showed some progression (Figure) and 11 lacunar infarcts were detected. At the age of 54 and 55 he experienced a stroke. At this time polycythemia (Hb 177 g/L, EVF 55%) was discovered. Venesection was conducted, and treatment with dipyridamol was initiated. His cognitive difficulties increased, he felt excessively exhausted wherefore he was no longer able to work full-time.

Twin B

Twin B was normotensive, his blood glucose and homocysteine levels were within reference values, and his alcohol consumption was light. Statin treatment was begun at the age of 48 after which his cholesterol values stayed within recommended values. He smoked daily from 14 to 38 years of age. He exercised lightly from young adulthood to the age of 54 when he had to stop because of physical exhaustion.

Twin B had the first stroke-like attack at the age of 39 with dysarthria as the main symptom. At the age of 47, several lacunar infarcts were visible in the MRI (Figure and Table). After the second stroke-like attack, at the age of 48, slight dysarthria remained. Dipyridamol treatment was started. Shortly thereafter he had difficulties in fixating the gaze. CADASIL diagnosis was established on the basis of clinical features, MRI findings, and presence of granular osmiophilic material (GOM) in skin biopsy. Further, at the age of 49 and 54, twin B had 2 ischemic attacks and his cognitive and motor functions continued to deteriorate. PET examination at the age of 50 showed significantly decreased CBF (over 2 SD in all cortical areas except for the visual cortex). FDG PET scan disclosed similar but milder changes for the $rCMR_{gluc}$. At the age of 53, MRI showed confluent WM hyperintensities and widened ventricles and perivascular spaces (Figure). At this time he had 16 lacunar infarcts visible on the MRI. Since the age of 53, twin B has been permanently on disability pension.

Genetics

Monozygocity was confirmed with 10 genetic markers (99.9%). Sequencing of *NOTCH3* exons 2 to 24 from both twins revealed a novel mutation, c.752G>A (p.Cys251Tyr), in exon 5. The twins' *apolipoprotein E* (*APOE*) genotype was identified as ε3/ε4.

Discussion

The *NOTCH3* mutation discovered in the twins, c.752G>A, p.Cys251Tyr, has not been previously reported. It replaces 1 cysteine residue in EGF repeat 6. Three other pathogenic mutations (p.Cys251>Arg,[8] p.Cys251>Ser,[9] p.Cys251>Gly[10]) have been described for this same codon.

Twin B experienced his first-ever ischemic attack 14 years earlier than twin A, and he experienced at least 4 attacks before twin A had his first definite attack. Furthermore, all twin B's symptoms, signs, and imaging findings (MRI and PET) were more severe than twin A's.

Genetic differences should not exist between monozygotic twins, but epigenetic alterations (DNA methylation and histone modifications) do increase during their lifetime as a result of nonshared environmental signals such as differences in nutrition, medications, physical and mental activity, use of alcohol, and smoking.[11] These and other environmental effects and lifestyle-related factors may have caused or contributed to the differences in our twins' phenotypes. The role of the environmental factors as a modifyer of CADASIL phenotype is also supported by our observation, that among the Finnish CADASIL patients with a common ancestral p.Arg133Cys mutation[12] the age at first-ever stroke has varied from 28 to 71 years. Even within a single Finnish family variation from 36 to 71 has been recorded. (M. Junna and M. Pöyhönen, unpublished data, 2008). However, variation in this cohort also includes the effects of genetic differences, whereas in the case of identical twins, we can focus exclusively on the environmental factors.

Smoking increases stroke risk in CADASIL patients,[13] and the effect of smoking is accentuated by *APOE* ε4 allele (M. Junna, unpublished data, 2007), both applying to twin B. Furthermore, twin A's longer use of statin[14] could also have contributed to the later onset of symptoms and milder phenotype. In addition, twin A's more intensive physical activity may be another beneficial factor.[15]

A further factor not commonly associated with CADASIL is increased blood viscosity, caused by polycythemia or dehydration, which appears to be harmful (Matti Viitanen, unpublished data, 2008). The former may be avoided by measuring patients' hemoglobin values regularly and the latter by advising the patients and their caregivers.

Table. Clinical Features of the Twins

	Twin A		Twin B	
Age at first ever stroke, years	53		39	
Age at assessment, years	48	55	48	55
No. of stroke-like attacks	0	3	2	4
Cognitive assessment*				
Similarities	−1.33	−2.00	−1.33	−1.33
FAS	−2.08	−1.69	−1.69	−1.92
Category fluency	−0.43	−0.91	−0.43	−2.57
Clock reading	0.50	0.50	0.50	0.50
Clock setting	−1.00	−0.96	−2.00	−1.96
Rey–Osterrieth copy	−1.36	−1.44	−1.76	−2.88
Block	0.33	−1.67	−0.67	−1.67
Block design	0.67	0.67	0.50	−0.33
Digit symbol	0.33	0.33	−0.67	−1.00
TMT A time	0.39	1.09	0.04	0.74
TMT B correct score	0.32	0.32	0.22	−0.20
TMT B time	0.39	1.04	0.22	0.46
Digit span	−0.33	−0.67	−1.00	−0.67
Digit span forwards	0.14	−0.39	−0.39	0.14
Digit span backwards	−0.78	−0.78	−0.78	−0.78
SGRC free recall	0.71	−0.28	−0.97	0.56
SGRC recognition	1.11	0.44	−1.51	0.37
RAVLT total learning	0.12	−0.60	−1.48	−0.63
RAVLT delayed recall	−1.19	−1.83	−0.52	−1.43
R–O immediate reproduction	0.00	−1.59	−0.36	−2.09
Disability (modified Rankin Scale)	0	1	1	2
MRI hyperintensities#	47 years	53 years	47 years	53 years
Deep white matter				
Occipital	1	1	5	5
Temporal	3	3	5	5
Internal capsule	1	3	5	5
Basal ganglia				
Caudate nucleus	0	1	3	3
Globus pallidus	1	1	2	2
Infratentorial				
Cerebellum	0	0	2	3
Mesencephalon	1	1	3	3
Lacunar infarcts	11		16	
Medication	Aspirin+dipyridamol simvastatin, vitamins B6, B12 and folic acid, antidepressant		Aspirin+dipyridamol, atorvastatin, vitamins B6, B12 and folic acid	

*Cognitive performance of the twins are given in z scores (z=0: mean reference value; z=1: 1 SD above mean; z = −1: 1 SD under mean; z=between −1 and 1: normal variation). Verbal functions: Similarities,[16] FAS=letter fluency,[17] Category fluency.[18] Visuospatial functions: Clock reading, Clock setting,[19] Rey–Osterrieth Complex Figure copying,[20] Block, Block Design, Block Design unpaced.[16] Executive functions: Digit Symbol,[16] Trail Making Test (TMT) A time, TMT B correct score and TMT B time.[21] Short-term memory: Digit Span; Digit Span forwards; Digit Span backwards.[16] Verbal episodic memory: Stockholm Geriatric Research Centre (SGRC) free recall and SGRC recognition[22]; Rey Auditory Verbal Learning Test (RAVLT) total learning and RAVLT delayed recall.[21] Visual memory: Rey–Osterrieth (R–O) Complex Figure immediate reproduction.[21]

Disability assessed according to modified Rankin Scale.[23,24]

#MRI hyperintensities are given as Scheltens scores[25]: 0 (no abnormalities), 1 (<3 mm and n≤5), 2 (<3 mm and n>5), 3 (4 to 10 mm and n≤5), 4 (4 to 10 mm and n>5), 5 (>10 mm and n≥1), 6 (confluent).

Because CADASIL is a late-onset disabling disease with no curative treatment, identifying any beneficial lifestyle choices would be advantageous. Our experience suggests that at least smoking and high blood viscosity should be avoided.

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Disclosures

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Hereditary multi-infarct dementia of the Swedish type is a novel disorder different from *NOTCH3* causing CADASIL

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Several hereditary small vessel diseases (SVDs) of the brain have been reported in recent years. In 1977, Sourander and Wålinder described hereditary multi-infarct dementia (MID) in a Swedish family. In the same year, Stevens and colleagues reported chronic familial vascular encephalopathy in an English family bearing a similar phenotype. These disorders have invariably been suggested to be cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy (CADASIL) but their genetic identities remain unknown. We used molecular, radiological and neuropathological methods to characterize these disorders. Direct DNA sequencing unexpectedly confirmed that affected members of the English family carried the R141C mutation in the *NOTCH3* gene diagnostic of CADASIL. However, we did not detect any pathogenic mutations in the entire 8091 bp reading frame of *NOTCH3* or find clear evidence for *NOTCH3* gene linkage in the Swedish DNA. This was consistent with the lack of hyperintense signals in the anterior temporal pole and external capsule in Swedish subjects upon magnetic resonance imaging. We further found no evidence for granular osmiophilic material in skin biopsy or post-mortem brain samples of affected members in the Swedish family. In addition, there was distinct lack of NOTCH3 N-terminal fragments in the cerebral microvasculature of the Swedish hereditary MID subjects compared to the intense accumulation in the English family afflicted with CADASIL. Several differences in arteriosclerotic changes in both the grey and white matter were also noted between the disorders. The sclerotic index values, density of collagen IV immunoreactivity in the microvasculature and number of perivascular macrophages were greater in the English CADASIL samples compared to those from the Swedish brains. Multiple approaches suggest that the Swedish family with hereditary MID suspected to be CADASIL has a different novel disorder with dissimilar pathological features and belongs to the growing number of genetically uncharacterized familial SVDs.

