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A DIAGNOSTIC VIEW OF THE GENETICS OF CADASIL

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ABSTRACT

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Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL, OMIM #125310) is an inherited vascular disease. The main symptoms include migraineous headache, recurrent strokes and progressive cognitive impairment. CADASIL is caused by mutations in the *NOTCH3* gene which result in degeneration of vascular smooth muscle cells, arteriolar stenosis and impaired cerebral blood flow.

The aims of this study were assessment of the genetic background of Finnish and Swedish CADASIL patients, analysis of genetic and environmental factors that may influence the phenotype, and identification of the optimal diagnostic strategy. The majority of Finnish CADASIL patients carry the p.Arg133Cys mutation. Haplotype analysis of 18 families revealed a region of linkage disequilibrium around the *NOTCH3* locus, which is evidence for a founder effect and a common ancestral mutation. Despite the same mutational background, the clinical course of CADASIL is highly variable between and even within families. The association of several genetic factors with the phenotypic variation was investigated in 120 CADASIL patients. *Apolipoprotein E* allele ε4 was associated with earlier occurrence of strokes, especially in younger patients. Study of a pair of monozygotic twins with CADASIL revealed environmental factors which may influence the phenotype, i.e. smoking, statin medication and physical activity. Knowledge of these factors is useful, since life-style choices may influence the disease progression. The clinical CADASIL diagnosis can be confirmed by detection of either the *NOTCH3* mutation or granular osmiophilic material by electron microscopy in skin biopsy, although the sensitivity estimates have been contradictory. Comparison of these two methods in a group of 131 diagnostic cases from Finland, Sweden and France demonstrated that both methods are highly sensitive and reliable.

Key words: CADASIL, autosomal dominant inheritance, founder effect, genetic diagnostics, *NOTCH3*, arteriopathy, GOM, stroke, vascular dementia

TIIVISTELMÄ

Kati Mykkänen

DIAGNOSTINEN NÄKÖKULMA CADASIL-TAUDIN PERINNÖLLISYYTEEN

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Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL, OMIM #125310) on periytyvä verisuonisto-tauti. Pääasialliset oireet ovat migreenityyppinen päänsärky, toistuvat aivohalvaukset ja kognitiivisten kykyjen heikkeneminen. CADASIL aiheutuu *NOTCH3* geenin mutaatioista, jotka aiheuttavat valtimoiden sileälihassolujen vaurioitumista, pienten valtimoiden ahtautumisen ja siten aivojen verenkierron heikkenemisen. Väitöskirjatutkimuksen tavoitteena oli selvittää suomalaisten ja ruotsalaisten CADASIL potilaiden geneettistä taustaa, tutkia mahdollisia taudinkuvaan vaikuttavia geeni- ja ympäristötekijöitä, sekä löytää hyvä menetelmällinen strategia diagnoosin varmentamiseksi. Suurin osa suomalaisista CADASIL-potilaista kantaa p.Arg133Cys-mutaatiota. 18 perheen haplotyyppianalyysi paljasti *NOTCH3*-lokuksen ympärillä kytkentä-epätasapainon, joka on osoitus perustajanvaikutuksesta ja yhteisestä esivanhemmilta peritystä mutaatiosta. Samasta mutaatiotaustasta huolimatta CADASIL-taudin kliininen kuva vaihtelee huomattavasti, niin perheiden välillä kuin sisälläkin. Eri geneettisten tekijöiden yhteyttä taudinkuvan vaihteluun tutkittiin 120 CADASIL-potilaan aineistossa. *Apolipoproteiini E:n* ε4-alleelilla todettiin olevan yhteys varhaisempaan aivohalvausten esiintymiseen etenkin nuorilla potilailla. CADASIL-tautia sairastavalla kaksosparilla tehty tutkimus paljasti myös ympäristötekijöitä, kuten tupakointi, statiinilääkitys ja liikunta, jotka voivat vaikuttaa taudinkuvaan. Tieto näistä tekijöistä on hyödyllinen, koska elämäntavoilla voidaan vaikuttaa taudinkulkuun. Kliininen CADASIL-diagnoosi voidaan varmentaa todentamalla joko patogeeninen *NOTCH3*-mutaatio tai ns. granulaarisen osmiofiilisen materiaalin kertymät ihobiopsian elektonimikroskooppisessa tutkimuksessa. Näiden menetelmien herkkyydestä on kuitenkin julkaistu ristiriitaisia arvioita, joten niitä vertailtiin Suomen, Ruotsin ja Ranskan yhdistetyssä 131 diagnostisen potilastapauksen aineistossa. Molemmat menetelmät todettiin herkäksi ja luotettavaksi.

Avainsanat: CADASIL, autosomaalinen vallitseva periytyminen, perustajan-vaikutus, geneettinen diagnostiikka, *NOTCH3*, arteriopatia, GOM, aivohalvaus, vaskulaarinen dementia

CONTENTS

ABBREVIATIONS

AGT	angiotensinogen
ApoE, *APOE*	apolipoprotein E (protein and gene)
CADASIL	cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CBF	cerebral blood flow
CBV	cerebral blood volume
CI	confidence interval
CVD	cerebrovascular disease
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EM	electronmicroscopic
eNOS	endothelial nitric oxide synthase
ESHG	European Society of Human Genetics
GDB	Genome Data Base
GOM	granular osmiophilic material
HR	hazard ratio
KcM	Kosambi centimorgan (sex-averaged genetic distance)
LD	linkage disequilibrium
LDB	Genetic Location Database
MRI	magnetic resonance imaging
mtDNA	mitochondrial DNA
N3ECD	Notch3 extracellular domain
N3ICD	Notch3 intracellular domain
OMIM	Online Mendelian Inheritance in Man
OR	odds ratio
PCR	polymerase chain reaction
PET	positron emission tomography
SD	standard deviation
TACE	TNF-α-converting enzyme
TIA	transient ischemic attack
VSMC(s)	vascular smooth muscle cell(s)
WM	white matter

LIST OF ORIGINAL PUBLICATIONS

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

I Mykkänen K, Savontaus M-L, Juvonen V, Sistonen P, Tuisku S, Tuominen S, Penttinen M, Lundqvist J, Viitanen M, Kalimo H, Pöyhönen M. Detection of the founder effect in Finnish CADASIL families. *European Journal of Human Genetics* 2004;12:813-819.

II Junna M, Mykkänen K, Pescini F, Rovio S, Roine S, Tuisku S, Kääriäinen H, Verkkoniemi A, Kalimo H, Pöyhönen M, Viitanen M. Genetic factors may modify the clinical course of CADASIL. Submitted.

III Mykkänen K, Junna M, Amberla K, Bronge L, Kääriäinen H, Kalimo H, Pöyhönen M, Viitanen M. Different clinical phenotypes in monozygotic CADASIL twins with a novel *NOTCH3* mutation. *Stroke* 2009; In press.

IV Tikka S*, Mykkänen K*, Ruchoux M-M, Bergholm R, Junna M, Pöyhönen M, Yki-Järvinen H, Joutel A, Viitanen M, Bauman M, Kalimo H. Congruence between *NOTCH3* mutations and GOM in 131 CADASIL patients. *Brain* 2009; In press. (Epub. Ahead of print: Jan 27)
* Equal contribution.

1. INTRODUCTION

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a dominantly inherited neurodegenerative disease and the most common cause of hereditary vascular dementia. Today, it has been over fifteen years since this disease was identified, named and the first diagnoses given (Tournier-Lasserve*, et al.*, 1993). Furthermore, for over ten years the causative mutation has been identifiable for single patients as well as for their families (Joutel*, et al.*, 1996). To a patient it is naturally psychologically important to obtain the correct diagnosis. Also for the health care professionals it is important to know the pathofysiology behind the symptoms in order that the correct medical treatment can be started and some possibly harmful treatments avoided. However, if the diagnosis is given in the form of an incurable hereditary disease with a devastating outcome of disability and dementia, it understandably raises worries and anxiety – not only in the patient, but also in the patient's relatives, who face a genetic risk of having the disease. Furthermore, improving the knowledge of this disease and the diagnostic services are essential means for reducing the economic and societal costs of this disease. The earlier the correct diagnosis can be given, the more resources can be saved through the reduction of the number of unnecessary tests and analyses.

In the future, we will also face the challenges of revealing the underlying pathomechanism of the *NOTCH3* mutations and discovering the possible leads for developing a curative treatment for CADASIL. Along the way our studies provide small – though nevertheless important – pieces of information related to the every-day life with the disease and to revealing ways with which to influence the course of the disease.

2. REVIEW OF THE LITERATURE

2.1. The history of CADASIL

In 1977 Sourander and Wålinder reported a Swedish family suffering from "hereditary multi-infarct dementia" and this paper was for a long time considered to be the first report of CADASIL. On the basis of more thorough scrutiny of the literature, the first CADASIL family is now believed to have been already described in 1955 by van Bogaert (Davous, 1998). At that time CADASIL was not known as a disease entity and the family was reported having a familial Binswanger's disease (van Bogaert, 1955). However, there might be even earlier reports of this disease, since van Bogaert referred to a report of similar disease described by Mutrux (1951). Since van Bogaert's report several similar disease descriptions were published in Europe: chronic familial vascular encephalopathy (Stevens*, et al.*, 1977) autosomal dominant syndrome with strokelike episodes and leukoencephalopathy (Tournier-Lasserve*, et al.*, 1991), familial subcortical dementia with arteriopathic leukoencephalopathy (Davous and Fallet-Bianco, 1991), a familial disorder with subcortical ischemic strokes, dementia and leukoencephalopathy (Mas*, et al.*, 1992) as well as slowly progressive familial dementia with recurrent strokes (Salvi*, et al.*, 1992). In 1987, inspired by the article of Sourander and Wålinder (1977), Sonninen and Savontaus (1987) described a large pedigree with multi-infarct dementia, which by means of molecular genetic analysis was confirmed in 1998 to be the first Finnish CADASIL family.

Tournier-Lasserve *et al.* studied a large French pedigree, which her group had previously described (Tournier-Lasserve*, et al.*, 1991), with linkage analysis and succeeded in mapping the disease locus to chromosome 19 (chr19q12) (Tournier-Lasserve*, et al.*, 1993). In this same report the group also suggested the name "cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy" to be used for this disease and mercifully also presented the acronym CADASIL for it. Three years later this same group refined the linkage region to chr19p13.1 (Ducros*, et al.*, 1996). Soon after, *NOTCH3* was identified as the defective gene behind the disease (Joutel*, et al.*, 1996), and the archetypical nature of pathogenic mutations were described (Joutel*, et al.*, 1997).

The identification of the genetic deficit behind the disease enabled not only the determination of the molecular background but also the definition of the clinical characteristics of CADASIL. Recently, the Swedish family described by Sourander

and Wålinder (1977) was studied in more detail. Neither *NOTCH3* mutation was identified nor CADASIL-specific granular osmiophilic material (GOM) detected in the patients' arteries (Low*, et al.*, 2007). Hence, this family suffers from some other inherited form of vascular dementia as yet unknown.

2.2. The *NOTCH3* gene

2.2.1. Gene and protein structure

In 1996 Joutel*, et al.* (1996) identified human *NOTCH3* (Notch homolog 3, *Drosophila*) as the defective gene behind CADASIL pathology. The *NOTCH3* gene is located in the short arm of the chromosome 19 (19p13.1), and it is a large (~41.3 kilobase) gene containing 33 exons. The gene product is the 2321 amino-acids-long NOTCH3, which is a membrane-bound receptor protein. The N-terminal end of the NOTCH3 protein contains a large extracellular domain (N3ECD), which consists of 34 tandemly repeated epidermal-growth-factor-like domains (EGF1-34) (Wharton*, et al.*, 1985). Each of these EGF-like domains contains six highly conserved cysteine residues within a certain distance of each other. The cysteines are vital for the correct folding of the EGF repeats and the function of the receptor. Six cysteines form three disulphide bridges in a certain order (1-3, 2-4 and 5-6) to create the spatial shape of each domain (Campbell and Bork, 1993, Artavanis-Tsakonas*, et al.*, 1995). Studies with *Drosophila* Notch have demonstrated that ligand binding occurs at the 11th and 12th EGF domain of the receptor (Rebay*, et al.*, 1991, Lawrence*, et al.*, 2000), although other EGF domains have also been suggested to influence the binding activity (Lawrence*, et al.*, 2000, Xu*, et al.*, 2005). In human NOTCH3, the corresponding EGFs are the 10th and 11th (Joutel*, et al.*, 2004). NOTCH3 also contains three Notch/Lin-12 repeats (LNR1-3) between the EGF repeats and the single transmembraine domain.

The intracellular part of the NOTCH3 receptor is responsible for the signal transduction from the neighbouring cell to the nucleus of the signal receiving cell. The C-terminal cytoplasmic part of the protein contains a RBP-Jκ-associated molecule region, at least five, possibly six, putative ankyrin-like repeats, two nuclear localization sequences, located on either sides of the ankyrin repeats and a PEST sequence (Stifani*, et al.*, 1992, Logeat*, et al.*, 1998, Weinmaster, 2000) (protein region enriched with proline (P), glutamic acid (E), serine (S) and threonine (T)), which carries a proteolytic signal characteristic of the nuclear proteins with a short half-time (Rogers*, et al.*, 1986). Schematic structure of the *NOTCH3* gene and the NOTCH3 receptor is presented in Figure 1.

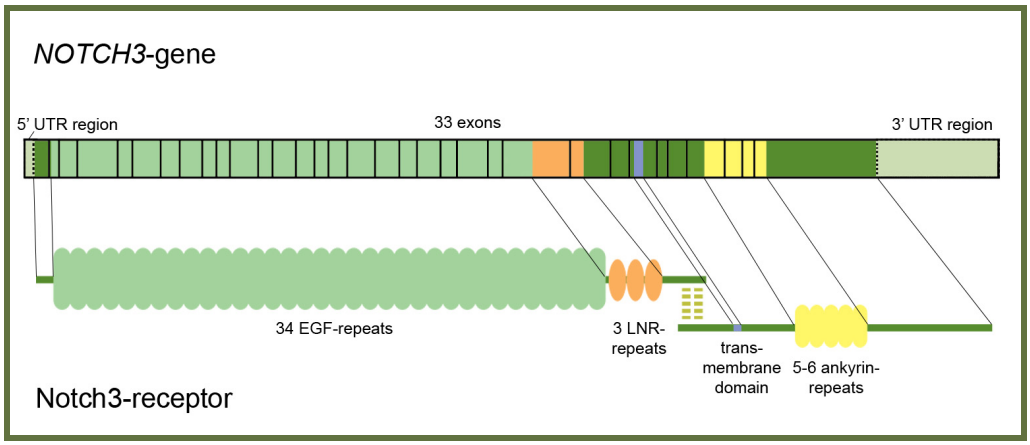

Figure 1: The exonic structure of the human *NOTCH3* gene and the domains of the NOTCH3 receptor protein.