Keywords: CADASIL; genetics; multi-infarct dementia; *NOTCH3*; vascular dementia

Abbreviations: CADASIL = cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy; EM = electron microscopy; MID = multi-infarct dementia; SI = sclerotic index; SVD = small vessel disease

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III

Introduction

Hereditary forms of neurovascular disease are estimated to comprise only a small proportion (<10%) of the burden of disease but they can cause extreme morbidity and mortality. To date several autosomal dominant conditions and at least one recessive disorder presenting with ischaemic or haemorrhagic strokes have been described (Grand *et al.*, 1988; Jen *et al.*, 1997; Terwindt *et al.*, 1998; Hassan and Markus, 2000; Kalaria, 2001; Ophoff *et al.*, 2001; Yanagawa *et al.*, 2002; Hagel *et al.*, 2004; Kalimo and Kalaria, 2005; Verreault *et al.*, 2006). Mutations in several genes involved in cardiovascular function or the circulatory system causing systemic pathologies and strokes are known but the molecular genetics of those disorders directly affecting cerebral vessels, whether endothelial or vascular smooth muscle structure and function remain virtually unclear. It would be important to understand how products of distinct genes cause cerebral infarction and white matter changes leading to cerebrovascular and post-stroke dementia syndromes.

Previous descriptions of cerebrovascular disease with familial traits included a report on a Swedish family suffering from recurrent strokes and cognitive dysfunction. Sourander and Wålinder (1977) had described it as hereditary multi-infarct dementia (MID). Soon after Stevens *et al.* (1977) concisely described chronic familial vascular encephalopathy in an English family. The genetic identities of these disorders have remained unclear. In the intervening years, Tournier-Lasserve *et al.* (1993) described cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy or cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy (CADASIL) in several French families suffering from migraine attacks and recurrent strokes. The clinical phenotype among both French (Chabriat *et al.*, 1995) and German (Dichgans *et al.*, 1998) families indicated CADASIL to be a subcortical small vessel disease (SVD) leading to cognitive impairment and progressive dementia. Mutations in the *NOTCH3* gene were linked to CADASIL (Joutel *et al.*, 1996). A subsequent study (Joutel *et al.*, 1997) reported strong clustering and stereotyped nature of *NOTCH3* mutations in unrelated CADASIL patients. All mutations are restricted to the EGF repeat domain of the extracellular portion of the NOTCH3 cell receptor and particularly concentrated within exons 3–5. In addition to single missense mutations, novel splice site and in-frame deletion mutations (Joutel *et al.*, 2000*b*; Dichgans *et al.*, 2001) have also been detected. Thus, far >100 *NOTCH3* mutations have been recorded (Kalimo *et al.*, 2002; Kalimo and Kalaria, 2005).

The aim of this study was to evaluate whether hereditary MID of the Swedish type (Sourander and Wålinder, 1977) and chronic familial vascular encephalopathy (Stevens *et al.*, 1977) were genetically and pathologically identical and akin to *NOTCH3*-linked CADASIL or novel disorders.

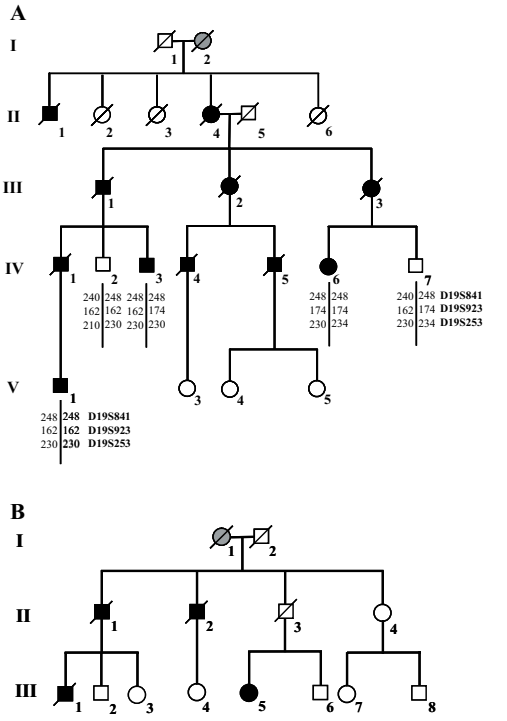

Fig. 1 (**A**) Tree of the Swedish family with hereditary MID. The family tree has been modified to preserve confidentiality. Filled symbols indicate affected individuals either living or since died (stroked symbol). The haplotypes, revealed by alleles amplified with three microsatellite markers, are shown in three affected (IV.3, IV.6 and V.1) and two unaffected (IV.2 and IV.7) members of the family. Dinucleotide microsatellite markers D19S841 and D19S253 flank the 5′ and 3′ ends of the *NOTCH3* locus, whereas D19S923 is localized within the gene locus (Fig. 2). Absence of shared haplotypes in affected and unaffected subjects suggests lack of linkage to the *NOTCH3* locus. (**B**) Tree of the English family with chronic vascular encephalopathy. The family tree structures have been modified to preserve confidentiality. Filled symbols indicate affected individuals either living or since died (stroked symbol). DNA from three affected family members revealed a mutation in exon 4 (R141C) of *NOTCH3* gene indicating the family is afflicted with CADASIL.

Material and methods

Families and subjects

A Swedish family presenting with stroke-like episodes, neuropsychiatric symptoms and progressive dementia was initially diagnosed having hereditary MID (Fig. 1A). The index patient (III.1) first presented in 1956 at the age of 32 years. In addition, seven other affected members (III.2, III.3, IV.1, IV.3, IV.4, IV.5, IV.6 and V.1) underwent clinical examination and evaluation for various diagnostic tests in subsequent years. Six unaffected family members were also interviewed at various times to establish the family tree

Table 1 Demographic details and clinical characteristics of the Swedish MID and English (CADASIL) families

Feature	Swedish family with MID (Sourander and Wålinder, 1977)	English (CADASIL) family (Stevens *et al.*, 1977)
No. of affected members clinically evaluated*	9	4
Gender	6 M, 3 F	3 M, 1 F
Mean age at onset (years)	34.6 ± 3.4	48 ± 3
Onset age range (years)	29–38	39–57
Mean age at death (*n*)	44.3 ± 4.3 (6)†	60 ± 7 (3)
Age at death range (years)	29.5–50	52–65
Duration of disease (years)	11 ± 2.1 (6)†	14.6 ± 4.5 (3)
Range of duration of disease (years)	0.5–15	10–15
Vascular risk factors	Absent	Absent
No. of stroke episodes	>4	>6
Migraine attacks§	—	—
White matter abnormalities	Prominent	Prominent
Bulbar symptoms	+	+
Behavioural symptoms	+	+
Progressive cognitive impairment	+	+

Numbers show mean (±SD) ages and duration in years. (*n*) Indicates mean (±SD) for number of subjects who died and were assessed. Symbols indicate: +, presence; —, absence. *For the Swedish family subjects included III.1, III.2, III.3, IV.1, IV.3, IV.4, IV.5, IV.6 and V.1, and for the English family II.1, II.2, III.1 and III.5 (per Fig. 1). †Includes the normotensive subject who presented at age 29 years and died six months later after a massive intracerebral haemorrhage involving the thalamus and striatum. §Migraine attacks with aura were not noted but three Swedish subjects reported occurrences of unilateral tension headaches.

and clinical phenotype. The principal clinical findings are summarized in Table 1. The age at onset was taken as the first time the individual presented with a stroke-like episode. Autopsies were performed on six affected family members (III.1, III.2, III.3, IV.1, IV.4 and IV.5). Clinical and neuropathological findings in four of these cases were reported previously by Zhang *et al.* (1994). For the current study, blood samples were collected from only six affected and unaffected individuals who gave consent.

An English family described with chronic familial vascular encephalopathy was discovered approximately a decade later (Fig. 1B) in 1967. Several members of the family underwent clinical examination over a 15 year period. The family tree was established after interviews with unaffected family members over three generations. Demographic and clinical features of affected members examined from this family were compared with those of the Swedish family (Table 1). Brains from three affected members of this family who came to autopsy (II.1, II.2 and III.1) were assessed in parallel with the Swedish family (Table 1). Informed consent was obtained from all the subjects following the guidelines for ethical research approved by the Ethics Committees of the University of Göteborg, Sweden, and the Bristol and Gloucestershire Hospitals NHS Trusts.

Neuroimaging

Computerized axial tomography (CT) or magnetic resonance (MR) imaging was performed in affected individuals at the time of clinical evaluation. MR imaging scans from two members of the Swedish family were further evaluated (OA and attending radiologist at Sahlgrenska Hospital) to establish whether changes were characteristic of CADASIL (O'Sullivan *et al.*, 2001).

NOTCH3 gene PCR and DNA sequencing

NOTCH3 gene sequencing was undertaken first to quickly establish whether the disorders were CADASIL (Joutel *et al.*, 1996). Genomic DNA was extracted from whole blood or brain tissue (in some cases) using standard methods. DNA was amplified by the PCR to screen the entire coding region of *NOTCH3* including exon–intron boundaries and splice sites of the 33 exons (Joutel *et al.*, 1996; Joutel *et al.*, 2000*b*; Low *et al.*, 2006). Larger exons such as exon 24 and 33 with high GC contents were divided into smaller fragments to facilitate easier screening. Special attention was paid to exons 3, 4, 5, 8, 11, 18 and 19 including the clustering region of *NOTCH3*. In addition, DNA from at least 50 patients suspected with CADASIL was screened in tandem as an internal control to reduce any false-negatives. The *NOTCH3* DNA and translated protein sequences were obtained from the Genbank database, Accession numbers NM_000435 and AH006054. Sequence data were used to design two different sets of primers for each exon. The PCR and sequencing analyses was carried independently at two sites (Newcastle and Turku) to ensure the findings. Details of primer sequences, expected PCR product size and conditions for annealing temperatures and PCR cycles are available upon request (R. N. Kalaria and H. Kalimo, unpublished data).

The Genius Thermal Cycler with HotStar Taq PCR mastermix kit (Qiagen) was used to screen the exons of *NOTCH3* except for exons 1, 24a, 24b, 33b and 33c in which case the GC2 advantage PCR kit (Clontech) was used in view of the high GC rich content within these exonic regions. At each site all findings were confirmed by either repeating the PCR screening at least twice, including DNA samples from affected and unaffected family members or including analysis of DNA samples from CADASIL subjects with known *NOTCH3* mutations. Entire PCR were run on 1.5% agarose–TBE gel. Amplicons of expected size for each PCR were then extracted and purified with the QIAquick gel extraction kit (Qiagen). DNA concentrations of PCR products measured at $UV_{260/280}$ were adjusted to about 200 ng per 100 bp sequences and sent for direct DNA sequencing (Department of Molecular Biology, Newcastle University and Genetics Department, University of Turku) employing the same forward and reverse primers previously used for PCR. The sequence data were subjected to BLAST search against GenBank database for verification of changes. Initially, the

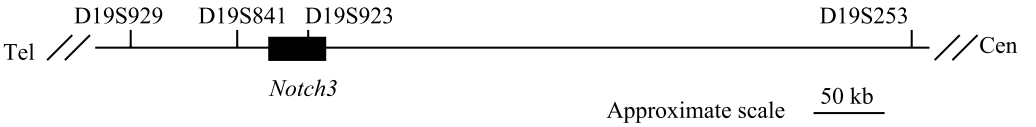

Fig. 2 Genetic and physical map of *NOTCH3* locus region on chromosome 19p13.1 shown as a horizontal line. The location of the *NOTCH3* gene is represented by the black box. Location of the microsatellite dinucleotide markers were as represented as approximate distance to the *NOTCH3* gene. D19S253 is located 418 Kbp downstream of the *NOTCH3* gene nearer to the chromosome centromere (Cen). D19S841 is located 20 Kbp upstream of the gene nearer to the telomere (Tel) end of the chromosome. D19S923 is located 2/3 of the 5′ end of *NOTCH3*. D19S929 and D19S253 were the two microsatellite markers originally used to specify the CADASIL criteria region.

amyloid precursor protein (*APP*) gene was also screened in the Swedish DNA for possible mutations essentially as described previously (Heckmann *et al.*, 2004).

NOTCH3 linkage analysis

Limited haplotype analysis was undertaken as a confirmatory measure to rule out the possibility that the *NOTCH3* gene was associated with the Swedish MID. CADASIL was originally mapped to a 2 cM interval of *NOTCH3* bracketed by D19S199 and D19S226 (Joutel *et al.*, 1996). We therefore selected three microsatellite markers, D19S253, D19S923 and D19S841, located immediately downstream, within and upstream of the gene from the genetic and physical map of the locus region (Fig. 2). The microsatellite markers were amplified by PCR carried out in the Genius Thermal Cycler with HotStar Taq PCR Mastermix kit (Qiagen) according to manufacturer's instructions. Amplified PCR products were directly used for Genescan analysis on the ABI Prism 1800 (ABI Biosystems). The analysis was repeated on three different occasions. Primer sequences for the microsatellite markers were obtained from the ENSEMBL website, and conditions used for PCR (product size in bp, annealing temperature in °C and number of cycles) were as follows: for D19S841 forward and reverse sequences were 5′ TCC TGA GCT CAG GCA AT 3′ and 5′ CCA AGC TTT GGA GAT GTC 3′ (264 bp; 50°C; 45 cycles), for D19S923 they were 5′ CTA GTC ACT GAG TTT GGA CAC TTT C 3′ and 5′ CGG CAG TAA GCC AAG ATT GT 3′ (168 bp; 59°C; 45 cycles), and for D19S253 they were 5′ ATA GAC AGA CAG ACG GAC TG 3′ and 5′ GGG AGT GGA GAT TAC CCC 3′ (228 bp; 55°C; 45 cycles). Forward primers for all three sets of markers were tagged with either Hex or Tet, fluorescent marker.

Autopsy, tissues and neuropathological analyses

To evaluate the cerebrovascular pathologies of the Swedish patients against those of the English family, we obtained brain tissue from three affected subjects of the Swedish family (III.2, III.3 and IV.1) and three subjects of the English family (II.1, II.2 and III.1). The mean (ages at death of these individuals were 60 (±7) and 44 (±4) years old (Table 1). At autopsy, brains were cut in the coronal plane, photographed and examined for gross morphological changes. While some of the findings in the Swedish subjects were reported previously (Sourander and Wålinder, 1977; Zhang *et al.*, 1994) we compared them with those in the English subjects to note apparent differences.

Ten micrometre paraffin embedded tissue sections were cut and stained by conventional histopathological and immunocytochemical methods from the frontal, temporal and parietal lobes, hippocampal formation, basal ganglia, cerebellum and the brainstem. For comparison with the vascular cases we also evaluated in parallel brain tissue sections from a 61-year-old CADASIL (R169C) patient, a 78-year-old male patient with Alzheimer's disease and five age-matched control subjects without any evidence of neurological disease or neuropathological diagnosis. The ages (and gender) of the controls were 43M, 51M, 51F, 56M and 58M years. These specimens were obtained from the Newcastle Brain Tissue Resource centre at the MRC building, Newcastle General Hospital. The length of fixation of the brain tissues from the patients in the two families and controls was between 1 and 5 months.

Tissue sections were stained with haemotoxylin and eosin (H&E), Luxol Fast Blue and the periodic acid Schiff methods. Immunocytochemical staining was performed on 10 μm sections essentially as described previously using the Vectstain in ABC System (Kalaria and Hedera, 1995). We compared the vascular pathology of the two disorders with various polyclonal and monoclonal antibodies to microvascular markers. These were to the following antigens (source): α-actin (Dako, UK), medin (courtesy of Dr P. Naslund, Karolinska Institute, Sweden), NOTCH3 N-terminal peptides upstream (N1) and downstream (N2) to EGF domain, NOTCH3 C-terminal peptide downstream to PEST sequence (Low *et al.*, 2006), collagen I (Dako), collagen III (Chemicon, USA), collagen IV (Sigma, UK), collagen VI (Chemicon) and glucose transporter 1 (GLUT1; Chemicon) (Kalaria and Hedera, 1995). Sections were also immunostained with antibodies to CD68 (Dako), the APP (22C11, Boehringer Mannheim, Germany) and ubiquitin (Dako). In addition to these markers, we checked tissue sections for amyloid β and neurofibrillary (tau) pathology. Specificity of the end-terminal NOTCH3 antibodies and the others has been well-established previously. Upon immunoblotting, the NOTCH3 antibodies recognized the expected band(s) of proteins in solubilized preparations of NOTCH3 cDNA transfected SHSYSY cells (Low *et al.*, 2006).

Electron microscopy on skin biopsy and post-mortem brain tissue

Skin biopsy taken from one affected member of the Swedish family (IV.3) was prepared for examination by electron microscopy (EM) as described previously (Miao *et al.*, 2004). For comparison, sural nerve and post-mortem brain tissue fixed in 0.2% glutaraldehyde and 5% formaldehyde from a CADASIL case with confirmed R169C *NOTCH3* mutation were also similarly examined. In addition, paraffin embedded tissue blocks (2 × 1 × 3 mm) from a brain sample of an affected Swedish subject (III.3) were processed for EM. The blocks were deparaffinized in xylene, hydrated in water

and then fixed in osmium tetroxide for 1 h. Subsequent to fixation, they were washed twice in 0.1 M phosphate buffer, pH 7.4, dehydrated, immersed in propylene oxide and embedded in agar 100 epoxy resin for 2 days at 60°C. One micrometre sections were cut and stained with 1% toluidine blue. Ultrathin sections were cut at 500–1000 Å on a diamond knife from the area of interest, transferred to a copper grid, stained with uranyl acetate–lead citrate and viewed in a Philips 201 transmission electron microscope.