2.2.2. NOTCH3-mediated signalling

The Notch receptor was originally identified and the *Notch* gene cloned from *Drosophila melanogaster* (Artavanis-Tsakonas*, et al.*, 1983). The NOTCH3 protein belongs to the evolutionarily conserved Notch receptor family, members of which are involved in the interactions and receptor-mediated signalling between adjacent cells. All Notch receptors are type I membrane proteins that interact with membrane-bound ligands (DELTA and JAGGED) which are presented at the cell surface of neighbouring cells (Artavanis-Tsakonas*, et al.*, 1995). Four different Notch receptors (Notch 1–4) and five Notch ligands (Delta-like-1,-3,-4, Jagged-1,-2) have been identified in mammals. In invertebrates and vertebrates, Notch signalling has an essential role in different developmental events during the embryonic development as well as in adult tissues. The best acknowledged function of Notch signalling is its involvement in cell differentiation and proliferation in the phases of tissue determination in *Drosophila* (Artavanis-Tsakonas*, et al.*, 1999). In mammals, Notch3 regulates cell differentation and is periodically expressed in many different tissues in several phases of the embryonic development. In the adult human, the *NOTCH3* gene is expressed almost exlusively in the vascular smooth muscle cells (VSMCs) (Joutel*, et al.*, 2000a).

All four mammalian Notch receptors are highly homologous and their maturation and signalling processes can be assumed to follow the same principles, even if in different studies several Notch receptors of different species have been used.

Generalized knowledge of the events in the course of Notch signalling is illustrated in Figure 2.

After the translation, full length proprotein of Notch3 is cleaved by furin convertase in Golgi network (cleavage site 1, S1). The resulting fragments are reassociated noncovalently (with Ca^{2+} ions) to form a heterodimeric molecule which is then transported to the cell membrane. Only the mature, cleaved and heterodimeric receptors are presented at the cell surface (Blaumueller*, et al.*, 1997, Logeat*, et al.*, 1998). The Notch receptors bind several proteins of the DSL family (Delta/Serrate/Lag2). Ligands (in mammalian cells Delta-1, -3, -4, Jagged-1 and -2) are presented at the surface of the neighbouring signal-sending cell (Weinmaster, 1997). The structure of the ligands resembles that of the Notch receptor itself; the ligands are also transmembrane proteins with several EGF-like repeats in the extracellular domain. Activation of the Notch-mediated signalling requires endocytosis of the ligand into the signal-sending cell. Ligands that cannot be internalized into the cell – either as a result of a mutation or inhibition of the endocytosis – are also unable to activate the Notch receptor (Seugnet*, et al.*, 1997, Parks*, et al.*, 2000, Itoh*, et al.*, 2003, Wang and Struhl, 2004). When a ligand binds to the Notch receptor, endocytosis of the ligand either physically pulls off the extracellular domain of the Notch3 receptor or creates a specific microenvironment to enable the release of the extracellular domain of Notch (NECD) (Parks*, et al.*, 2000, Wang and Struhl, 2005, Nichols*, et al.*, 2007a). The ligand-NECD complex is then fully internalized to the signal-sending cell where the ligand is recycled back into the membrane and the NECD is degraded (reviewed by (Nichols*, et al.*, 2007b).

The removal of the NECD is implemented by the second proteolytical cleavage (S2) of the Notch protein by a TNF-α-converting enzyme (TACE) which belongs to a family of metalloproteases called a disintegrin and metalloprotease (ADAM) (Brou*, et al.*, 2000). Finally, the third cleavage by γ-secretase (S3) releases the intracellular signalling domain of the Notch protein (NICD) (De Strooper*, et al.*, 1999). The γ-secretase, which lies on the cell memrane, is a complex formed by presenilin, nicastrin, Aph-1 and Pen-2 (Chyung*, et al.*, 2005). After its release, NICD is subsequently transported to the nucleus, where it associates with a DNA-binding transcriptional regulator CSL (the name is originally derived from *Drosophila*: CBF1, Supressor of Hairless and Lag-1; in mammals this regulator is called CBF1/RBP-Jκ) (Jarriault*, et al.*, 1995), displacing the co-repressor (CoR) proteins from the complex and recruiting co-activators (CoA). In mammalian cells, this complex activates the expression of gene families called Hairy-Enhancer of Split (HES) and HES-related with YPRW motif (HEY, also called HESR or HERP). (Lasky and Wu, 2005). Recently, a new target gene for NOTCH signalling was identified: *PDGFR-β* (platellet-derived growth factor receptor-β)

(Jin, *et al.*, 2008). PDGF signalling is an essential element in vascular development, homeostasis of blood vessels, and it also has a role in restenosis in response to angioplasty (Heldin and Westermark, 1999, Levitzki, 2005).

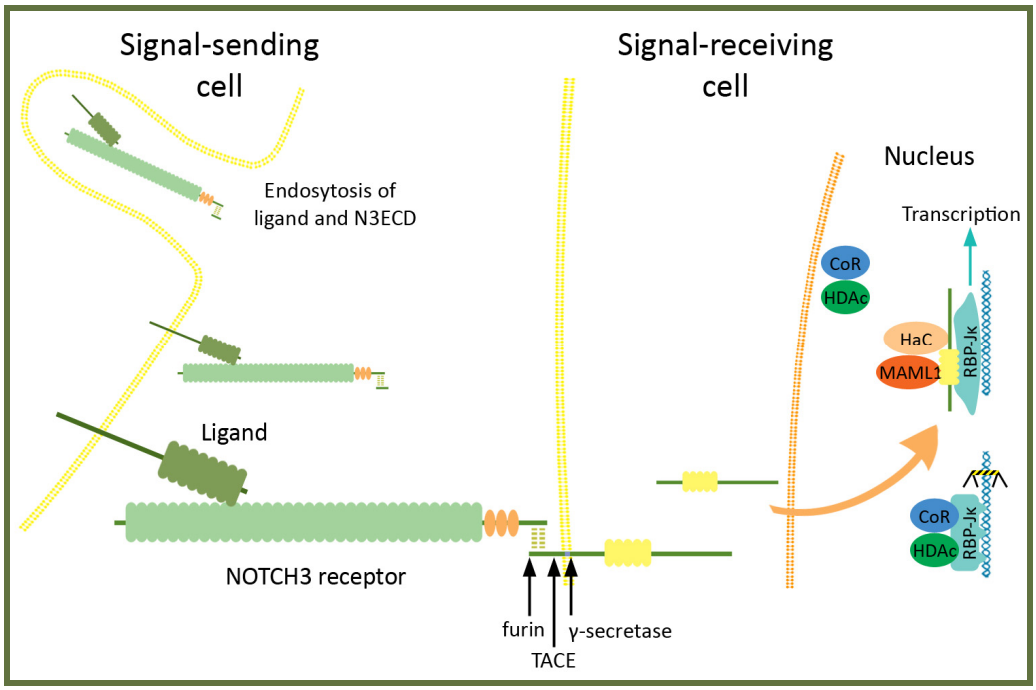

Figure 2: A schematic presentation of the Notch signalling. After cleavage by furin (S1), a mature NOTCH3 receptor is transported to the cell surface (at the lowest part of the figure). A ligand is presented at the surface of the neighbouring cell (left). After the ligand binding, the second cleavage (S2) by TACE enables the removal of the NECD. Endocytosis of the ligand-ECD-complex is presented left. The third cleavage by γ-secretase (S3) releases the NICD, which is transported to the nucleus (right). There the NICD associates with a DNA-binding transcriptional regulator CBF1/RBP-Jκ displacing the repressor proteins and binding activators. The complex activates the expression of the mammalian HES and HEY gene families.

NOTCH3 signalling, in addition to the involvement in tissue determination during embryonic development, also regulates cell fate in adult tissues. Recent studies have demonstrated that NOTCH3 signalling regulates growth, migration and apoptotic cell death of the VSMCs. Wang, *et al.* (2002) revealed that NOTCH3-mediated signalling promotes VSMC survival via inhibition of FasL-induced cell death by upregulating c-FLIP expression through the ERK/MAPK pathway, independent of the previously established classic CBF1/RBP-Jκ pathway. Sweeney, *et al.* (2004) demonstrated that a constitutional expression of

N3ICD resulted in an increased proliferation, inhibition of migration and inhibition of apoptosis in rat VSMCs *in vitro*. NOTCH3 expression is also required in the normal T cell differentation (Felli*, et al.*, 1999). Furthermore, NOTCH3 has a tumorigenic potential, and its overexpression has been associated with the development of lymphoblastic T cell leukemia in mice (Bellavia*, et al.*, 2000, Felli*, et al.*, 2005).

2.2.3. *NOTCH3* mutations linked to CADASIL

In 1997, Joutel*, et al.* reported on the quintessential nature of the *NOTCH3* mutations that cause CADASIL. In 45 unrelated patients the group identified 25 different mutations, all of which lead to an altered number of cysteine residues in one of the extracellular EGF-like repeats of the NOTCH3 receptor. To date, at least 170 different mutations following this stereotyped pattern have been described (Table 1, for references see the Supplemental table 1 in original publication IV). A great majority of the reported pathogenic *NOTCH3* mutations are missense point mutations leading to a replacement of one cysteine with another amino acid or *vice versa*. Six pathogenic deletions, one combined deletion and insertion (or two adjacent nucleotide substitutions), two duplications and two splice site mutations have also been described. In addition to these common types of cysteine-affecting CADASIL mutations, seven mutations not altering the number of cysteines have been reported. One of these mutations is a deletion, which removes the amino acids between two cysteines, and the remainder are missense mutations leading to one amino acid substitution. Whether these substitutions truly are pathogenic mutations or merely polymorphisms is as yet unresolved. So far, only three reports of patients homozygous for a pathogenic *NOTCH3* mutation have been published (Tuominen*, et al.*, 2001, Dotti*, et al.*, 2003, Liem*, et al.*, 2009). In addition, two confirmed *de novo* mutations in CADASIL patients have been reported (Joutel*, et al.*, 2000c, Coto*, et al.*, 2006).

Table 1: Different types of the pathogenic *NOTCH3* mutations causing CADASIL.

Mutation type in DNA level		Effect of the mutations in protein level	
Missense	166 (93.8%)		
		Cysteine +/-	160 (90.4%)
		Non-cysteine	6 (3.4%)
Deletions	6 (3.4%)		
		In frame	5 (2.8%)
		Frame shift	1 (0.6%)
Deletion+insertion	1 (0.6%)		
Duplications	2 (1.1%)		
Splice site	2 (1.1%)		
	n=177		

2.2.4. Pathogenic effect of the *NOTCH3* mutations

The exact mechanisms and events during the pathogenesis of CADASIL are still unrevealed, although several possible pathogenic effects have been suggested. The mechanism may involve a signalling defect as a result of an impaired receptor (loss of function) or a toxic effect from a faulty receptor protein (gain of function).

The striking majority of the pathogenic *NOTCH3* mutations alter the number of the conserved cysteine residues in the target EGF domain. (Dichgans*, et al.*, 2000) illustrated the misfolding of the NOTCH3 receptor as a result of a disruption of the disulfide bridging of the EGF repeats with 3D modelling of the mutations. This effect is clearer when the mutation deletes one of the cysteines forming these bridges. Whether the mutations creating an excess cysteine have the same effect is not as evident. However, both types of mutations leave one unpaired cysteine residue in the target EGF domain, which as such could lead to misfolding of the EGF domain.

Some of the mutated NOTCH3 receptors are processed in the usual manner and transported to the cell surface in a matured heterodimeric form, whereas some of the mutations result in a reduction in the number of receptor proteins at the membrane (Joutel*, et al.*, 2004). Peters*, et al.* (2004b) found that part of the mutated NOTCH3 receptors were properly cleaved by furin, and heterodimeric

forms of the receptors were presented at the cell surface, but the portion of uncleaved receptor proteins was elevated. The signalling activity of the mutated NOTCH3 receptors differs according to the different mutation locations. Predictably, mutations located at the ligand-binding area of the receptor (EGFs 10 and 11) fail in the signal mediating task, whereas mutations outside this area usually do not influence ligand binding and the receptors activate the CBF1/RBP-Jκ signalling cascade normally (Joutel*, et al.*, 2004, Peters*, et al.*, 2004b, Monet*, et al.*, 2007). These results from *in vitro* studies on the different *NOTCH3* mutations and the fact that the homozygous patients' phenotypes are comparable to that of heterozygous patients (Tuominen*, et al.*, 2001, Liem*, et al.*, 2009) suggest that loss of signalling function is not the fundamental pathogenic effect of the CADASIL-causing mutations. This conclusion is further supported by the observation that no null allele of the *NOTCH3* gene has been detected.

One gain of function mutation of *NOTCH3*, p.Leu1215Pro, has been identified in exon 25 of the gene (Fouillade*, et al.*, 2008). This mutation affects the conserved leucine at the heterodimerization domain of NOTCH3 and the mutated receptor is constitutively active, independently of the ligand binding. However, the mutation caused neither GOM deposits nor accumulation of N3ECD in the arteries, which are the key elements associated with CADASIL pathogenesis. Therefore, this mutation can be assumed to cause another cerebral small vessel disease, distinct from CADASIL.

Joutel*, et al.* (2000a) discovered that in CADASIL patients N3ECD of the Notch receptor accumulates on the cytoplasmic membrane of the VSMCs. In addition, Ishiko*, et al.* (2006) revealed that N3ECD is one component of the GOM deposits seen exclusively in CADASIL. These GOM deposits seem to be the decisive common feature of the pathogenic mutations and supports the gain of function effect of the mutated receptor protein. Progressive accumulation also fits well with the adult onset nature of the CADASIL disease. However, the mechanism that leads to the accumulation of N3ECD and whether the accumulation itself leads to a toxic effect in the VSMCs needs futher clarification.

Several possible theories for the pathogenic effects of the mutated and misfolded NOTCH3 receptor can be considered. Since some of the mutations lead to a heterodimerized receptor at the cell membrane and normal signalling activation, disruption of these mechanisms is unlikely to be the primary effect of the mutations. Since misfolded proteins as such cause stress in cells, and cultured CADASIL VSMCs have been shown to be under oxidative stress, this is one of the possible contributors for the progressive stress of the VSMCs (Sayeed and Ng, 2005, Ihalainen*, et al.*, 2007). However, stress caused by the misfolded proteins

and their degradation is not sufficient to explain the accumulation of N3ECD, and therefore other explanations need to be explored. The predominant pattern of the mutations that produce one free cysteine residue in one of the EGF domains potentially leads to a dimerization of the mutated NOTCH3 receptors with each other or oligomerization with other proteins (Joutel*, et al.*, 2000a). This theory would agree with the accumulation of receptors, and possibly also other proteins, on the cell surface. However, whether the dimerized/oligomerized receptors would be able to activate the signalling is not clear.

Even if the unpaired cysteines would not result in a dimerization or oligomerization, the transendocytosis of the ligand and misfolded N3ECD may fail. If the endocytosis fails, it is likely unable to induce the S2 cleavage by TACE and consequent N3ECD release. This would lead to a defective removal of the receptor molecule from the cell surface. As illustrated in a review by Kalimo*, et al.* (2008), accumulation of the receptors would result, which eventually could also lead to weakened NOTCH3 signalling. Whether the S2 cleavage is required for the S3 cleavage to occur is still unclear, but since several CADASIL mutations retain the signalling activity, S3 cleavage most likely occurs regardless. It is also possible that S2 cleavage occurs normally, but the internalization of the misfolded N3ECD into the ligand-expressing cell still fails. In this case the N3ECD would accumulate in the intercellular space between the adjacent cells.

Several studies have also suggested mitochondrial aberrations in CADASIL patients. Decreased complex I activity of the mitochondrial respiratory chain, ragged-red fibres with decreased cytochrome c oxidase (COX) staining (de la Pena*, et al.*, 2001), core-like lesions and mitochondrial abnormalities (Malandrini*, et al.*, 2002) and subsarcolemmal aggregation of muscle mitochondria (Dotti*, et al.*, 2004) have been detected in CADASIL patients, although without any symptoms of myopathy. In addition, Finnilä*, et al.* (2001) described a Finnish patient suffering from CADASIL (p.Arg133Cys) and also from myopathy caused by the m.5650G>A mutation in the mitochondrially encoded tRNA alanine gene (*MT-TA)* in mitochondrial DNA (mtDNA). The frequency of mtDNA mutations has been studied in 77 Finnish CADASIL patients with the c.397C>T (p.Arg133Cys) mutation in the *NOTCH3* gene (Annunen-Rasila*, et al.*, 2006). The sequencing analysis of the coding region of mtDNA revealed eight novel mtDNA mutations. Comparisons of nucleotide diversity indices showed an increased variation among CADASIL sequences compared to that among 192 control sequences. The results suggest an increased mtDNA sequence variation in CADASIL. One explanation is that the *NOTCH3* mutation leads to decreased mitochondrial oxidative phosphorylation, an increased amount of oxidative radicals, and consequently, secondary mutations in mtDNA. This hypothesis is also supported by the observation that mitochondrial superoxide dismutase is

upregulated in cultured VSMCs from the umbilical cord of a newborn carrying the p.Arg133Cys mutation (Ihalainen*, et al.*, 2007).

2.3. The clinical picture of CADASIL

CADASIL (in Online Mendelian Inheritance of Man, OMIM #125310) is an autosomally dominantly inherited, slowly progressive disease manifesting in early adulthood. The diseases clinical course is characterized by four main features: (1) migraineous headache, (2) recurrent cerebral ischemic attacks, (3) psychiatric symptoms and (4) cognitive decline progressing into subcortical vascular dementia (Chabriat*, et al.*, 1995b).

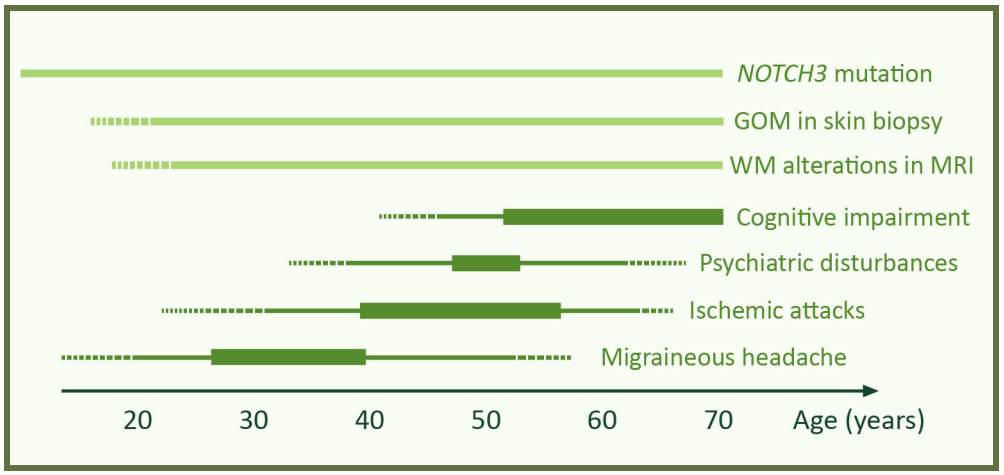

Figure 3: The symptom manifestation and findings in CADASIL during the disease course. WM; white matter, MRI; magnetic resonance imaging, GOM; granular osmiophilic material. Revised from a figure by Chabriat and Bousser (2007).

2.3.1. Clinical findings

Migraineous headache

In most clinical studies of CADASIL, patients are documented to suffer from migraine or recurrent headaches; different studies report occurence of migraine from 22% up to 64% among the patients (Chabriat*, et al.*, 1995b, Davous, 1998, Dichgans*, et al.*, 1998, Markus*, et al.*, 2002, Vahedi*, et al.*, 2004). Usually, migraine is the first symptom with the mean age of onset in the late twenties. However, the age of onset can vary from early childhood to the middle age (6–54 years of age) (Dichgans*, et al.*, 1998, Vahedi*, et al.*, 2004). In CADASIL migraine is typically associated with aura. Most often the aura is typical (71%), i.e. symptoms are visual, sensory, aphasic or motor features, and these may also appear in different combinations. However, migraine attacks may also be associated with atypical aura, be basilar or hemiplegic or they may be prolonged or exceptionally severe. (Vahedi*, et al.*, 2004). The frequency of the attacks, in general, is highly variable among patients, and it can also change during the disease course. Most patients have reported an increase in the frequency of migraine attacks before the first ischemic event and a decrease in the frequency after the first stroke episode (Dichgans*, et al.*, 1998).

Cerebral ischemic attacks

The most common and charasteristic feature of CADASIL are the recurrent cerebral ischemic attacks, i.e. transient ischemic attacks (TIAs) or strokes. In the largest clinical studies, 83% (172/206) of the patients in the symptomatic phase had experienced TIAs or strokes (Chabriat*, et al.*, 1995b, Dichgans*, et al.*, 1998, Peters*, et al.*, 2004a). Patient age at the onset of ischemic attacks varies from circa 25 to over 60 years, the mean being slightly under 50 years (Chabriat*, et al.*, 1995b, Dichgans*, et al.*, 1998, Opherk*, et al.*, 2004). In nearly half of the patients, an ischemic event is the first symptom of CADASIL (Dichgans*, et al.*, 1998, Desmond*, et al.*, 1999). At the early stage of the disease, ischemic events are typically milder TIAs which may be difficult to distinguish from severe migraineous aura or hemiplegic migraine attacks. The majority of the ischemic attacks are classic lacunar strokes presenting as pure motor or sensory symptoms, sensorimotor symptoms, dysarthria, clumsy hand syndrome, expressive dysphasia, visual field defects or ataxic hemiparesis (Kalimo*, et al.*, 2008). As the strokes recur in the course of the disease, different physical deficits, for example gait disturbances and pseudobulbar palsy, accumulate and lead to a stepwise progression towards a severe disability. By the age of 60 over 50%, and by the age of 65, 63% of the patients reach the disability degree 4 on the Rankin Scale (Rankin, 1957), which means inability to walk and attend to own bodily needs without assistance (Dichgans*, et al.*, 1998).

Progressive cognitive decline and dementia

An impaired cerebral blood flow (CBF) and consequent ischemic injuries result in progressive cognitive symptoms in CADASIL patients. In general, cognitive symptoms begin with deterioration of executive functions, later followed by a decline of multiple cognitive domains. Although some studies have failed to detect cognitive defects in neurologically asymptomatic patients (Trojano*, et al.*, 1998), other studies have shown that before the clinical strokes or TIAs, neuropsychological tests can already reveal deterioration in certain cognitive functions (Taillia*, et al.*, 1998, Amberla*, et al.*, 2004). Amberla*, et al.* (2004) detected a significant difference between CADASIL patients in a prestroke and poststroke stage in tests for working memory, executive functions and mental speed. A similar profile of cognitive impairment was also found in a study of 65 CADASIL patients by Peters*, et al.* (2005b). Usually, cognitive deterioration progresses slowly, corresponding to the occurence of the recurrent ischemic attacks, although in 5–15% of the patients deterioration develops into a vascular dementia without clinical strokes. However, in these patients as well, the magnetic resonance imaging (MRI) of the brain revealed silent infarcts which contribute to the impairment of the cerebral white matter (Dichgans*, et al.*, 1998, Mellies*, et al.*, 1998, Kalimo*, et al.*, 2008). The cognitive deterioration primarily affects the frontal lobe functions, whereas memory functions are usually not affected until at the later stages (Chabriat*, et al.*, 1995b, Dichgans*, et al.*, 1998). Approximately 65% of the patients have reached the state of vascular dementia by the age of 65 (Dichgans*, et al.*, 1998).

Psychiatric disturbances

Psychiatric disturbances are diagnosed in 34% of the CADASIL patients (Chabriat*, et al.*, 1995b, Dichgans*, et al.*, 1998, Valenti*, et al.*, 2008). Usually, psychiatric symptoms manifest themselves after other symptoms have already appeared. Mood disorders are the most common form (69%) of reported psychiatric manifestations and only a small number of CADASIL patients suffer from other forms of psychiatric disturbances (anxiety disorders, adjustment disorders, behavioural or personality disorders, addictions and psychotic episodes) (Valenti*, et al.*, 2008). The great majority of mood disorders is accounted for by major depression (Desmond*, et al.*, 1999, Amberla*, et al.*, 2004, Peters*, et al.*, 2005b, Singhal*, et al.*, 2005). More rarely, mild chronic depression (dysthymia), bipolar disorder or attempted suicides have been reported (Valenti*, et al.*, 2008).

Other findings

In CADASIL patients, the general laboratory findings (i.e. general blood status, cerebrospinal fluid, liver, kidney and thyroid findings as well as parameters of memory related investigations) are usually within the normal limits, and vascular risk factors are present at approximately the same frequency as in the general population (Kalimo*, et al.*, 2008).

However, some more uncommon symptoms and findings have been reported in CADASIL patients. Relatively rarely, in about 10% of the patients, epileptic seizures occur at the later phase of the disease (Malandrini*, et al.*, 1997, Dichgans*, et al.*, 1998, Haan*, et al.*, 2007, Valko*, et al.*, 2007). Spontaneous cerebral haemorrhages have occasionally been observed in CADASIL patients (Maclean*, et al.*, 2005, Choi*, et al.*, 2006), and in a few patients anticoagulation treatment may have caused fatal haemorrhages (Kalimo*, et al.*, 2002). Cerebral microbleeds are also fairly common in CADASIL patients, but usually they are silent events without any clinical manifestations and they occur almost always outside the ischamic lesions (Lesnik Oberstein*, et al.*, 2001, Dichgans*, et al.*, 2002). Microbleeds have been considered either to imply an increased risk for later brain haemorrhages or to be largely independent manifestations of the underlying angiopathy. Some cases of reversible coma-like episodes and confusion have also been documented (Joutel*, et al.*, 2000b, Feuerhake*, et al.*, 2002, Le Ber*, et al.*, 2002, Schon*, et al.*, 2003). Several studies have reported abnormalities in retinal vessels of CADASIL patients, but they seem to cause only minor functional deficits (Robinson*, et al.*, 2001, Cumurciuc*, et al.*, 2004, Haritoglou*, et al.*, 2004, Harju*, et al.*, 2004, Rufa*, et al.*, 2005, Roine*, et al.*, 2006). These findings are concordant with the sparing of cerebral cortex since the retina and its blood vessels are analogous to cerebral cortex and its vasculature.

Mitochondrial abnormalities and related neuromuscular symptoms have also been observed in CADASIL patients (de la Pena*, et al.*, 2001, Finnila*, et al.*, 2001, Malandrini*, et al.*, 2002, Dotti*, et al.*, 2004, Schroder*, et al.*, 2005). Mitochondrial involvement is further elucidated by the discovery that in CADASIL patients the frequency of mutations in mtDNA is higher than in the control subjects (Annunen-Rasila*, et al.*, 2006). Whether this is a result of a general ischemia and an increased mutational frequency due to the secondary oxidative stress in mitochondria, or whether there is some other pathological mechanism contributing the mitochondrial defect remains unresolved.

Since CADASIL is a systemic arteriopathy, it might be expected that the patients suffer from myocardial manifestations. Lesnik Oberstein*, et al.* (2003a) found

evidence of myocardial infarctions in 10 out of 41 CADASIL patients. On the other hand, Cumurciuc*, et al.* (2006) found no evidence of myocardial infarctions in ECG of 23 CADASIL patients. Thus, this question has not yet been settled.

Disease progression

Manifestation of the main symptoms and the detection of diagnostic findings during the disease course are presented in Figure 3. In the early stages CADASIL is a slowly progressive disease which usually proceeds with steps of aggravated deterioration as a consequence of recurrent ischemic attacks. In earlier studies, the disease duration after symptom manifestation (most often determined from the first definite stroke or TIA) has been reported as being usually about 20 years and the mean age at death slightly over 60 years (Chabriat*, et al.*, 1995b, Dichgans*, et al.*, 1998). Opherk*, et al.* (2004) reported statistically significant differences between men and women in the disease duration from the first stroke to death (6.6 years difference; 19.3 vs. 25.9 years) and in the age at death (6.1 years difference; 64.6 vs. 70.7). However, the increasing knowledge of the CADASIL disease, better diagnostic possibilities and improving health care resources will likely lead to an earlier identification of the symptoms as well as earlier diagnosis, which would result in the observation of a longer disease duration in the future.

2.3.2. Variation in the CADASIL phenotype

In a study population of 102 individuals from 28 German families and 1 Austrian family, Dichgans*, et al.* (1998) found considerable inter- and intrafamilial variation in the phenotype of CADASIL. A difference of over 20 years (ranging from 37 to 59) was observed between families in the mean onset of ischemic deficits and a similar difference was found also within individual families. Similarly, a difference of 12 years (ranging from 19 to 31) was reported in the mean age at onset of migraine between and within families. Disability scores were age-related, but nevertheless intrafamilial variation in the degree of disability was evident. In addition, considerable variation in the disease progression has been detected among patients (Peters*, et al.*, 2004a). Similar variation has also been detected among Finnish CADASIL patients (M. Viitanen, personal communication). Opherk*, et al.* (2006) studied variation and heritability of the extent of ischemic lesions in MRI in 151 CADASIL patients. The heritability estimates in this study strongly suggested that genetic factors, other than the causative mutation, affect the volume of ischemic lesions. Although in this study the *NOTCH3* mutations seemed not to contribute to the variation, a few cases of phenotype-genotype correlations have been suggested: Opherk*, et al.* (2004)

found the p.Cys117Phe and p.Cys174Tyr mutations to be associated with an earlier deterioration in the late phase of the disease; Joutel*, et al.* (2000b) reported a splice site mutation leading to an in-frame deletion of seven aminoacids, p.Gly114_Pro120del, which might contribute to the family's phenotype with few strokes, high migraine frequency and episodes of confusion or coma; Lesnik Oberstein*, et al.* (2001) found a connection between the p.Arg153Cys mutation and cerebral microbleeds; Arboleda-Velasquez*, et al.* (2002) described a CADASIL family carrying the p.Cys455Arg mutation with early occurence of strokes (the mean age at onset was 31 years). In the clinical description of a CADASIL patient homozygous for the p.Arg133Cys mutation of *NOTCH3*, the clinical presentation corresponded to the severe end of the phenotypic spectrum but did not differ significantly from the age-matched controls who were heterozygous for the same mutation (Tuominen*, et al.*, 2001). Furthermore, this homozygous CADASIL patient's heterozygous son experienced his first TIA at the same age as his father. The other reported CADASIL patient homozygous for a *NOTCH3* mutation (p.Arg578Cys) presented a mild CADASIL phenotype that did not differ from the disease phenotype of the heterozygous sibling (Liem*, et al.*, 2009).