Arteriosclerotic changes, quantitative microvascular pathology and *in vitro* digital imaging

To determine the degree of small vessel arteriopathic changes in the Swedish and CADASIL samples, we compared the sclerotic index (SI) of arterial vessels (Lammie *et al.*, 1997) <300 μm in the grey and white matter from the frontal lobe sections of both diseases (Miao *et al.*, 2004). Briefly, the outer and inner diameters of 60–80 arterial vessels in cross-section were measured from multiple digital images of H&E stained 10 μm tissue sections and substituted in the formula (SI = 1 − internal diameter/outer diameter) to obtain mean SI for the grey and white matter for three cases each of Swedish MID and CADASIL. Where oblong vascular profiles in cross-section were encountered at least two sets of measures across the longest and shortest diameters were taken and averaged to provide one value. SI values below 0.3 were considered as normal as apparent in control subjects without vascular risk (Miao *et al.*, 2004). From the same tissue sections we also quantified the number of perivascular cells in 40–63 microvessels of >100 μm diameter that showed arteriosclerotic changes. Only cells at the abluminal layer (edge of vessel wall) previously verified to be CD68 positive by comparing with adjacent sections were counted. The mean number of perivascular cells in the grey matter and white matter for each of the Swedish and CADASIL groups were determined.

Tissue sections from Swedish and CADASIL cases and controls immunostained with α-actin, collagen IV, GLUT1, NOTCH3 N1 or C2 immunoreactivity were coded and used for quantitative analysis. Digital images of sections stained with the antibody were acquired through a ×10 Zeiss plan objective using Kohler illumination. Images of 10 randomly selected fields of known area within each brain region of interest (grey or white matter) were captured using a JVC KY-F55B three-chip CCD colour video camera. Image capture analysis (Image Pro Plus package, version 4) was used to measure the percentage immunostained area within each field (microvessels including capillaries) which comprised the entire width of the neocortex or equivalent area of white matter as appropriate. A mean value was determined for at least 10 randomly selected fields within each region of interest per section per subject sample. Quantification of the NOTCH3 N1 or C2 immunoreactivity was determined by simply recording the presence or absence of antibody immunoreaction in a total of 100 arterial vessels within the grey and white matter of each case or control. All of the above analyses were performed blind to the diagnosis.

Statistical analysis

Standard statistical methods including non-parametric techniques were appropriate for the data analysis using the SPSS v.13 software (Chicago, IL, USA). One-way analysis of variance (ANOVA) was performed to assess the disease effect and where appropriate using Tukey's test for multiple *post hoc* pair-wise comparisons. Differences between the groups were compared using the Mann–Whitney *U*-test. Mean values of SI, per cent α-actin, collagen IV or GLUT1 immunoreactivities were determined after repeated measures for each case so that a matched analysis could be performed to examine disease differences. Standard deviation (SD) is represented by the ± symbol. Statistical significance was considered at a probability (*P*) value ≤0.05.

Results

Clinical features

Upon screening the DNA from the English family as the first step, we identified the common mutation R141C in exon 4 of *NOTCH3* gene in all three affected members. The disorder in the English family with chronic familial vascular encephalopathy (Stevens *et al.*, 1977) was henceforth designated as CADASIL. Comparison of the demographic and clinical features of the Swedish MID subjects with the CADASIL subjects in the English family revealed some differences (Table 1). The mean ages at onset were 35 and 48 years in the two families. The mean ages at death of subjects who came to autopsy were 44 years for the Swedish and 60 years for the English family members. The duration of disease was ∼11 and 14 years. The mean age at death in the Swedish subjects was likely skewed by one subject who died 6 months after presentation at age 29 years (Table 1). While recurrent strokes were very common among both families migraine episodes were not apparent as described in CADASIL. Only three affected subjects in the Swedish family reported having mild to severe headaches, which did not appear entirely characteristic of CADASIL. They were unilateral, usually non-pulsating but preceded by light phenomena and relieved with aspirin or paracetamol. Interestingly, depressive illness and behaviour symptoms including mood changes were evident in both the families. There was also evidence of moderate to severe cognitive impairment in affected members of both families.

Neuroimaging

To establish if affected members in the Swedish family exhibited similar cerebral changes upon MRI as do CADASIL patients, we examined head MR images of affected members in the Swedish family. Affected members revealed mild cerebral atrophy with white matter hyperintensities in subcortical regions as well as multiple lesions in the basal ganglia and thalamus (Fig. 3). However, upon further scrutiny the images were not entirely consistent with abnormalities characteristic of CADASIL, particularly white matter hyperintensities in the anterior temporal pole (Fig. 3). There were no apparent changes in the external capsule or corpus callosum. Thus, upon MRI there were clear differences in the abnormalities between Swedish MID and typical CADASIL.

NOTCH3 gene screening in the Swedish family

A definitive diagnosis of CADASIL was dependent on sequencing the entire 8091 bp of *NOTCH3* exonic sequences

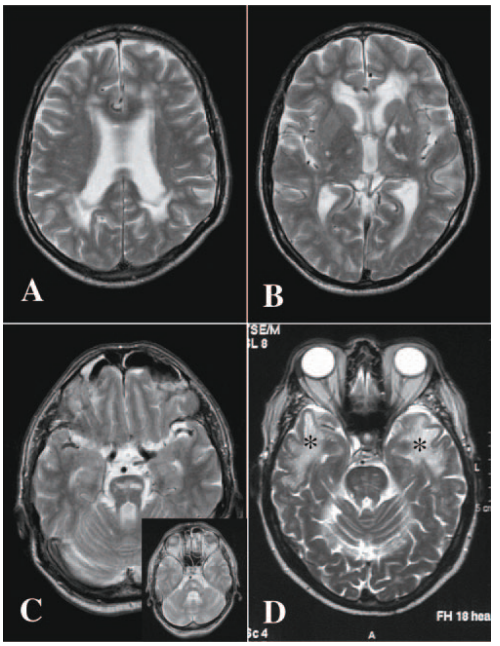

Fig. 3 Axial MR images of an affected subject from the Swedish family with hereditary MID and a CADASIL patient. (**A**) T_2-weighted MR image at the level of basal ganglia showing periventricular hyperintensities in the 'caps' of the ventricles. Infarcts were also evident in left putamen. (**B**) MR image at the level of thalami with more lesions evident in the basal ganglia. (**C**) (and inset), MR images showing lack of hypersignals in the temporal poles and no profound lesions in basal part of brain. There were no clear hypersignals in the temporal horns comparable to those in CADASIL. (**D**) For comparison note high signal in white matter of temporal poles in the 61-year-old CADASIL patient.

rather than limiting to the 23 exons encoding the EGF domains. Extensive and repeated screening of the gene in at least four affected members showed no pathogenic mutations in any of the exons. Sequencing of some PCR amplicons, however, revealed common silent polymorphisms not involving an amino acid change (data not shown). These polymorphisms were found within exons 3, 4 and 16 and were detected in different members of the family as well as other non-related individuals with no specific pattern of change. Therefore, these polymorphisms were a random occurrence. The possibility of false-negative results was reduced by using DNA from other patients suspected to be suffering from CADASIL as additional internal controls. We also ruled out mutations in the *APP* gene by direct sequencing. We found no apparent sequence variation in any of the four affected compared to all the unaffected siblings or cousins in the fourth generation and two disease free individuals from the third generation.

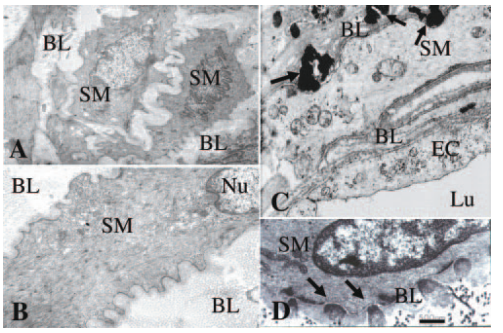

Fig. 4 Lack of granular osmiophilic material (GOM) in skin vessels of an affected Swedish family member. (**A** and **B**) Two views of vascular muscle cells in skin with irregular indentations and without any visible GOM. (**C** and **D**) Typical GOM deposits in brain and sural nerve of a CADASIL patient with R169C mutation. Brain microvessels from another Swedish subject showed no GOM (data not shown). Magnification bar, (**A**) = 900, (**B**) = 400 nm, (**C**) = 500 and (**D**) = 500.

NOTCH3 locus is not associated with the disorder affecting the Swedish family

A full-scale linkage-based analysis could not be undertaken due to availability of limited DNA samples from the family. However, the haplotype analysis with albeit a few samples clearly showed an absence of a shared allele in the D19S923 marker even though there were shared alleles of the two other markers for the three affected members (Fig. 1A). Similarly, no distinctive haplotype could be attributed to only the diseased members of the family as similar haplotypes were also found to occur in the unaffected individuals. The most distinctive clue for non-linkage was from the haplotype of individual IV.6 who is the first cousin of IV.3 and aunt of V.1 where no shared alleles for D19S923 marker were noted between these three affected members. Since the D19S923 marker is located within the *NOTCH3* locus itself, it is highly improbable that there would be any independent cross-over event during independent meiosis that did not affect *NOTCH3*. Taken together, the genetic analysis rejected the possibility of *NOTCH3* causing CADASIL as the primary disorder affecting this family.

Pathological findings

EM of skin biopsy and brain tissue

EM examination of several sections of a skin sample from an affected Swedish family member (IV.3) showed degenerative smooth muscle cells with irregular contours, thickened basement membrane and increased collagen fibres but no evidence for the presence of granular osmiophilic material (GOM) or other granular material in association with the muscle cells (Fig. 4C and D). Similarly, examination of at least 30 vessels in brain tissue from another Swedish subject

(III.3) revealed no GOM deposits. In contrast, we observed distinct GOM deposits juxtaposed to smooth muscle cells of small and medium sized arteries upon EM of peripheral nerve and brain tissue from a CADASIL case with a known R169C mutation (Fig. 4).

Gross examination and histopathology

Cerebral hemispheres of brains from the Swedish family revealed necrotic lesions in the subcortical structures and the pons. The white matter was reduced and there was secondary dilatation of the ventricles. Similar changes with no clear distinction in laterality or size distribution were seen in coronal slices from affected subjects of the English cases with CADASIL. Whereas lacunes, microinfarcts and degrees of perivascular changes in the subcortical structures including the thalamus were evident in both disorders, lesions in the neocortex were rare (data not shown). There was moderate to severe arteriopathy, hyalinization, fibrosis and perivascular necrosis in all the cases of both disorders (Fig. 5). Several vessels in vicinity of infarcts were completely stenosed. Fragmentation of the elastic lamina and perivascular polymorphonuclear leucocytes and macrophages were common in both disorders (Fig. 5A and B). Loss of arterial smooth muscle cells was prominent in the white matter compared to grey in both hereditary MID and the CADASIL cases. We determined the SI values of arterial microvessels (<300 μm) in the three Swedish and CADASIL cases. Mean (±SD) values of SI for the grey and white matter for the Swedish cases were 0.54 (±0.13) and 0.62 (±0.12) ($P = 0.001$, Mann–Whitney *U*-test). Similarly, values for the grey and white matter for the CADASIL cases were 0.60 (±0.13) and 0.64 (±0.14) ($P = 0.09$). Further analysis indicated significant differences between the Swedish and CADASIL cases in the grey matter (0.54 versus 0.60; $P = 0.008$) but not in the white matter in the grey matter (0.62 versus 0.64; $P = 0.243$). Thus, white matter tended to exhibit a higher degree of sclerosis and the grey matter in the Swedish cases showed significantly less sclerosis compared to that in CADASIL.

Immunocytochemical findings

α-Actin immunostaining of small vessels in both disorders showed scattered deposits within vessel walls representing disrupted smooth muscle cells that could not be distinguished readily between the disorders (Fig. 5C and D). The white matter was consistently more affected. This was reflected in the mean per cent α-actin immunoreactivity determined by *in vitro* digital imaging. Also consistent with the SI values, α-actin immunoreactivity was reduced in both the Swedish MID (0.34%) and CADASIL (0.25%) compared to the controls (0.42%, ANOVA, $P = 0.04$). Immunoreactivity to medin, an arterial muscle cell marker, similarly revealed severe disarray and depletion of the wall cell contents (Fig. 5E and F). There were no apparent qualitative differences in immunostaining for collagen I, III or VI between the two disorders although overall there appeared a greater number of stained vessels in the CADASIL cases. Strong collagen IV immunoreactivity was particularly evident in the medial layers of many vessels (Fig. 5G and H). Quantitative immunocytochemical imaging showed that total collagen IV immunoreactivity in the grey matter (frontal) was significantly increased in CADASIL cases

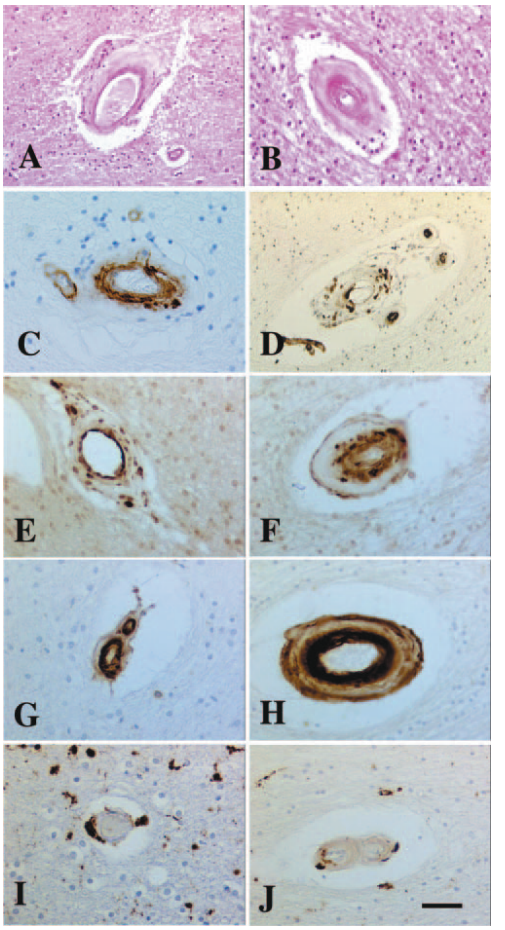

Fig. 5 Comparison of the microvascular pathology of the Swedish hereditary MID (**A**, **C**, **E**, **G** and **I**) and CADASIL (English family) (**B**, **D**, **F**, **H** and **J**). (**A** and **B**), (**H** and **E**) stained vessels showing hyalinization, fibrosis, narrowing of lumen and loss of smooth muscle cells in the Swedish and CADASIL cases. (**C** and **D**), immunostaining with α-actin antibodies demonstrating disruption and loss of smooth muscle cells. (**E** and **F**), staining with medin antibodies demonstrating disruption of smooth muscle cells. (**G** and **H**), anti-collagen IV showing intense reactivity in both cases. (**I** and **J**), distribution of CD68 positive perivascular microglia/macrophages in the two disorders. Magnification bar: (**A** and **D**) = 50 μm; (**B**, **C**, **E**–**J**) = 100 μm.

compared to the Swedish MID cases and controls. The per cent collagen IV immunostaining in CADASIL was 5.26 compared to 3.17 in Swedish and 3.73 in controls (ANOVA, $P = 0.01$ and *post hoc* Tukey's, $P = 0.03$). These basement membrane changes between the CADASIL and Swedish MID cases were not age (at death) dependent as there were no differences in collagen IV immunoreactivities between the Swedish cases and controls.

Sections stained for the cerebral endothelium with GLUT1 antiserum showed abnormalities in form of 'blebs' in the neocortex of Swedish cases but this was not readily evident in the CADASIL cases. Quantitative immunocytochemistry indicated total GLUT1 immunoreactivity to be significantly increased in the Swedish cases compared to controls (2.5% versus 1.3%, $P = 0.017$). This observation was also supported by increased GLUT1: collagen IV ratios (basic elements of the microvasculature) in the Swedish samples (0.79%) compared to controls (0.37%; $P = 0.04$) and to a lesser degree in the CADASIL cases (0.39%; $P = 0.08$). We also undertook quantification of perivascular cells (mostly CD68 +ve microglia) in the grey and white matter of the two groups (Fig. 5I and J). While the mean number (±SD) of perivascular cells was not different between the grey and white matter in either the Swedish or CADASIL cases we noted significantly increased perivascular cells in both white (6.4 ± 3.7 versus 4.4 ± 4.1; $P = 0.001$) and grey (7.7 ± 6.3 versus 3.2 ± 3.5; $P < 0.001$; Mann–Whitney *U*-test) matter in CADASIL compared to the Swedish samples. In addition, we noted diffuse ubiquitin immunoreactivity within vessel walls, especially in the absence of smooth muscle, to be comparable in both disorders (data not shown). None of the cases exhibited amyloid β plaques or neurofibrillary tangles which are diagnostic of Alzheimer's disease.