Other than these few study results, very little is known about the reasons behind the clinical variation in CADASIL. The potential factors that affect the variation may be either environmental or genetic. Naturally, the *NOTCH3* gene itself may contain genetic features that contribute to the phenotypic variation. Not only the different mutations, but also the polymorphisms – either silent or amino acid changing – of the gene may cause variation directly in the pathogenic events. At least 30 different polymorphisms have been identified in the *NOTCH3* gene. In three studies, no association with certain *NOTCH3* polymorphisms and cerebrovascular disease (Ito*, et al.*, 2002, Dong*, et al.*, 2003) or migraine (Borroni*, et al.*, 2006) has been detected, but Schwaag*, et al.* (2006) found an association between migraine and the G allele of a silent *NOTCH3* polymorphism, c.684G>A. In addition, other genes than *NOTCH3* can have effects on the CADASIL phenotype. Since the fundamental pathology in CADASIL arises from arterial deficits which cause cerebral ischemia, the genes affecting arterial and/or brain functions may also influence CADASIL pathogenesis – either directly or indirectly. Apolipoprotein E (ApoE) has been shown to influence many neurological processes as well as lipid transportation activity, and the influence may vary according to the different ApoE isoform (ε2, ε3 and ε4) (Cedazo-Minguez and Cowburn, 2001, Mahley*, et al.*, 2006). Allele ε4 of the *APOE* gene (chr19q13.2) is a known cardiovascular risk factor (Martins*, et al.*, 2006) and it also associates with the Alzheimer's disease and neurodegeneration (Cedazo-Minguez and Cowburn, 2001). Another possible candidate as a modifier of the CADASIL phenotype is angiotensinogen (AGT), which is a protein involved in a renin-angiotensin pathway system that regulates

blood pressure, vascular muscle tone and vasoconstriction. Furthermore, the p.Met235Thr polymorphism in the *AGT* gene (chr1q42–q43) has been associated with hypertension as well as lacunar infarcts (Jeunemaitre*, et al.*, 1992, Takami*, et al.*, 2000). In addition to genetic factors, environmental factors can influence the clinical manifestation of CADASIL. Several aspects of one's life-style and the living environment contribute to the overall health as well as to the maintenance of normal vascular and neuronal functions. Since CADASIL is a late onset disease, identifying these types of factors would be beneficial to the patients and their family members.

2.3.3. Imaging findings

Magnetic resonance imaging

The most characteristic imaging feature of CADASIL is leukoencephalopathy, which is revealed as hyperintesities in subcortical white matter (WM) in T2 weighted (T2w) MRI (leukoaraiosis; Figure 4) (Chabriat*, et al.*, 1998). Signal abnormalities are already visible in the younger, as yet asymptomatic patients. The characteristic early alterations are seen in temporopolar and periventricular WM (mostly in frontal lobes) as well as in the capsula externa (Coulthard*, et al.*, 2000). In the symptomatic phase, WM lesions become more confluent, and hyperintensities are most frequently seen in periventricular WM and deep white matter (Chabriat*, et al.*, 1998). Auer*, et al.* (2001) and O'Sullivan*, et al.* (2003) suggested that involvement of the temporal pole is the most distictively characteristic feature of CADASIL in comparison to lesions seen in sporadic ischemic leukoencephalopathies. White matter impairment and lacunar infarcts can also be seen as hypointensities in T1w MRI (Chabriat*, et al.*, 1998) and with computed tomography (CT) (Kalimo*, et al.*, 2008). In T2w gradient echo MRI, clinically silent cerebral microbleeds can be detected in 31–61% of CADASIL patients as small areas of signal loss (Lesnik Oberstein*, et al.*, 2001, Dichgans*, et al.*, 2002). Susceptibility weighted imaging (SWI) is a new magnetic resonance technique, which is also effective in detecting microbleeds (Santhosh*, et al.*, 2009).

Imaging of cerebral blood flow

Several functional imaging tools can be used to provide evidence of the cerebral circulatory dysfunction in CADASIL patients. A positron emission tomography (PET) study by Chabriat*, et al.* (1995a) revealed a 40% decrease in CBF in a 58-year-old asymptomatic patient and a 50% reduction in CBF and oxygen consumption rate ($CMRO_2$) in a 63-year-old demented patient. In the same study, the symptomatic patient had normal $CMRO_2$, and increased oxygen extraction fraction (OEF), which indicates a compensatory effect for the decreased CBF at the earlier stages of the disease (Kalimo*, et al.*, 2008). Tatsch*, et al.* (2003) investigated regional cortical glucose metabolism ($rCMR_{gluc}$) using 2-[^{18}F]-fluoro-2-deoxyglucose (^{18}F-FDG) PET in CADASIL and found a reduced rate of cerebral glucose metabolism in patients aged 46 to 65 years (mean 55.8). In the latest PET study, Tuominen*, et al.* (2004) found significantly reduced CBF and slightly lower, yet relatively normal, $rCMR_{gluc}$ in 14 younger (mean 32.8, range 19–41 years) CADASIL patients. The results from these studies suggest that impaired CBF arises from the arterial dysfunction rather than being secondary to neuronal loss (Kalimo*, et al.*, 2008).

MRI bolus tracking is another method to measure CBF, and also cerebral blood volume (CBV). In two studies, both regional CBF and CBV were discovered to be lowered in CADASIL patients in areas where T2w MRI showed hyperintensities (Chabriat*, et al.*, 2000, Bruening*, et al.*, 2001). A reduction in CBF and CBV correlated negatively with the patients' disability and cognitive impairment. These findings are furthermore supported by the observations in studies using the transcranial Doppler sonography: impaired cerebral vasoreactivity and reduced middle cerebral artery blood flow velocity were detected in CADASIL patients compared to control subjects (Pfefferkorn*, et al.*, 2001). In addition, the prolonged arteriovenous cerebral transit time in CADASIL patients can be demonstrated with transcranial colour-coded duplex sonography (Liebetrau*, et al.*, 2002). Cerebral angiography should be avoided in CADASIL patients since consequent neurological complications have been reported in several cases (Dichgans and Petersen, 1997).

2.3.4. Vascular pathology

CADASIL is a systemic arteriopathy, and vascular abnormalities can be detected by immunohistochemical or electron microscopic analysis. Arterial changes are mainly restricted to small and medium-sized arteries and the primary target tissue is VSMCs. Even though the symptoms are almost purely neurological, vasculopathy is generalized throughout the organs, which makes the detection of the chacteristic changes possible in more accessible tissues, for example in a skin

biopsy (Ruchoux*, et al.*, 1995). Vascular changes can be demonstrated prior to the appearance of any other signs or symptoms, even before 20 years of age (Tuominen*, et al.*, 2001, Brulin*, et al.*, 2002). The main findings in the electron microscopic (EM) studies include thickened arterial walls, degenerating VSMCs and the accumulation of granular osmiophilic material (GOM) on or between the VSMCs (Baudrimont*, et al.*, 1993, Ruchoux*, et al.*, 1994) (Figure 4). GOM has been exclusively detected in CADASIL, but its origin, full composition or role in the whole pathogenesis of this disease is not yet fully understood. With immunohistochemical analyses, Joutel*, et al.* (2000a) discovered that N3ECD accumulates on the cytoplasmic membrane of the VSMCs. In a later article, these authors also tested the immunostaining technique as a diagnostic tool for CADASIL and noted it to be a sensitive and specific method (Joutel*, et al.*, 2001), but results questioning the specificity have been published (Lesnik Oberstein*, et al.*, 2003b). Recently, based on the immunoelectron microscopy study on skin biopsies from two CADASIL patients, Ishiko*, et al.* (2006) suggested that N3ECD is one of the components of GOM.

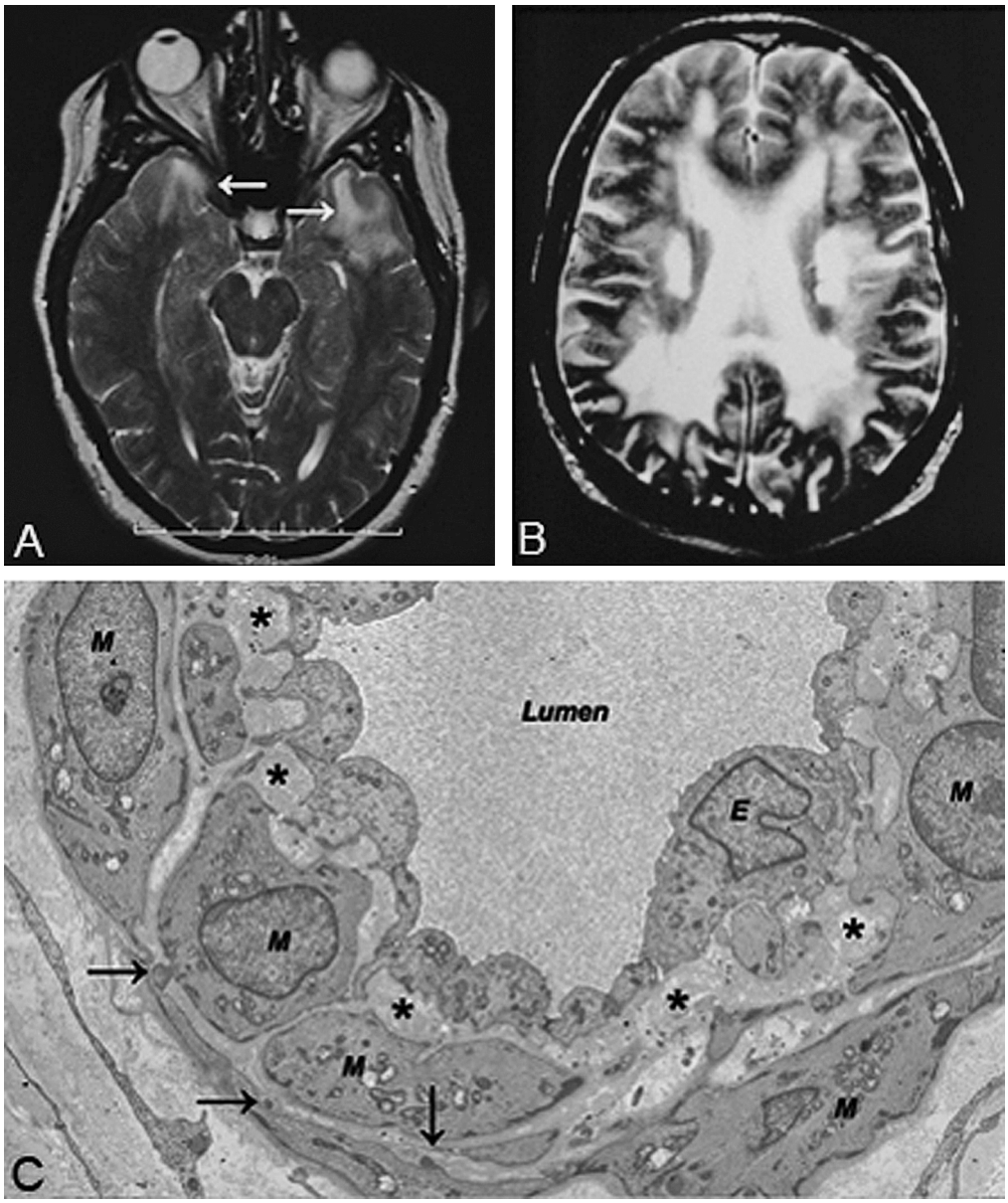

Figure 4: MRI and electronmicroscopic EM findings in CADASIL patients. **A**. Characteristic early alterations in CADASIL: hyperintensities in the anterior tempolar lobes in T2w MRI of a 29-year-old patient at the time of the first TIA. **B.** Extensive white matter hyperintensities of a patient in an advanced stage of dementia. **C.** An electron micrograph of a dermal artery from a 28-year-old patient at an early stage of the disease. Note the widened subendotelial spaces (asterisk) and irregular smooth muscle cells (M), on which there are three deposits of GOM (arrows). Figures from the article by Kalimo*, et al.* (2008). The figures has been reproduced with the permission of the copyright holder.

2.4. Diagnosing CADASIL

2.4.1. A clinical and differential diagnosis

Since CADASIL is a progressive adult onset disease with gradually increasing and worsening symptoms, a clinical diagnosis at early stages of the disease can be challenging if CADASIL has not been previously diagnosed in the family. CADASIL patients have often sought medical care for their migraineous headache, but since migraine is such a common ailment, diagnostic examinations are not necessarily done. When CADASIL patients are seeking medical care after the first ischemic episode, the patients usually have a history of migraine episodes and occasionally also mood changes or mild cognitive defects. An MRI examination is then most likely performed. If the patient is relatively young and no major vascular risk factors are present, white matter alterations (described more in detail in Chapter 2.3.3. Imaging findings) in MRI is suggestive of CADASIL. If similar symptoms or findings exist in relatives, CADASIL should definitely be considered, although a negative family history reported by the patient should not be considered a reason to exlude CADASIL.

The first diagnostic criteria were proposed by Davous (1998). The main aspects of diagnostic evaluation for probable CADASIL include:

- onset at young age (<50 years)
- two of the following clinical features: stroke-like episodes, migraine, mood changes or cognitive deficits
- absence of vascular risk factors
- positive family history
- white matter abnormalities in MRI

Several other diseases can create similar symptoms or imaging findings. The familial hemiplegic migraine (FHM, OMIM #141500) may sometimes include similar stroke-like symptoms as seen in CADASIL. Furthermore, FHM is also dominantly inherited and one of its forms (FHM1, *CACNA1A*) maps near the *NOTCH3* gene (Ophoff*, et al.*, 1996). White matter infarcts associated with hypertension or type II diabetes mellitus might arise from microangiopathy. Strokes at young age can be caused by the MELAS syndrome (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) (Nakamura*, et al.*, 1995, Melone*, et al.*, 2004). Akhvlediani*, et al.* (2007) described a patient with genetically proven mitocondrial encephalopathy whose brain white matter

hyperintensities in MRI strongly resembled those seen in CADASIL. Similarly, imaging signs mimicking multiple sclerosis (MS) have been described (Pandey and Abubacker, 2006), and, earlier, MS was a relatively common misdiagnosis for CADASIL patients. All unverified diagnoses resembling the CADASIL phenotype should be taken into account when assessing family history and searching for patients with appropriate symptoms in the pedigree.

Clinical diagnosing can produce only a suspicion of the CADASIL disease that then needs to be confirmed with method that specifically proves either the genetic or the pathological characteristics of the disease.

2.4.3. The skin biopsy

A CADASIL diagnosis can be confirmed from the skin biopsy of a clinically suspected CADASIL patient with an immunohistochemical identification of the accumulated N3ECD in ateries (Joutel*, et al.*, 2001). However, in Finland, this method has not been used in diagnostic services. Similarly, an EM analysis can be used to search for GOM deposits in arterial vessels (Ruchoux*, et al.*, 1995). Although the pathological meaning of the accumulated GOM is not fully solved, it is considered a definite proof of CADASIL as it has not been detected in any other disease (Ruchoux*, et al.*, 1995, Kalimo*, et al.*, 2008).

GOM deposits have been detected only in patients that are either clinically or genetically diagnosed to have CADASIL. GOM has not been detected in any other disease entity, and the specificity of GOM detection for CADASIL is well acknowledged. The reports on the sensitivity of detecting GOM in skin biopsies of patients with genetically verified CADASIL have been contradictory. Two early studies on a relatively small number of patients (Ebke*, et al.,* 1997; one family with 8 patients, mutation not specified; Mayer*, et al.*, 1999; three families, 14 patients, mutations not specified) suggested 100% sensitivity and specificity, whereas two more recent papers have reported significantly lower figures for sensitivity: Markus*, et al.* (2002) reported a sensitivity of only 44.4% (8 out of 18), while Razvi*, et al.* (2003) suspected that the sensitivity might be even lower. In the largest study so far, Peters*, et al.* (2005a) identified a *NOTCH3* mutation in 120 of 125 GOM positive patients, although the study design was the opposite; sensitivity of the *NOTCH3* mutation analysis was studied in a cohort where GOM detection was used as a criterion for the CADASIL diagnosis. When using the GOM detection as a diagnostic tool, it should be noted that even if GOM is not found, CADASIL cannot be definitely excluded, since it is always possible

that a single focal skin biopsy does not contain enough proper-sized arteries, or the quality of the biopsy is not adequate for the detection of GOM.

2.4.3. Genetic testing and genetic counselling

A definite diagnosis of CADASIL is obtained through genetic testing by showing the presence of a characteristic *NOTCH3* mutation (Joutel*, et al.*, 1997). If the pathogenic *NOTCH3* mutation in the suspected patients' family has been previously identified, that particular mutation can be tested directly with the most suitable method. If there is no previous CADASIL diagnosis in the family, the mutation can be sought by means of different approaches. If a mutation is identified in the index patient, the test can be then also be performed as a predictive test for any asymptomatic members of the family who are at risk of having the disease. An unknown mutation can be searched for either by testing directly for the known *NOTCH3* mutations or by screening (e.g. using sequencing, the SSCP or DHPLC analysis) the exons 2–24 for the CADASIL-type mutations. Since the mutational spectrum of *NOTCH3* is dependent on the population in question, different approaches are used in different countries.

Usually, the most practical and cost effective method for the detection of a particular mutation is a standard amplification of the target region with PCR and a restriction enzyme analysis of the amplicon with a suitable restriction enzyme. The enzyme that either cuts the mutated or normal allele creates a distinct restriction pattern visible in the agarose gel electrophoresis. These kinds of tests should always include negative and positive control samples, which can be used to ensure the reliability of the test. As a mutation screening method, the single strand conformation polymorphism (SSCP) analysis is no longer very widely used. Its sensitivity varies between 60 and 94% (Gross*, et al.*, 1999). Denaturing high performance liquid chromatography (DHPLC) is usually a sensitive method in mutation screening, but a high GC content may lower the sensitivity in case the of the *NOTCH3* gene (Escary*, et al.*, 2000). Direct sequencing is the most sensitive method in mutation screening, but nevertheless, its sensitivity is not 100% since, for example, large deletions and, depending on the screening approach, intronic mutations can be missed using this method.

During the years 1998–2004, in Finland and Sweden the routine diagnostic molecular genetic analyses have been limited to restriction enzyme analysis of the two previously found mutations in Finland, p.Arg133Cys and p.Arg182Cys. From 2005 onwards, it has been possible to complement this analysis with the sequence analysis of exons 3 and 4.

The genetic testing of an inherited disease should always include counselling on the meaning of the result – either negative or positive. The European Convention on Human Rights and Biomedicine (Council of Europe, 1997) includes a requirement of appropriate genetic counselling prior to predictive or carrier testing. However, most of the member countries have not ratified the agreement. The European Society of Human Genetics (ESHG) has investigated the national legislations and guidelines concerning the genetic counselling and produced recommendations regarding the process of genetic testing and counselling, which are reviewed below to the extent applicable to CADASIL.

Genetic counselling, which should be performed by a health care professional who is trained for genetic counselling, should be given in a language understood by the counsellee. The main aspects that should be included are the clinical course of the disease, treatment prospects, pattern of inheritance, and the reliability and limitations of the testing. The counsellees should be informed about their right to know or not-to-know the test result, their disease risk, how to inform their relatives about the result, and the consequences of the result for the counsellees and their families. Similarly, the psychological and emotional impacts of the result should already be taken into account in the pre-testing counselling, but especially in the post-testing counselling after the counsellee has heard the test result. When the patient is symptomatic and the genetic testing is used for verifying the clinical CADASIL diagnosis (i.e. diagnostic testing), counselling prior to the testing is not always necessary. However, testing should be based on the patient's consent after being given the adequate information about the testing, its purpose, and the meaning of either result. If the result of the diagnostic test is positive, the patient and their family members should always be offered genetic counselling. If an asymptomatic family member wishes to be tested, both pre- and post-test counselling has to be offered.

It should be noted that even though the *NOTCH3* mutation, and the GOM deposits in the EM examination of a skin biopsy can be detected before the age of 18, examination of samples from under-age patients should be refrained. In general, when there is no treatment or preventive measures available for the late onset disease, minors should not be tested, and this is applicable also for CADASIL.

2.4.4. Therapeutic possibilities

For the present, only symptomatic treatment is available for CADASIL. Acetazolamide treatment is suggested to improve impaired CBF in CADASIL

(Chabriat, *et al.*, 2000), and at least it has been succesfully used as a treatment for frequent and severe migraine attacks (Weller, *et al.*, 1998, Forteza, *et al.*, 2001). On the other hand, ergotamines and triptans should be avoided in the treatment of migraine because their vasoconstrictive effect can reduce the already impaired CBF in CADASIL patients (Kalimo, *et al.*, 2008). Some clinical trials with cholinesterase inhibitors have shown slight benefits for cognitive performance in vascular dementia (Kavirajan and Schneider, 2007), and the effects of treatment with donepezil were recently also studied in CADASIL patients (Dichgans, *et al.*, 2008). In this study, Dichgans *et al.* (2008) observed a slight improvement in executive functions, but the results were not statistically significant, and the clinical value of the treatment remained uncertain. Since the vascular pathology has been suspected to cause local thromboses, anticoagulants or antiaggregants have also been experimented with, but definite positive effects have not been achieved. In fact, in some patients these medicaments may have caused fatal parenchymal brain haemorrhages (Werbrouck and De Bleecker, 2006). In a few studies, L-arginine has been shown to induce an endothelium-dependent increase in CBF by vasodilatation (Reutens, *et al.*, 1997, Micieli, *et al.*, 1999, Zimmermann, *et al.*, 2004), but the effects in patients with a cerebrovascular disease have been contradictory. Zimmermann, *et al.* (2004) found that L-arginine increased the brain blood flow in patients with a history of strokes or TIAs and Koga, *et al.* (2005) have reported a decreased frequency and symptoms of strokes in MELAS, whereas Pretnar-Oblak, *et al.* (2006) found no difference in the L-arginine induced vasoreactivity between stroke patients and healthy controls. However, a recent study by Peters, *et al.* (2008) revealed enhanced L-arginine-induced vasoreactivity in CADASIL patients compared to controls. This may be due to an impaired endothelial function (Stenborg, *et al.*, 2007) and consequent decreased activity of endothelial nitric oxide synthase (eNOS). Recently, Rufa, *et al.* (2008) detected elevated levels of asymmetric dimethylarginine (ADMA), an inhibitor of eNOS, in CADASIL patients. L-arginine is the substrate for eNOS and its application may have a beneficial effect on a low eNOS activity. Thus, these studies suggest that L-arginine may have therapeutic potentials in CADASIL (Peters *et al.*, 2008), but the clinical relevance of the treatment is still open.

2.5. Epidemiology

Since the clinical characteristics and genetic background of CADASIL have been established and knowledge of the disease has increased, cases have been reported from all over the world and from all ethnic groups at an ever increasing rate. Altogether, well over 600 CADASIL families have been identified worldwide (Table 2). A great majority of the cases have been reported in Europe while surprisingly few cases have been reported in North America. However, a patient organization for the CADASIL patients (CADASIL Together We Have Hope) maintains an online registry which contains information on about five hundred CADASIL cases from the USA and Canada. This information is, however, based on personal notifications of patients and relatives and thus is not evidence-based.

There are no exact global incidence or prevalence figures available for CADASIL. In the west of Scotland, the prevalence of confirmed CADASIL cases in 2004 was 1.98 / 100 000 and an estimated prevalence based on the pedigree information was 4.15 / 100 000 (Razvi, *et al.*, 2005). However, the authors noted that these numbers are only minimum figures and likely to be underestimates. In Finland, a similar prevalence can be estimated (Kalimo, *et al.*, 2008), but it is noteworthy that until recent years, diagnostic genetic testing has been markedly restricted and the true number of CADASIL patients in Finland is still unknown. Due to the clinicians' lack of awareness of the disease and the associated lack of routine diagnostic services and criteria in many countries, CADASIL can still be regarded as a widely underdiagnosed disorder.

Table 2: The verified CADASIL cases in different countries.

	Country	Number of verified cases / families	LA / GOM / *NOTCH3* mutations	References
1	Finland	139 / 39	8 mutations	**This study**
2	Sweden	29 / 15	11 mutations	**This study**
3	Norway	10 / 3	2 mutations	1
4	Denmark	2 / 2	2 mutations	2, 3
5	UK	>220 / >70	at least 18 mutations	4-6
6	France	At least 80 / -	several mutations / GOM	7
7	Netherlands	- / 44	23 mutations	8
8	Belgium	3 / 1	p.Arg182Cys	9
9	Germany	> 450 / > 260	> 60 mutations	10
10	Austria	- / 1	GOM	11
11	Poland	3 / 2	GOM, 1 mutation	12
12	Romania	1 / 1	p.Arg141Cys	13
13	Portugal	40 / 30	13 mutations	14
14	Spain	- / 3	3 mutations	15-17
15	Italy	- / 68	28 mutations	18-25
16	Greece	10 / 4	4 mutations	26-29
17	Tanzania	1 / 1	p.Cys174Ser	30
18	Turkey	12 / 4	3 mutations	31, 32
19	Iran	1 / 1	p.Arg90Cys	33
20	Kuwait	9 / 3	LA, p.Arg110Cys	34, 35
21	Saudi-Arabia	3 / 1	p.Arg110Cys	35
22	Yemen	2 / 1	p.Arg133Cys	35
23	India	2 / 1	GOM	36
24	Thailand	- / 1	p.Arg110Cys	37
25	China	7 / 5	4 mutations	38, 39
26	Taiwan	11 / 8	5 mutations	40, 41
27	Japan	17 / 10	9 mutations	33, 42-46
28	Korea	34 / 13	7 mutations	33, 47-49
29	Australia	9 / 6	GOM, 2 mutations	50-52
30	USA	- / 8	GOM, 4 mutations	53-57
31	Puerto Rico	1 / 1	GOM	58
32	Columbia	26 / 2	2 mutations	59
33	Argentina	1 / 1	p.Arg169Cys	60
34	Chile	4 / 1	p.Arg141Cys	61

LA = linkage analysis, GOM = granular osmiophilic material. References: 1: Rein Gustavsen *et al.,* 2006 2: Tfelt-Hansen *et al.,* 2008 3: Binzer *et al.,* 2000 4: de Lange *et al.*, 2000 5: Thomas *et al.*, 2000 6: Markus *et al.*, 2002 & H.S. Markus, pers. com. 7: Joutel *et al.*, 1997 8: Lesnik Oberstein, 2003 9: Werbrouck & De Bleecker, 2006 10: Opherk *et al.*, 2004 & M. Dichgans, pers. com. 11: Dichgans *et al.*, 1998 12: Rafalowska *et al.*, 2003 13: Pescini *et al.*, 2007 14: Viana-Babtista *et al.*, 2007 15: Posada *et al.*, 2003 16: Rojas-Marcos *et al.*, 2004 17: Rojas-Marcos *et al.*, 2007 18: Dotti *et al.*, 2005 19: Ragno *et al.*, 2006 20: Mazzei *et al.*, 2007 a, b 21: Ragno *et al.*, 2007 22: Mazzei *et al.*, 2008 23: Pescini *et al.*, 2008 24: Pradotto *et al.*, 2008 25: Ungaro *et al.I,* 2008 a, b, c 26: Mandellos *et al.*, 2005 27: Vikelis*, et al.I,* 2006 28: Vikelis *et al.*, 2007 29: Andreadou *et al.*, 2008 30: K. Huoponen, pers. com. 31: Utku *et al.I,* 2002 32: Uyguner *et al.,* 2006 33: Santa *et al.*, 2003 34: Pandey & Abubacker, 2006 35: Bohlega *et al.*, 2007 36: Panagariya *et al.*, 2004 37: Suwanwela *et al.*, 2003 38: Wilder-Smith *et al.*, 2004 39: Wang *et al.,* 2004 40: Tang *et al.I,* 2005 41: Lee *et al.I,* 2006 42: Kotorii *et al.*, 2001 43: Murakami *et al.*, 2001 44: Matsumoto *et al.*, 2005 45: Nakamura *et al.*, 2005 46: Oki *et al.*, 2007 47: Moon *et al.*, 2003 48: Kim *et al.*, 2006 a, b 49: Oh *et al.*, 2008 50: Grigg *et al.*, 2000 51: Chuah *et al.*, 2001 52: Kleinig *et al.*, 2007 53: Hedera & Friedland, 1997 54: Desmond *et al.*, 1998 55: Lanska & Markesbery, 1999 56: Van Gerpen *et al.*, 2003 57: Maclean *et al.*, 2005 58: Rubio *et al.*, 1997 59: Arboleda-Velasquez *et al.*, 2002 60: Zurru *et al.*, 2002 61: Miranda *et al.*, 2006

3. THE AIMS OF THE STUDY

This study focuses on the genetic background of CADASIL, but is also closely linked to the patients' view of the diagnostic services. Although the *NOTCH3* mutations were already identified as the underlying defect behind CADASIL in 1996, the diagnostic services in Finland have been strongly limited for a long time. An offshoot of this study was the goal to improve the methods of genetic diagnostics in Finland. The sequencing protocol of the *NOTCH3* exons 2–24 (IV) was also established in order to complement the previously available diagnostic tools.

The specific aims of the study were:

I to investigate whether the Finnish CADASIL patients with the p.Arg133Cys mutation descend from a common ancestor in the past and represent a founder effect.

II to explore two candidate genes, *APOE* and *AGT*, as well as *NOTCH3* polymorphisms as the possible genetic factors, which could contribute to the phenotypic variation in CADASIL.