NOTCH3 immunolocalization

In addition to GOM accumulation, NOTCH3 ectodomain deposition in the microvasculature was previously reported as a distinct characteristic (96% sensitivity and 100% specificity) of CADASIL (Joutel *et al.*, 2001). Immunocytochemical staining of brain sections with antibodies to NOTCH3 ectodomain showed intense accumulation of the NOTCH3 N-terminal fragment within walls of the microvasculature in both grey and white matter of CADASIL cases from the English family but virtually absent in the Swedish samples (Fig. 6). N1 antibodies immunostained 81 (±1)% of the microvessels in the CADASIL cases but there were no vessels with distinct staining in any of the Swedish samples. N2 antibody also showed lack of staining in Swedish MID but was similar to that of N1 in CADASIL cases (data not shown). Specificity of the lack of immunostaining in Swedish cases was further demonstrated by the presence of similar levels of C2 immunostaining in the controls, CADASIL and Swedish cases (82, 87 and 80%). Double-immunostained sections with NOTCH3 N1 and α-actin antibodies similarly revealed disrupted smooth muscle cells but no NOTCH3 ectodomain reactivity in the Swedish cases (Fig. 6E). Brain sections from age-matched controls and the Alzheimer's disease patient, the different CADASIL subject served as negative and positive controls (data not shown). Thus, in addition to the subtle differences in the microvascular pathology there was distinct lack of immunoreactivity to the NOTCH3 N-terminal specific antibodies in the Swedish samples.

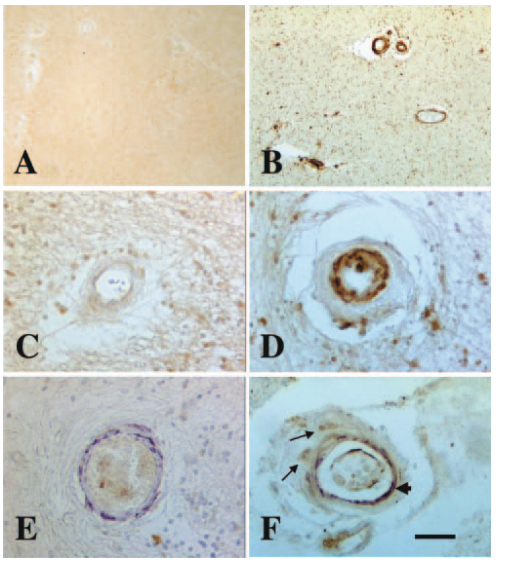

Fig. 6 Immunolocalization of NOTCH3 protein in cerebral vessels in the Swedish family with hereditary MID (**A**, **C** and **E**) and English family with CADASIL (**B**, **D** and **F**) originally labelled as chronic familial vascular encephalopathy. (**A** and **C**), lack of NOTCH3 N-terminal immunoreactivity in cerebral vessels in the Swedish family compared to positive (**B** and **D**) immunoreactivity in the English family. (**E**) Lack of NOTCH3 N-terminal immunoreactivity in cerebral vessel wall also double immunostained for α-actin (purple). (**F**) NOTCH3 N-terminal (brown, arrows) and α-actin (purple, arrowhead) in affected Swedish and English family members. Magnification bar, (**A** and **B**) = 250 μm; (**C**–**F**) = 100 μm.

Discussion

We suggest that despite previous suspicions the original Swedish family described by the eponym hereditary MID (Sourander and Wålinder, 1977) is not *NOTCH3* gene causing CADASIL. While several of the clinical, radiological and pathological features in the brain closely resemble CADASIL (Zhang *et al.*, 1994) and those of the first English family (Stevens *et al.*, 1977), our evidence including absence of temporal pole hypersignals on MRI (O'Sullivan *et al.*, 2001) and most pertinently genetic evidence indicates the Swedish disorder is a novel SVD. We failed to detect a single

mutation within the entire exon coding region of *NOTCH3* in affected members of the Swedish family. Although the possibility of a large frame deletion mutation could not be excluded based on the PCR screening method used, any possible splice site missense mutations or any novel missense mutation in other domain of the receptor would still have been detected. However, linkage analysis using three affected and two non-affected members of the Swedish family showed absence of shared allele or haplotype within the three markers of the *NOTCH3* locus. This indicated that the *NOTCH3* gene locus is not linked to the disorder affecting this Swedish family.

Whether a change in the regulatory elements of *NOTCH3* gene is responsible for MID in the Swedish family is worthy of consideration. There are no reports on polymorphisms or mutations affecting the regulation of NOTCH3 expression but the limited RT–PCR in the Swedish MID samples indicated that at least the RNA expression level was not different between affected and non-affected family members. While we cannot entirely exclude *NOTCH3* allelic variation based on our genetic data the clinical, radiological and pathological findings strongly imply otherwise.

It is further possible that the Swedish MID is caused by mutations in the receptor ligands JAGGED and DELTA (Gridley, 2003). While there are no known mutations or polymorphisms in *JAGGED* or *DELTA* that resemble the phenotype described, several reports indicate that all mutations in *JAGGED* result in the Alagille syndrome. The syndrome is characterized by developmental abnormalities of the liver, heart, eye, skeleton and patients typically present with neonatal jaundice and cholestasis, which results from a paucity of intrahepatic bile ducts (Gridley, 2003). There is a remote possibility that the *DELTA* gene is responsible for the Swedish MID.

Until the DNA from the Swedish patients could be exhaustively screened using current genetic analysis, it was readily plausible that based on the clinical phenotype the Swedish cases were suspected to be CADASIL. CADASIL patients express a varied clinical phenotype (Joutel *et al.*, 1997; Dichgans *et al.*, 1998) and several similarities in neuropathological features such that a misdiagnosis may occur. Phenotypic variation in CADASIL has previously been mistaken for several disorders including multiple sclerosis, cerebral vasculitis, viral encephalitis, Binswanger's disease, leucoencephalopathy of undetermined cause and Alzheimer's disease. This study also emphasizes the import of genetic screening for diagnosis of suspected CADASIL cases (Peters *et al.*, 2005) and successful management of the patient.

Our pathological findings indicated distinct absence of GOM in the skin and the cerebral microvasculature, and NOTCH3 N-terminal peptide accumulation in cerebral microvessels of affected Swedish subjects. While the absence of GOM deposits in itself may not be used for the definitive diagnosis of CADASIL (Ruchoux *et al.*, 1994, 1995) it was previously suggested that accumulation of NOTCH3 N-terminal fragments within the vessels wall is highly specific for CADASIL (Joutel *et al.*, 2000*a*, 2001). It is possible we may have missed blood vessels containing GOM in the Swedish skin specimen but unlikely that the abundant microvessels in brain tissue examined in parallel were missed if they exhibited GOM. On the other hand, cases examined in parallel from the English family described with chronic familial vascular encephalopathy (Stevens *et al.*, 1977) were consistent with the diagnosis of CADASIL in every manner including the relatively common mutation in exon 4 of *NOTCH3*.

The precise pathogenesis of SVD in either CADASIL or the Swedish cases remains largely unknown. The loss of arterial smooth muscle cells in CADASIL linked to mutations in *NOTCH3* has been proposed to be due to a gain of toxic function (Donahue and Kosik, 2004). Whether gain of toxic function also occurs in hereditary MID of the Swedish type is unclear. However, it is conceivable that the onset of the smooth muscle cell degeneration in the Swedish disorder occurs downstream of the effect of *NOTCH3* receptor signal activation (Low *et al.*, 2006). This would be consistent with the absence of mutations and lack of ectodomain accumulation in the vasculature as is characteristic of CADASIL. Although severe arteriopathy was manifest in both diseases we observed subtle differences in the composition of the microvascular components including vascular smooth muscle, endothelium, basement membrane and perivascular infiltrates in the two disorders. The increased SI values and collagen IV immunoreactivity in CADASIL compared to the Swedish disorder suggests different mechanisms may be occurring in the breakdown/remodelling of the basement membrane that could be attributed to reduced activity of the matrix metalloproteinases (Liu and Rosenberg, 2005) or increased endothelial secretion of the collagens. Sourander and Wålinder (1977) had originally suggested that the pathogenesis of hereditary MID could be attributed to either an autoimmune process or metabolic disturbance of the vascular connective tissues. We previously reported that an immune response, implicated by complement factor B reactivity in the CSF (Unlu *et al.*, 2000), was involved in CADASIL. This is supported by the finding of complement 3 (or factor B) reactivity in the brain microvasculature of CADASIL patients but not those with the Swedish disorder (W. C. Low and R. N. Kalaria, unpublished data). That different immunopathogenic mechanism appear to be involved in CADASIL and Swedish hereditary MID is also suggested by the fewer perivascular cells in the Swedish disorder.

CADASIL is clearly the most common form of familial SVD. Other hereditary SVDs have been identified including hereditary endotheliopathy retinopathy nephropathy and stroke or HERNS (Jen *et al.*, 1997), cerebroretinal vasculopathy (Ophoff *et al.*, 2001), hereditary vascular retinopathy (Terwindt *et al.*, 1998; Ophoff *et al.*, 2001), cerebral autosomal recessive arteriopathy with subcortical infarcts and leucoencephalopathy or CARASIL (Yanagawa *et al.*, 2002), hereditary infantile hemiparesis, retinal arteriolar

tortuosity and leucoencephalopathy (Vahedi *et al.*, 2003). However, the clinical features in the Swedish family are not consistent with any of the characteristics of these disorders suggesting the hereditary MID is another autosomal dominantly inherited SVD. Recessive transmission, retinal abnormalities or infantile hemiparesis were not described in any of our Swedish family members. More recently, Chabriat and colleagues (Verreault *et al.*, 2006) have reported a novel autosomal dominant with incomplete penetrant SVD of the brain characterized by motor hemiplegia, memory deficits, executive dysfunction and white matter lesions upon MRI in the general absence of vascular risk factors. It is premature to suggest that this SVD is the same as the Swedish disorder we describe.