III to detect possible environmental factors or life-style choices which may affect the phenotype of CADASIL by investigating the diagnostic cases of a pair of monozygotic twins with marked differences in the onset and course of their CADASIL disease.

IV to compare the mutational analysis of the *NOTCH3* gene and the electronmicroscopic examination of a skin biopsy as diagnostic tools in CADASIL.

4. MATERIALS AND METHODS

4.1. Subjects

4.1.1. The Finnish p.Arg133Cys families (I)

Altogether, 60 patients from 18 Finnish CADASIL families carrying the p.Arg133Cys mutation were haplotyped for ten microsatellite markers around the *NOTCH3* locus. Ten of the families originated from Mid-Ostrobothnia, six from Savo-Carelia and two from Satakunta. The CADASIL diagnosis of the patients was based on the genetic confirmation of the p.Arg133Cys mutation in exon 4 of the *NOTCH3* gene.

Ten healthy family members were included in the analysis to elicit the allele segregation in the families. The alleles from patients' non-disease chromosomes, patients' spouses and healthy siblings, as well as alleles of 25 Finnish control samples were used as controls.

4.1.2. The 120 Finnish and Swedish CADASIL patients (II)

Altogether, 111 Finnish (from 24 families) and 9 Swedish (from 5 families) CADASIL patients were analysed. All 120 patients were analysed for the *APOE* alleles, *AGT* polymorphism and neutral intragenic *NOTCH3* c.684G>A polymorphism. A smaller subgroup of 50 patients was analysed for the seven intragenic amino acid changing polymorphisms of the *NOTCH3* gene. The CADASIL diagnosis of the patients was based either on the detection of a mutation in the *NOTCH3* gene or on the detection of GOM in the skin biopsy.

4.1.3. The Swedish monozygotic twins with CADASIL (III)

The clinical symptoms, imaging, electron microscopic and genetic background of a pair of Swedish monozygotic twins were studied. The twin brothers had a marked discordance in their clinical picture of CADASIL. The diagnosis of the twins was based on the detection of GOM deposits in an EM examination of the skin biopsy.

4.1.4. The 131 diagnostic cases from Finland, Sweden and France (IV)

The study was conducted as an investigation (mainly retrospective) of diagnostic cases. The subjects comprised 131 CADASIL patients diagnosed in three different countries (38 from Finland, 13 from Sweden and 80 from France) and 26 control subjects (4 from Finland, 2 from Sweden and 20 from France), in whom both the analysis of the *NOTCH3* gene and the EM examination of the skin biopsy had been adequately performed. Confirmation of the CADASIL diagnosis was based on a positive result in either analysis.

4.1.5. Clinical data (II, III)

The clinical data of the 120 CADASIL patients (II), including the Swedish twin brothers (III), were collected using the medical records and questionnaires filled in by the patients or their caregivers to assess the patients' health status, health behaviour, and medical history. In the questionnaire, the main areas of information enquired about were the occurrence of and age at the time of the first ever TIA and/or stroke (Aho*, et al.*, 1980), as well as the occurrence of migraineous headache, the history of myocardial infarction and vascular risk factors, such as hypertension (>140/90mmHg or antihypertensive medication), diagnosis of diabetes mellitus, and high total serum cholesterol (>6.2mmol/l or statin therapy). Height and weight were also asked about, the body mass index (BMI) was calculated and >25.0kg/m^2 was considered overweight (World Health Organization, 2000). The patients were grouped into smokers and non-smokers according to their current smoking habit, and into heavy drinkers (men >280g of alcohol/week, women >190g/week), moderate drinkers (men <280g/week, women <190g/week) and non-drinkers according to their current alcohol consumption (Sillanaukee*, et al.*, 1992).

4.2. Ethical aspects

The study was approved by the ethics committee of the Hospital District of Southwest Finland and Turku University Hospital in Finland and by the ethical committees of Huddinge University Hospital and Uppsala Academic Hospital in Sweden. All samples were sent to the Department of Medical Genetics at Turku University during the years 1996–2006 either as research samples or for diagnostic CADASIL testing. Use of diagnostic and research DNA samples in the study and the collection of patients' medical information was licensed by the National Authority for Medicolegal Affairs (TEO) and the Ministry of Social Affairs and Health (STM).

4.3. Methods

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

The patients' and controls' leukocyte DNA was extracted from etylenediaminetetraasetic acid (EDTA) blood samples, or in one case from a *post mortem* brain tissue sample, by the standard proteinase K/phenol-chloroform method or with the Nucleon BACC3 Genomic DNA Extraction Kit (GE Healthcare, Buckinghamshire, UK).

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

The presence of the two mutations previously identified in Finnish CADASIL patients, p.Arg133Cys and p.Arg182Cys, was analysed by means of amplification of exon 4 with specific primers (Table 3) in a standard PCR reaction, and using a restriction analysis of the amplicons with the MspAI1 restriction enzyme. Both mutations destroy a recognition site of the MspAI1 enzyme and the presence of the mutations can be concluded on the basis of the restriction pattern on agarose gel electrophoresis.

4.3.3. Genealogical investigation (I)

The genealogies of the families were studied using the Finnish church records. Many of the families could be traced backwards up to ten generations and some of the families coalesced into larger pedigrees during the process. After the investigation 18 pedigrees without evident ancestral connection remained. Related nuclear families were considered independent in age estimation analyses and at least six separating meiotic events was used as the criterion for distinct haplotypes.

4.3.4. Haplotype analysis (I)

Ten polymorphic microsatellite markers flanking the *NOTCH3* gene were selected in order to haplotype the patients and controls. Four markers were located telomeric and six markers centromeric of the *NOTCH3* gene. All markers were dinucleotide repeats, with the exception of one tetranucleotide marker. The markers covered a DNA region of 12.3 Kosambi cM (KcM, sex-averaged). The order of the markers and genetic distances were obtained from the Genetic Location Database (LDB) and the Marshfield sex-averaged genetic map for chromosome 19. Information on primer sequences (Table 3), allele frequences and allele sizes was obtained from the Genome Data Base (GDB).

The microsatellite regions were amplified in a standard PCR reaction with a radioactive ^{32}P-label. The lengths of the microsatellite repeats were determined using denaturing polyacryl gel electrophoresis (PAGE). The haplotypes linked to CADASIL were determined by the allele segregation in each family.

4.3.5. Age estimation of the mutation (I)

To assess the age of the p.Arg133Cys mutation in the Finnish CADASIL cohort the linkage disequilibrium (LD) data from 18 families were analysed with the DMLE+ program (version 2.14) (Rannala and Reeve, 2001, Reeve and Rannala, 2002). The program uses a Bayesian inference with the Markov chain Monte Carlo method to calculate the mutation age from LD data of the markers. The following parameters were used: (1) the location of the *NOTCH3* locus within the haplotype, $\theta = 0.06$; (2) an estimated population growth parameter λ, with $\lambda = 0.14$ selected as the final value based on Finnish population history and census data. The program was tested for variation sensitivity to this parameter

with the values 0.10 and 0.20. (3) For the proportion of the p.Arg133Cys alleles in our sample, of all the p.Arg133Cys carriers in the Finnish population, a final value of 0.12 was used (based on the assumption that the real number of carriers was ca 200, given the dominant model of inheritance and presumed underdiagnosis). The sensitivity to this parameter was also tested using the values 0.05, 0.10 and 0.20. In each of the settings, either one of the parameters was kept at its final value and an identical seed was used for the pseudorandom number generator. Ten million iterations were performed. As all of the used markers located outside the gene region, uniform priorities were used.

4.3.6. Sequencing of the *NOTCH3* exons 2-24 (II, III, IV)

Since the skin biopsy had shown the presence of GOM in 9 Finnish and Swedish suspected CADASIL patients without the mutation being detected, an extended genetic analysis for *NOTCH3* was established. For the detection of the CADASIL type mutation in these patients and their 3 relatives, exons 2-24 of *NOTCH3* were amplified with 16 sets of specific primer pairs (Table 3), and subsequently amplicons were sequenced using an automated sequencing system (Applied Biosystems, CA, USA). The quality of the sequences was checked and preliminary analyses were conducted with Sequence Scanner v1.0 (Applied Biosystems) and individual sequences were also analysed manually against the reference sequence.

4.3.7. *APOE* and *AGT* genotyping (II)

Two candidates for the genetic contributor for the clinical picture of CADASIL were studied: *Apolipoprotein E* (*APOE*) and *Angiotensinogen* (*AGT*). 120 patients were genotyped for the three most common isoforms of *APOE* (ε2, ε3 and ε4), and the *AGT* p.Met235Thr (c.704C>T) polymorphism. The *APOE* isoforms were genotyped with a standard PCR reaction with specific primers (Table 3) that amplify the DNA region containing the codons 112 and 158 followed by an automated sequencing of the amplicons with the ABI3100 system. The *AGT* region coding for the codon 235 was amplified in a PCR reaction where the other one of the primers contains a mismatch nucleotide (Table 3) which, with the c.704C>T transition, produces a restriction site for the Tth111I enzyme (Russ, *et al.*, 1993). Genotypes were inferred on the basis of the restriction pattern after agarose gel electrophoresis.

4.3.8. The analysis of *NOTCH3* intragenic polymorphisms (II)

A subgroup of 50 CADASIL patients were analysed for seven amino-acid-changing polymorphisms of *NOTCH3*: p.His170Arg, p.Gly288Ala, p.Pro496Leu, p.Ser497Leu, p.His1133Gln, p.Val1183Met and p.Ala1852Thr. For 20 patients, the polymorphisms were genotyped with a heteroduplex analysis in an automated denaturing high performance liquid chromatography (DHPLC) system (Wave™, Transgenomics, Omaha, USA), and for 30 patients with direct sequencing using an automated ABI3100 system. In addition, a neutral *NOTCH3* c.684G>A polymorphism was analysed from all 120 patients by means of PCR amplification of exon 4 and restriction analysis with the HhaI restriction enzyme, which cuts the G allele of the polymorphism.

4.3.9. Association analysis (II)

The Cox proportional hazards regression model was used to analyse the associations between the *APOE* genotype or *AGT* p.Met235Thr polymorphism and the clinical course of CADASIL. The first ever stroke was used as the outcome representing the clinical course of CADASIL. First, the analyses were conducted unadjusted (the crude model), and then adjusted for age and sex (model 1). Furthermore, the analyses were additionally adjusted for hypertension, hypercholesterolemia, and history of myocardial infarction (model 2). Finally, the analyses were adjusted additionally for smoking and alcohol consumption (model 3). All the analyses were also repeated with a linear mixed model with siblings as a random effect. The correlation between the neutral c.684G>A *NOTCH3* polymorphism and migraine was analysed with logistic regression with siblings as a random effect. All of the Cox proportional hazards regression analyses were conducted separately for a subgroup of patients carrying the p.Arg133Cys mutation (n = 106) and for all CADASIL patients (n = 120). The analyses were also conducted separately for younger (age <50 years) and older (age ≥50 years) patients. All statistical analyses were conducted using either the SPSS program, version 15.0 (SPSS Inc. Chicago, USA) or the SAS program, version 9.1 (SAS Institute, Cary, USA).

4.3.10. Electron microscopy (IV)

The skin biopsies of 51 Finnish and Swedish CADASIL patients and 6 controls were taken at the hospitals where the patients or their relatives were examined. The skin biopsies were fixed in phosphate buffered 3–4% glutaraldehyde and sent

to the Laboratory of Electron Microscopy at the University of Turku or Helsinki in Finland, or to the Laboratory of Electron Microscopy at the Department of Pathology, University of Uppsala in Sweden. In France, the biopsies, fixed in either Carson or Trump liquids or as above, were sent to the Laboratories of Electron Microscopy at Hôpital Roger Salengro, University of Lille or at Hôpital Bretonneau, University of Tours, France. The samples were postfixed in buffered osmium tetroxide, dehydrated in ascending grades of ethanol, and embedded in Epon. Semithin sections (0,5–1 μm) were cut and stained with toluidine blue for selecting arteries of appropriate size (external diameter 20–40 μm) for thin sectioning. Thin sections were double stained with uranyl acetate and lead citrate and then examined in transmission electron microscopes at the EM laboratories mentioned above. The skin biopsies were analysed by professional electron microscopists in Finland and in France.

Table 3: The primer sequences and PCR conditions.

	Primer sequences (5'→3')	T_{ann} and special PCR conditions
D19S221	GCAAGACTCTGACTCAACAAAA CATAGAGATCAATGGCATGAAA	58°, 2% formamide, 0.5 μCi [α-^{32}P]-dCTP
D19S840	ATAGGCCAAGACTGTCTAAAACAA GCCCTAACTGCTGTAAGAGAACT	60°, 10% glycerol, 0.5 μCi [α-^{32}P]-dCTP
D19S415	GATGTTGTTGCATTGGC GGGTCATGGTATGCTCC	55°, 10% glycerol, 0.5 μCi [α-^{32}P]-dCTP
D19S929	TGATTTGGTGGATATTAGCCT CAACAGTGTGGCAGGG	60°, 2% formamide, 0.5 μCi [α-^{32}P]-dCTP
D19S411	AAAATTTAAAAACAGTAGGCTTCAG GATAAAATATCACTGAGGAGTTGC	50°, 10% glycerol, 0.5 μCi [α-^{32}P]-dCTP
D19S885	CTGGGTGACAGAGTGGG TAATGGAGAAACTGGCTCG	55°, 2% formamide, 0.5 μCi [α-^{32}P]-dCTP
D19S930	GTGGGCACATGGGTGA GGTGCTGTGTGAGCCC	60°, 2% formamide, 0.75 μCi [α-^{32}P]-dCTP
D19S593	TAGAGATGACAGATGAAGAGATGG CCACCCCAGAGAGTGAGC	55°, 2% formamide, 0.5 μCi [α-^{32}P]-dCTP
D19S410	ACCTCTCCAGCAGTACCATCTG CTGACAGCAGCCCCCA	57°,2% formamide , 0.5 μCi [α-^{32}P]-dCTP
D19S215	CATGCATTAAAAATGACAACTGT GCTCTGCANTCCATTACTCA	57°, 2% formamide, 0.5 μCi [α-^{32}P]-dCTP
N3ex2	GAGGGGGTTTGTCACTTGG AACACAGAGGCAGAGGGAGA	62°, 1 × GC-RICH Solution
N3ex3	TGTGCTGCCCAACCAAGCCA TCCAGACTCTTCCCCTCTCA	65°
N3ex4	TAGTCGGGGGTGTGGTCAGT CCTCTGACTCTCCTGAGTAG	65°
N3ex5+6	TGAGTGAGCCCTACTCAGGA GCCCTCACTAAAAACCATCC	58°
N3ex7+8	TGGGCAGAGCAGGAAGAT GCCCCCTGCCTCAGGAC	63°
N3ex9+10	ACCCCGTTCACACCATAGG CCGCCTCCTGATTCTTGTC	62°
N3ex11+12	AAGTGGGCGGAGCCTGAC TCGATGTAAGGACCCCCTCT	64°
N3ex13+14	CTGGTTGTCCCTGCTGACTT AGAAGGCCCATGGTGTTG	62°
N3ex15	GGGAGTCCCTCAAGGCTATC GCAGAGGAGATGGAGAGGAG	63°
N3ex16	CCCTGCTCTGTACCCTGTAA TGTTCCCCAGAGCAGCAC	62°
N3ex17	CTAATGGGGGCAAGGTAGGT AAGCCAGAGTCCCTGCTCTC	64°
N3ex18+19	GATCCTCCCTCCCACTCCT CTTCCCAAGGCCCCACAC	62°, 1 × GC-RICH Solution
N3ex20	TGGGGTTACCTCTGTTCCTG CCCACCTCCTCTTCCCTCT	62°
N3ex21+22	GGTCTGTGTCCCACTAAGCTG CAGCCACAATGGGGGAAT	62°
N3ex23	CTGTCATTCCCCCATTGTG GCCCCTACTCCTCCTCCA	61°
N3ex24	CCCCACCCTCATTTTTATCC AAACAGACTGGGATGGATGC	56°, 1 × GC-RICH Solution
APOE	GGCACGGCTGTCCAAG GCGGATGGCGCTGAGG	59°, 10% DMSO
AGT	CAGGGTGCTGTCCACACTGG<u>AC</u>CCC CCGTTTGTGCAGGGCCTGGCTCTCT	69°, final extension 10 min

T_{ann}, annealing temperature; DMSO, dimetyl sulfoxide; GC-RICH Solution (Roche, Basel, Switzerland); mismatch sequence in *AGT* primer underlined.

5. RESULTS AND DISCUSSION

5.1. The founder effect and age of the ancestral p.Arg133Cys mutation in Finland (I)

Linkage disequilibrium in the haplotype data showed that the analysed 18 CADASIL families carrying the p.Arg133Cys mutation share a similar haplotype linked to the *NOTCH3* gene. The region of the strongest linkage disequilibrium covers six markers and spans 6.47 KcM. The consensus haplotype shared by the families is clear evidence of a common ancestor and a single founder effect among the Finnish families carrying the p.Arg133Cys mutation. The most common haplotype in the families for all markers represents the founder haplotype inherited from a common ancestor. This founder effect for the p.Arg133Cys mutation is the first reported in CADASIL worldwide. The relatively long region of the linkage disequilibrium in the families suggests a relatively young founder mutation. However, the genealogical research of the family pedigrees reaches back as far as 10 generations without one common ancestor and therefore the origin of the ancestral mutation must arise from an earlier event.

The data for the age estimation of the founder mutation consisted of 24 mutation-linked haplotypes and 48 control haplotypes, among which 12 and 28 were complete, respectively. Each haplotype was included once from each family. The age analysis of the founder mutation using the most likely parameters resulted in a maximum age at 12.2 generations. The parameter changes for population growth and for the proportion of p.Arg133Cys chromosomes did not produce any significant changes in the results. When the generation span is considered to be 25 years, the introduction of the ancestral mutation to the Finnish population would date back to the late 1600s or the beginning of the 1700s.

Finnish population history offers one logical explanation for the clustering pattern of the families carrying the p.Arg133Cys mutation and age estimation of the founder mutation. From the mid 1500s through the 1600s, people from the South Savo region migrated as a result of taxation favouring the settlement of unpopulated regions. At first the settlers moved mainly to the eastern, central and northern parts of Finland (Norio, 2003), but some continued onwards to sparsely populated Central Ostrobothnia, and smaller groups continued even to Sweden and Norway (P. Virrankoski, 2003, personal communication). In the light of this internal migration (Figure 5) and clustering of the CADASIL families in the

western coast as well as the Savo-Karelia region, it can be hypothesized that the mutation could have originated in the area of South Savo and then migrated northwards and also to the western coast. The p.Arg133Cys mutation has been found also in Sweden and Denmark. Whether these mutations also represent the same founder effect, remains unresolved. It cannot be distinguished whether the original mutation event occurred in Finland or whether the mutation has been introduced into the Finnish population from elsewhere. Migration of the mutation in the opposite direction is also possible but more unlikely considering the Finnish population history combined with this age analysis.

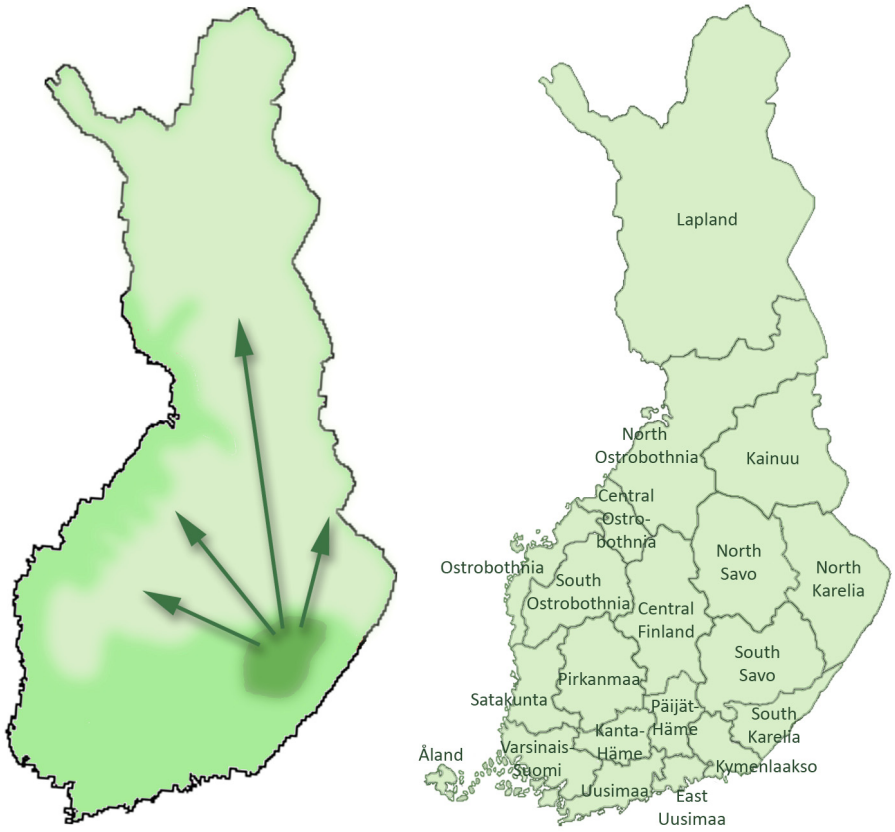

Figure 5: Population expansion in South Savo and the following migration from the mid-1500s through the 1600s (left). The area of early settlement (light green) concentrated in along the southern and western coast of Finland. The late settlement (dark green and arrows) expanded northward and westward from South Savo. Regions of Finland are presented on the right. Revised from a figure by Norio (2003).

Founder or major mutations may ease the genetic diagnostics if the majority of the patients belong to the families carrying these mutations. In Finland, the vast majority of the families and patients carry the p.Arg133Cys mutation (see Table 7 in Chapter 5.4.), and it is practical to start the diagnostic work with the relatively simple analysis of this mutation, especially if the patient or the patients' family originates from the regions mentioned above. The genealogy of the families in this study was extensively researched, and a brief enquiry into the family history might even reveal a connection to a pedigree with a known mutation.

5.2. Association of the *APOE, AGT* and *NOTCH3* polymorphisms with the CADASIL phenotype (II)

The study cohort included 60 females and 60 males. The mean age was 54, ranging from 18 to 93 years. Among the patients analysed, 109 had had TIA, a stroke or migraineous headache. For all the patients, the CADASIL diagnosis was confirmed by mutation analysis of the *NOTCH3* gene (IV). The number of patients with the different *NOTCH3* mutations in the two patient groups for genetic analyses is presented in Table 4.

Table 4: The spectrum of *NOTCH3* mutations in the two patient groups.

Mutation	Patients analysed for: *APOE*, *AGT* and *NOTCH3* c.684G>A	Patients analysed for: 7 *NOTCH3* polymorphisms
p.Arg90Cys	1	1
p.Arg133Cys	106	45
p.Cys174Arg	3	3
p.Arg182Cys	1	-
p.Cys206Tyr	2	-
p.Cys251Tyr	2	1
p.Tyr1069Cys	5	-
	n=120	n=50

Abbreviations: *APOE*, *Apolipoprotein E*; *AGT*, *Angiotensinogen*; G, guanine; A, adenine; p, protein; Arg, Arginine; Cys, Cysteine; Tyr, Tyrosine.

5.2.1. The *AGT* and *NOTCH3* polymorphisms

The frequencies of the *AGT* p.Met235Thr genotypes in the cohort were 19.2% for Met/Met, 56.7% for Met/Thr and 20.0% for Thr/Thr. None of the genotypes produced a statistically significant association with the first ever stroke (e.g. all patients, Thr/Thr genotype, crude model: hazard ratio, HR, 0.76 95 %, confidence interval, CI, 0.40–1.46, and model 3: HR 0.81 95% CI 0.35–1.90). The result suggests that the *AGT* polymorphism does not contribute to the variation in the clinical course.

No association was detected between the neutral *NOTCH3* c.684G>A polymorphism and migraineous headache (A/A vs. G/G, odds ratio, OR, 0.71 95%, CI 0.01–40.59; A/G vs. G/G, OR 1.15 95%, CI 0.27–4.86). None of the seven analysed amino-acid-changing *NOTCH3* polymorphisms was detected in the studied fifty patients. This suggests too a small frequency of these alleles in the Nordic population for any conclusions about their influence on the clinical course of CADASIL to be made.

5.2.2. *APOE*

The relative *APOE* allele frequencies in the cohort were 5.4% for ε2, 77.5% for ε3 and 17.1% for ε4, which corresponds well with the frequencies previously detected in the Finnish population (Schiele*, et al.*, 2000). Among all patients, a trend for *APOE* ε4 genotype association with earlier occurrence of the first ever stroke was observed after full adjustments (HR 1.68 95%, CI 0.83–3.42). In the age-stratified analysis adjusted according to model 2, a significant predisposing effect of the *APOE* ε4 allele to the earlier occurrence of the first ever stroke was found in younger patients (HR 6.62 95%, CI 1.46–30.13). The association was further strengthened when additional adjustments for life-style factors were made (HR 9.71 95%, CI 1.92–49.19). A corresponding trend, but not statistically significant, was also observed for the older patients (model 2: HR 1.13 95%, CI 0.59–2.16 and model 3: HR 1.74 95%, CI 0.71–4.28). Analysis replication with a linear mixed model and sibling as a random effect did not produce any significant changes (e.g. age stratified analysis, model 3 for the younger patients, β coefficient 6.40 95%, CI 1.82–11.00).

In this study, the *APOE* ε4 allele was shown to predispose to earlier occurrence of stroke in young patients after adjusting for cardiovascular risk factors. In the

two previous CADASIL studies, no influence of *APOE* ε4 on the CADASIL phenotype was found (Singhal*, et al.*, 2004, van den Boom, *et al.*, 2006); thus this is the first study showing that *APOE* ε4 can influence the clinical course of CADASIL. Several differences can be detected between these studies, which may contribute to the discordant results. In the previous investigations, the numbers of *APOE* ε4 carriers were smaller (16 and 8) than in our cohort (39). Singhal *et al.* (2004) found no association between the *APOE* ε4 allele and stroke/TIA, dementia or disability scores (Rankin scale). TIA together with stroke was used as the end-point in the previous studies instead of the definite first ever stroke as in this study. TIA might be difficult to distinguish from other neurological deficits (i.e. migraineous aura), which could lead to different data and consequently also to altered results. TIA together with stroke was also tested as the end-point in this cohort and the association was clearly less evident. Moreover, van den Boom, *et al.* (2006) focused on the neuroradiological signs of the disease rather than on the clinical manifestations.

The age-dependent effect of *APOE* ε4 could be explained by the fact that in the early phase of CADASIL the arteries are less severely altered (Ruchoux and Maurage, 1997, Miao*, et al.*, 2006), and genetic factors other than the *NOTCH3* mutation, such as the *APOE* ε4 allele, can still have an aggravating effect on the endothelial dysfunction in arteries. In the later stages, the alterations in the arterial walls caused by CADASIL could be so advanced that other factors can no longer have a significant influence on the arteriopathy. Similarly, sporadic cerebral ischemia associates with *APOE* ε4 in young and middle-aged (Kokubo*, et al.*, 2000, Pezzini*, et al.*, 2004) but not in elderly patients (Basun*, et al.*, 1996, Zhu*, et al.*, 2000). In a vascular disease like CADASIL, the increasing effect of ApoE on stroke risk is most likely mediated by its involvement in vascular functions. For example, ApoE acts as a signalling molecule in the vessel walls (Hui, 2004), and in CADASIL, endothelium-dependent vasodilatation is impaired (Stenborg, *et al.*, 2007). *APOE* ε4 is also known to associate with elevated levels of low-density lipoprotein cholesterol (Stojakovic, *et al.*, 2004) exacerbating the atherosclerosis of the arterial walls, and might also thereby increase the stroke risk. *APOE* ε4 has been shown to associate with more severe brain damage in traumatic brain injuries (Smith*, et al.*, 2006) and also with cognitive impairment (Shi*, et al.*, 2008) and a faster progression rate (Fazekas*, et al.*, 2001) in multiple sclerosis (MS). All these associations suggest that *APOE* ε4 allele has a wide-ranging effect on general function – as well as malfunction – of the brain.

A limitation of this association study is the small sample size, which is an inevitable consequence of the low incidence of CADASIL. The small number of subjects in this study leads to compromised statistical power in the analyses, which in this study is reflected as the large confidence intervals, especially in the

analyses for the smaller subgroups (younger vs. older patients). However, simultaneously with widened confidence intervals the point estimates increased noticeably indicating a clear increase also in the actual hazard ratios. Therefore, even if the wide confidence intervals limit the inferential value of the observations, the high point estimates clearly suggest an association between the *APOE* ε4 allele and earlier stroke among CADASIL patients. An advantage of this study cohort is the large number of patients with the same p.Arg133Cys mutation. In addition to the mutational homogeneity, Finnish CADASIL patients can be expected also to have other similarities in their genetic background due to the founder effect and the relatively small size of the Finnish population. This may make detection of the differential genetic factors more challenging, but nevertheless, the associations found can be considered reliable.

5.3. Environmental factors and the CADASIL phenotype (III)

To evaluate the clinical variation and reveal possible candidates for contributing environmental factors, this study investigated the symptoms and clinical findings in a Swedish pair of identical twins suffering from CADASIL (referred as twin A and B).

Genetic studies

The monozygosity of the twin brothers was confirmed with a DNA test with 10 genetic markers (99.9% likelihood, National Public Health Institute (KTL), Finland). A previous analysis of *APOE* revealed that the genotype is ε3/ε4 for both twins. The CADASIL diagnosis was established first in twin B, which prompted a search for the *NOTCH3* mutation. Sequencing analysis revealed a substitution c.752G>A in exon 5. The mutation leads to replacement of a cysteine with a tyrosine in amino acid position 251 (p.Cys251Tyr) in the 6th EGF repeat of the NOTCH3 receptor. The mutation is novel and previously unreported. Three other mutations have been described for this same codon (Markus*, et al.*, 2002, Lesnik Oberstein, 2003, Vikelis*, et al.*, 2007). The new p.Cys251Tyr mutation follows the archetypal principle of CADASIL-causing mutation, resulting in an odd number of cysteine residues in one of the EGF repeats; thus, it most likely is the pathogenic cause of the disease in the twins.