In summary, collective evidence bearing particularly on genetic and pathological features suggests hereditary MID of the Swedish type is not CADASIL in contrast to the disorder in the English family fittingly described as chronic familial vascular encephalopathy. We propose that it is a novel form of hereditary SVD leading to vascular dementia. Another gene appears responsible for the disorder that displays certain phenotypic features and disease manifestation resembling *NOTCH3* causing CADASIL. Interestingly, these may belong to the group of several SVDs recently reported in European and Japanese families (Santa *et al.*, 2003; Hagel *et al.*, 2004; Tomimoto *et al.*, 2006) (R. N. Kalaria, W. C. Low and T. Mizuno, unpublished data). Until further genetic identity of the original hereditary MID (Sourander and Wålinder, 1977) is revealed we suggest this eponym describes another distinct SVD of the brain.

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Seeking the pathogenic mutation in a family with hereditary multi-infarct dementia of Swedish type.

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IV

Running title: Seeking the pathogenic mutation in a Swedish hMID family

Abstract

The Swedish multi-infarct family reported in 1977 by Sourander and Wålinder was for long believed to represent the first report of Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencelopathy (CADASIL). However, in 2007, sequencing of the coding area of the *NOTCH3* gene, partial haplotype analysis and electron microscopic studies confirmed that the family suffers from another form of dominantly inherited progressive small vessel disease.

To search for the pathogenic gene in the family, we used a whole-exome sequencing approach coupled with a parametric multipoint linkage analysis. As a result we found three variants located in the areas representing LOD score >2. Two of these variants were excluded since their global minor allele frequency was too high for an autosomal dominant trait. The remaining variant was a 3'UTR variant (c.*32G>A) on the collagen4A1 (*COL4A1*) gene. *In silico* analyses predicted the variant to cause a splice site alteration. The previously reported splice site variants in *COL4A1* are suggested to cause a less severe phenotype than missense mutations. The phenotype of the Swedish hMID family shares similarities with other published COL4-related disorders. Hence, the *COL4A1* variant is the best plausible candidate gene behind the disease. Nevertheless, further studies are needed to assess the functionality of this variant.

Introduction

In 1977, Sourander and Wålinder described a family with cerebrovascular disease manifesting with stroke-like episodes, neuropsychiatric symptoms and progressive dementia. The disease was described to be fully penetrant in three generations with almost 1:1 gender ratio suggesting an autosomal dominant inheritance pattern (Sourander, Wålinder 1977). For almost 30 years, the family was thought to present a phenotypic variant of the most common form of inherited vascular dementia: cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) (Zhang et al. 1994). However, in 2007 it was shown that the family does not have CADASIL-causing mutation in the *NOTCH3* gene, the disease is not linked to same chromosomal region as CADASIL and lastly, granular osmiophilic material (GOM) was not detected in the patients skin arterioles

(Low et al. 2007). Hence, it was concluded that the family does not have CADASIL, but suffers from a novel small vessel disease: hereditary multi-infarct dementia (hMID) of the Swedish type.

In the pathologic examination Swedish hMID is characterized by multiple necrotic lesions seen in the subcortical structures and the pons (Low et al. 2007, Zhang et al. 1994). The amounts of white matter (WM) (Sourander, Wålinder 1977) as well as the number of arterial smooth muscle cells in the WM (Low et al. 2007) have shown to be reduced. Several vessels located near the infarcts were shown to be completely stenosed and the lamina elastica was fragmented (Low et al. 2007). Together these findings suggest that Swedish hMID is caused by microvascular disease that disrupts arteries by thickening their wall and obliterates the lumen. Due to the defects in arteries, the cerebral blood flow is reduced, which causes lesions in the brain.

Materials and methods

Altogether 21 members of the Swedish hMID family participated in this study. Ten of them were affected, ten unaffected and one participant was unrelated spouse used as a control (Figure 1)

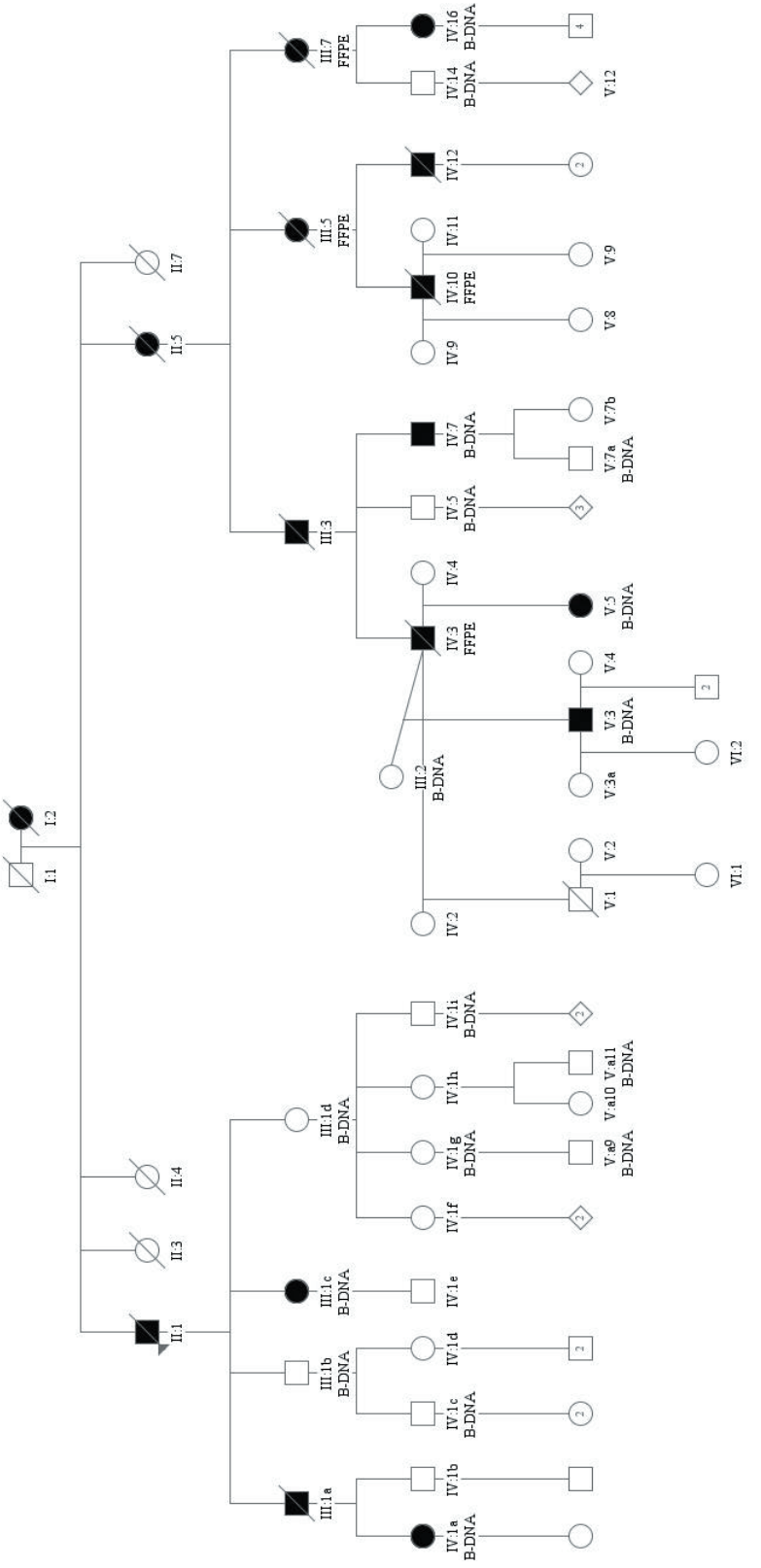

Figure 1: *Pedigree of the Swedish hMID family. The family members included in the study are marked with B-DNA (blood derived DNA) or FFPE (formalin fixed paraffin embedded tissue) according to the sample type available.*

Genetic Analyses

Blood and saliva samples were collected and DNA was extracted from peripheral blood lymphocytes of 17 individuals and additionally from saliva of 8 individuals (III:1b, III:1c, III:1d, IV:1c, IV:1g, IV:1i, V:a9, V:a11). For four patients only formalin fixed paraffin embedded (FFPE) tissues were available.

Four patients (III:1c, IV:7, IV:16 and V:3) and 2 controls (IV:5 and IV:14) were selected for the whole-exome sequencing (WES). The sequencings were done in the Institute of Molecular Medicine in Finland (FIMM, Helsinki, Finland) (IV:5, IV:7, IV:14, IV:16,V:3), in the National Institutes of Health (NIH, Bethesda, Maryland, USA) (IV:16, V:3) and in the University College London (UCL, London, UK) (III:1c). Exomes were prepared using the SeqCap EZ Human Exome Library version 2.0 (Roche Nimblegen Inc., Basel, Switzerland) and sequencing run was performed on the HiSeq 2000 (Illumina, San Diego, CA, USA). The reads were aligned to GRCh37/hg19 and SIFT (Kumar, Henikoff & Ng 2009) and PolyPhen (Adzhubei et al. 2010) were used to predict the functional effects of coding non-synonymous variants. Data analysis was based on an autosomal dominant mode of inheritance and the hypothesis that the underlying mutation was not present in neurologically healthy control individuals. Also the variants that are common in the general population were ruled out. Variants not shared between all affected individuals in the family and variants that were non-autosomal, homozygous or synonymous were excluded. For the linkage study, whole genome microarray was performed for all except the FFPE-samples using HumanOmniExpress Bead chip (Illumina). Parametric multipoint linkage analysis was performed using Allegro (Gudbjartsson et al. 2000) with a fully penetrant autosomal dominant model. Validation of variants found with exome sequencing was done using Sanger sequencing with the BigDye Terminator version 3.1 chemistry (Applied Biosystems, Foster City, CA, USA).

Results

Parametric multipoint linkage analysis identified four peaks on chromosomes 10, 11, 12 and 13 achieving LOD scores >2 (Table 1). When these regions were compared to the data from WES we were able to identify three variants (Table 1). For two of the variants (*SPOCK2*

c.*11G>A and *COL4A1* c.4470C>T) global minor allele frequencies (Gmaf) in the Exome Aggregation Consortium (ExAC) data set were so high, that it is unlikely that they would cause a dominantly inherited rare disease. Hence, the most interesting finding was a 3'UTR variant c.*32G>A in *COL4A1* gene. The segregation of *COL4A1* c*32G>A with the disease was analysed with Sanger sequencing. The results confirmed, that all known affected cases had the variant and none of the older unaffected cases (age > 40 years) carried it. However, one young (33 years of age), currently unaffected, family member had the *COL4A1* c.*32G>A.

Table 1: *Linkage regions with LOD >2 and the variants located in these chromosomal areas*.

Chromosome	Chromosomal location	dbSNP accession numbers	LOD-score	Variants in the region	Gmaf
10	7349891-76372030	rs10823837-rs4746209	2,352	SPOCK2 c.*11G>A	0,379
11	47929846-49000550	rs6485795-rs11040198	2,294		
12	85165879-87281210	rs11116595-rs7316774	2,348		
13	109327788-111067000	rs9284246-rs10851243	2,407	COL4A1 c.*32G>A COL4A1 n.249C>T, c.4470C>T	 0,376

As our finding is a 3' prime UTR variant, we assessed its functionality *in silico.* Both MutationTaster (mutationtaster.org) and Human splicing finder v.2.4.1 (http://www.umd.be/HSF/#) predict the variant to be pathogenic through altering the splicing. At present (May 2015) *COL4A1* c.*32G>A is not reported in the public databases 1000 Genomes (1000 Genomes Project Consortium et al. 2012) and dbSNP, nor in up to 6503 exomes provided by NHLBI Exome Sequencing Project (ESP) (Exome Variant Server, Seattle, WA). However, most of the UTR-variants are not included in these databases that are concentrated on exonic variants.

Discussion

The *COL4A1* gene locates on the chromosome 13q34 (Emanuel et al. 1986) and encodes the proα1(IV) chain (Mayne et al. 1984), which is one of the six collagen type IV α-chains. Two of these proα1(IV) subunits form a heterotrimeric helix together with a proα2(IV) chain coded by *COL4A2* gene (Mayne et al. 1984). Unlike collagens I-III, type IV does form a meshwork rather than ordered fibrillary structure (Khoshnoodi, Pedchenko & Hudson 2008). This nonfibrillary collagen is a main constituent of the basement membranes of many tissues, among them vascular endothelia. The network formed by collagen IV is an important part of the inter- and intramolecular interactions influencing cell adhesion, migration and differentiation (Khoshnoodi, Pedchenko & Hudson 2008).

In 2005, animal models let to the discovery of *COL4A1* mutations behind the autosomal dominant porencephaly (Gould et al. 2005). Later on, it has become clear that mutations in this gene cause systemic disease but the phenotype has great variation depending on the exact mutation (Lemmens et al. 2013). To date, *COL4A1* mutations are reported in a wide variety of autosomal dominant diseases, including porencephaly, small-vessel disease and haemorrhagic stroke, leukoencephalopathy, hereditary angiopathy with nephropathy, aneurysms and muscle cramp (HANAC) syndrome, and Walker-Warburg syndrome (Meuwissen et al. 2015). Swedish hMID is a small vessel disease characterized by multiple infarctions affecting the cerebral blood flow, so clinically it fits to the expanding group of *COL4A1* related disorders. Also, one of the Swedish hMID family members died at the age of 29 after a massive cerebral hemorrhage (Sourander, Wålinder 1977) thus representing yet another symptom associated with *COL4A1* mutations.

The exact mechanism by which COL4A1 causes ischemic infarction and white matter disease remains unsolved (Lemmens et al. 2013). One hypothesis is that the mutations in the *COL4A1* could cause structural problems in the basement membrane resulting in thickening of the vessel wall. This could lead to the destruction of small penetrating vessels, which can then present as multiple lacunar infarctions and progressive white matter disease. In the Swedish hMID family, the pathological examinations showed that the disease is caused by microvascular disease affecting arterioles by thickening their wall and obliteration of the lumen (Zhang et al. 1994, Zhang 1997, Low et al. 2007). Hence, the pathogenesis of Swedish hMID is suggested to be similar to that in COL4 related cerebral small vessel disease.

At present most of the reported *COL4A1* mutations cause a substitution of glycine at the triple amino acid repeat sequence (Gly-Xaa-Yaa, where Xaa and Yaa = any residue) (Meuwissen et al. 2015) in the evolutionary conserved triple helical domain of the COL4 protein (Khoshnoodi, Pedchenko & Hudson 2008). The repeated sequence is interrupted with several cysteine residues, which give flexibility to the collagen IV meshwork and provide binding sites for other molecules (Meuwissen et al. 2015). Majority of the *COL4A1* mutations reported are missense mutations in highly conserved regions of this triple helix domain. These mutations are assumed to induce structural disruptions in the basement membrane potentially resulting in weakening of the vessels due to increased intracellular accumulation of COL4A1 and/or decreased extracellular COL4A1 (Weng et al. 2012). Based on this observation, the autosomal dominant inheritance pattern and lack of a phenotype in mice heterozygous for the null alleles of *Col4a1* and *Col4a2* (Poschl et al. 2004), it has been suggested that mutations in the *COL4A1* cause the disease through a dominant-negative effect.

In 2013, it was suggested that frameshift mutations and splice site alterations could cause a *COL4A1* related disorder trough haploinsufficiency (Lemmens et al. 2013). As the variant we found is located on the 3'UTR, it is likely to either mutate a regulatory region or cause alternative splicing. According to *in silico* analyses, our variant is predicted to alter splice site. This suggests that if the 3'UTR variant in COL4A1 is pathogenic, it could cause the disease through haploinsufficiency. Earlier studies have suggested that the clinical phenotype may be more severe in patients with missense mutations resulting in a dominant-negative effect than in patients with mutations leading to haploinsufficiency (Lemmens et al. 2013). This could partially explain the fact that hMID of Swedish type is an adult-onset disease, whereas many other *COL4* related diseases have earlier, even prenatal, onset (Meuwissen et al. 2015). Nevertheless, this hypothesis needs further studies, since Lemmens *et al.* (2013) have described haploinsufficiency also in two families whose phenotypes were similar to phenotypes described in patients with missense mutations.

A limiting factor in our study arises from the sequencing of the whole-exome and not the whole-genome. As only the currently known coding regions of the genome are targeted, it is possible (although unlikely) that we have missed another, possibly causative variant in the non-coding areas of genome or even in the introns of *COL4A1*.

The 3'UTR *COL4A1* variant was found to segregate with the disease in this Swedish hMID family as all of the patients carried the variant, and none of the older (>40 years of age) unaffected family members had it. One of the young, currently asymptomatic family members carried the *COL4A1* c*32G>A variant. However, he is only 33 years old and the mean age at the onset of the disease has been reported to be approximately 34 years (range 29-38 years of age) (Sourander, Wålinder 1977). Hence, he could still be in the asymptomatic phase of the disease. Since we were unable to study the expression of *COL4A1* in RNA level, the possible functional consequences of the variant are still unknown. Further studies are needed to confirm the functional effects of the 3'UTR variant c.*32G>A in *COL4A1*.

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There are no conflicts of interest.

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