Retrospective investigation of clinical data in the twins and their parents

From the age of 70, the twins' mother suffered from episodes of dysarthria, had difficulties in concentrating, developed progressive memory deficits and became ever increasingly passive. The neuropsychological examination at the age of 73 showed generally declined cognitive functions. In addition, her brain MRI showed widely scattered white matter hyperintensities as well as multiple small infarcts, on the basis of which vascular dementia was diagnosed. She died some months later of a gastrointestinal malignancy. The *post mortem* examination of the brain did not show any macroscopic pathological alterations. Neuropathological examination was not performed, but in periodic acid Schiff (PAS) staining, positivity characteristic of CADASIL was observed in the histopathology of the kidney arteries. The twins' father died at the age of 81 after suffering from a dementing disease. Based on clinical assessment and a *post mortem* neuropathological examination, he was diagnosed as having Alzheimer's disease. The twins are the only children in the family. The pregnancy and delivery were uneventful. The nature of their placentation is unknown. The birth weight of twin A was 2340g and of twin B 2150g, and the height for both was 46.5cm. The main clinical symptoms and findings in the twins are presented in Table 5.

Twin B went to a normal elementary school and high school (a total of 12 years). He later worked most of the time as a salesman. His alcohol consumption was light, but he smoked daily from the age of 14 to the age of 38. At the age of 52 he was normotensive and while on atorvastatin treatment his fasting plasma lipid levels were within recommendations. Regarding physical exercise, he took light exercise regularly from early adulthood to the age of 54 when he had to stop due to physical exhaustion. Since the age of 53, twin B has been permanently unable to work and on disability allowance.

Twin A went to normal elementary and vocational schools (11 years). His alcohol consumption was moderate and he has never smoked. He has been physically active since youth. He has been on simvastatin treatment from the age of 43 due to a hypercholesterolemia. He works in the field of computer system development and is still working part-time.

Table 5: Clinical symptoms and findings in Swedish monozygotic twins with CADASIL.

Symptom / finding	**Twin A**			**Twin B**		
Ischemic attacks (age), **Symptoms**	2 strokes (53y, 55y), TIA (54y) Dysarthria, dysphasia, loss of consciousness, nausea, abdominal pain, dizziness, spell of confusion			2 strokes (39y, 48y, 49y, 54y), TIA (48y) Dysarthria, visual symptoms, loss of consciousness, nausea, chills, cold sweat		
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
No of lacunar infarcts			11			16
PET **at the age of 50**	CBF decreased 2 SD only in the pons.			CBF decreased over 2 SDs in the cerebral WM and in all 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. **At the age of 55 years**: deterioration in verbal episodic memory, complex visuospatial abilities and visual memory.			**At the age of 48 years**: 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**: further deterioration in verbal fluency, visuospatial abilities, verbal delayed memory, visual memory. Recovery in verbal learning and memory retrieval tasks.		
Medication	Simvastatin (from the age of 43) Aspirin, low dosage Antidepressants Folic acid B vitamins Dipyridamol			Atorvastatin (from the age of 47) Aspirin, low dosage Folic acid B vitamins		
Other	Polychytemia, treated with venesection					

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)

Phenotypic and life-style factors differentiating the twins

Despite the identical genetic background, the age at onset and the course of the disease in these twins is different. In twin B, the first ever ischemic attack occurred 14 years earlier, and all the symptoms, including cognitive decline, signs and imaging findings were more severe than in twin A. Twin B had experienced at least four ischemic attacks before twin A had his first definite attack. Furthermore, the MRI and PET findings were clearly more severe in twin B. In neuropsychological examinations, the twins showed, in general, similar profiles, but differed in the level of performance and progression. Although the localisation of the cerebral infarcts may have an effect on the neuropsychological profiles, the twins' individual profiles were similar, which suggests that there are no other major contributors besides CADASIL. In twin A, a clear deterioration of cognitive skills was observed after the onset of ischemic attacks. However, in twin B, some recovery was seen in the evaluation at the age of 55, which is relatively frequently seen among CADASIL patients (K. Amberla, personal communication).

Since we can assume that there are no genetic differences between monozygotic twins, the variation in the onset and manifestation of symptoms and signs may be ascribed to environmental factors and life-style choices. Differences in intrauterine environment can already induce developmental differences which modify disease severity later in life. Even though the twins' birth weights differed only slightly and the post partum periods were uneventful, we cannot exclude the influence of other possible differences, such as intrauterine placement or asymmetric placental blood flow (Rose, 2005). All environmental factors, also later in life, may have direct effects at the tissue level. On the other hand, it has been shown that differences in DNA methylation and histone modifications increase between monozygotic twins during their lifetime (Fraga*, et al.*, 2005). These alterations store different external signals caused by non-shared environmental elements as epigenetic information. Possible external signals include differences in nutrition, physical and mental activity, smoking and use of alcohol. Statin treatment (Di Mascio*, et al.*, 2000, Asahi*, et al.*, 2005) as well as physical activity (Gillum*, et al.*, 1996, Hu*, et al.*, 2000) has been shown to reduce stroke risk. Smoking is a well-known risk factor for stroke in the general population (Shinton and Beevers, 1989) and increased stroke risk caused by smoking has also been verified in CADASIL patients by Singhal*, et al.* (2004). In this study (II), the *APOE* ε4 allele was shown to even accentuate the effect of smoking. Since twin B had smoked in early adulthood for several years, this, accentuated by one *APOE* ε4 allele, is a likely contributing factor for his earlier ischemic attacks. Furthermore, twin A's longer regular use of a statin (simvastatin) could also have contributed to the delay in the appearance of his symptoms and milder disease phenotype, although, in a study with CADASIL patients, no significant effects of another statin (atorvastatin) on haemodynamics was detected

(Peters*, et al*., 2007). In addition, twin A's longer duration and higher intensity of physical activity can be considered one possible beneficial factor.

CADASIL is a late onset disease with a drastic outcome and for the moment no curative treatment is available. Therefore identifying any potentially beneficial life-style choices would be important for the families suffering from this disease. However, these observations, based as they are on a single pair of monozygotic twins are only indicative and further studies with more powerful sample sizes would be needed to confirm the influence of these factors.

5.4. Genetic testing and skin biopsy as diagnostic tools in CADASIL (IV)

The study cohort comprised of 131 CADASIL patients (38 from Finland, 13 from Sweden and 80 from France), in whom the genetic analysis and EM examination of a skin biopsy had been performed. In France, GOM was detected in all 80 *NOTCH3* mutation-positive patients. Among these patients, 26 different *NOTCH3* mutations were identified. In Finland and Sweden, GOM was detected in 51 patients from 28 families with verified *NOTCH3* mutations. Altogether, 12 different *NOTCH3* mutations were identified, four of which were previously unpublished (p.Cys67>Ser, p.Cys251>Tyr, p.Tyr1069Cys and p.Glu434_Leu436dup). In one Finnish patient with a strong suspicion of CADASIL based on the clinical picture and MRI findings, repeated skin biopsies were positive for GOM, although no mutation was found in the screening of the *NOTCH3* exons 2–24. Furthermore, GOM was also detected in this patient´s sister with similar symptoms. This prompted genetic analysis with another set of primers and the samples were sent to the Hôpital Lariboisière in France for analysis. This second analysis revealed a heterozygous c.1582G>T, p.Gly528Cys mutation in both patients. The analysis was then repeated with the original primers and the mutation was then detected, which suggests that the missing of the mutation in the first analysis may have resulted from an uneven amplification of alleles in that particular PCR. This experience further emphasizes the value of skin biopsy examinations as a guide before moving forward with the more comprehensive genetic analyses.

In summary, GOM was detected in skin biopsies from all 131 patients, who were also determined to carry the *NOTCH3* mutation for CADASIL. Representative skin biopsies were negative for GOM in 26 subjects, who were also tested to be negative for the *NOTCH3* mutation detected in their family. Therefore, these subjects were identified as definite negative cases for CADASIL. GOM was

detected mainly in arteries, whereas the veins and capillaries were either GOM negative or, only rarely, GOM positive. In most positive cases, GOM was detected in the first biopsy performed, but in a few cases a repeated biopsy was needed.

The inevitable limitation of this study was its retrospective nature, although the genetic analysis and EM detection of GOM were primarily made simultaneously. However, since the analyses have been performed as a part of the diagnostic work, the EM analysis was used in Finland to support the restricted diagnostic genetic testing (only two mutations tested routinely) and, therefore, blind analyses with these two methods were not achieved in all cases. In some cases, GOM was found in the biopsy but the limited mutational analysis gave a negative result, which led to screening of the exons 2–24. During the examinations of suspected patients, in some cases no representative vessels were detected. A repeated biopsy was requested, and if the biopsy was still non-representative the case was excluded from the cohort. Most often the parallel, although restricted, genetic analysis of these patients proved to be negative, and in only one *NOTCH3* mutation-positive patient could representative arteries not be found in the only available biopsy. Since the presence of GOM could be neither ascertained nor excluded, on the basis of the inclusion criteria this patient was excluded from this cohort.

In this patient cohort, detection of GOM from skin biopsy was a highly reliable method as a diagnostic tool for CADASIL. GOM was detected in the skin biopsy of all 131 patients in whom the *NOTCH3* mutation had been identified and a representative skin biopsy was available. The detected *NOTCH3* mutation was the criterion for the true CADASIL case; thus, the sensitivity of GOM detection with EM was 100% in this study. However, the specificity cannot be calculated in this context since the obtaining the correct estimation of false positive results would require more blind analyses of true negative cases. In a retrospective investigation of diagnostic cases this is rarely achieved.

The EM analysis proved to be a very sensitive method in this study, but nevertheless the quality and proper analysis of the skin biopsy should be ensured. The vessels in which GOM is best detected are generally medium-sized or small arterioles in the deep dermis or upper subcutis, but in a few cases GOM has been detected also in veins. In toluidine blue semithin sections, the detection of lamina elastica interna as dark blue dots is a good marker of representative arterioles (see Figure 1 in original article IV). Technical aspects in the processing of the samples also influence the results and should be noted. Electron stains containing heavy metals increase the contrast in EM, and the osmium tetroxide treatment should be done in such a way that GOM becomes sufficiently

contrasted. Furthermore, if GOM is not found in the first vessel inspected, other vessels and, if necessary, a new biopsy should be performed and analysed. The first negative result might be an unfortunate result of an unrepresentative sample or improper sample handling. In addition, it must be noted that the identification of GOM as well as distinguishing it from possible fallacious deposits and cellular debris requires experience in EM analysis and GOM appearance.

The most logical and efficient strategy to confirm the clinical suspicion of CADASIL depends on the patient's family history and the mutational background in the population. In several populations, a different spectrum of pathogenic *NOTCH3* mutations has been reported (Markus*, et al.*, 2002, Lesnik Oberstein, 2003, Dotti*, et al.*, 2005, Peters*, et al.*, 2005a), and this should be taken into account when planning the diagnostic protocol. In families with a known mutation, the method of choice is naturally to analyse directly the presence of that particular mutation. In the case of an unknown mutation, the diagnostic workup is best begun by first searching for the possible founder or major mutations in the population. If no founder or other determined mutations are known in the population, molecular screening of the mutational hot spot region of the *NOTCH3* gene should be the first molecular genetic method used to search for CADASIL-type mutation. Of all the reported pathogenic *NOTCH3* mutations, 62% locate in exons 3, 4, 5 and 8. Furthermore, to obtain 80% coverage, further investigation of exons 2, 6, 11 and 18 is required (see the supplement table 1 in original article IV). Since mutation screening covering the whole region coding for EGF repeats (exons 2–24) is not realistic for all patients and for most laboratories, at the latest after the analyses of hot spot regions of *NOTCH3*, the EM examination for GOM is highly recommended.

On the basis of this study, the analysis for GOM is reliable and can be strongly recommended as saving time and unnecessarily expensive genetic analyses. Although some of the estimates of the sensitivity of GOM detection have been relatively low (Markus*, et al.*, 2002, Razvi*, et al.*, 2003), Peters*, et al.* (2005a) were able to identify a *NOTCH3* mutation in 120 of their 125 GOM-positive patients. In cases with at least a fair amount of accumulated N3ECD, the immunostaining has also been found to be a remarkably reliable method for detecting CADASIL (Joutel*, et al.*, 2001). However, at the early stage of the disease, when only a small amount of the N3ECD has accumulated, nonspecific staining may be problematic (Lesnik Oberstein*, et al.*, 2003b). At the early stage, the ultrastructural resolution and characteristic appearance of GOM most likely make EM analysis more reliable since GOM has been detected even in patients below the age of 20 years. Moreover, EM examination may also provide information about other pathological changes in the arterial wall, such as those due to hypertension, ageing and possibly even other hereditary arteriopathies

(Ruchoux, *et al.*, 2000, Brulin, *et al.*, 2002, Ruchoux, *et al.*, 2002, Low, *et al.*, 2007).

5.5. CADASIL diagnostics in Finland and Sweden

In Finland, since the identification of the genetic background of the disease, the genetic diagnostic services for CADASIL have been concentrated mainly in the Diagnostic DNA laboratory of the Department of Medical Genetics at the University of Turku. From 1998 to 2004 the genetic testing of CADASIL was restricted to the analysis of two previously detected mutations, p.Arg133Cys and p.Arg182Cys. Later, the sequencing analysis of exons 3 and 4 was added to the selection. In the light of today's knowledge, this covers only 43% of the pathogenic *NOTCH3* mutations reported worldwide. Furthermore, there were 5 Finnish and 4 Swedish patients with a positive GOM finding in their skin biopsy but no mutation identified with the restricted mutational analysis. Three of these patients also had a first-degree relative who had a clinically similar disease and findings, but a skin biopsy was not performed. Thus, there were 12 patients with very strong evidence for CADASIL diagnosis although no mutation was found, which suggested a broader mutational spectrum of *NOTCH3* in Finland and Sweden than earlier had been evidenced.

In order to improve the service of genetic testing for CADASIL, to shed light on the true mutational spectrum of *NOTCH3* in Finland and Sweden, and to test the sensitivity of GOM detection in skin biopsy as a diagnostic method (IV), the sequencing analysis of the whole *NOTCH3* gene was established. The analysis of 9 GOM-positive patients was begun with the sequencing of the exons 2–24, which code for the EGF repeats of N3ECD. In 8 of the patients and in two relatives with a similar clinical picture the disease-causing mutation was found (p.Tyr1069Cys in 5 Finnish patients, p.Cys206Tyr in 2 Swedish patients, p.Cys251Tyr in 2 Swedish patients). In one patient and this patient's sibling, the mutation (p.Gly528Cys) was found in the second analysis in France (see Chapter 5.4. above). In one Swedish patient, the mutation (p.Cys67Ser) was detected in Uppsala during study IV. During this study, several *NOTCH3* exons (in addition to the previous 3 and 4) were added to the scope of the sequence analysis of the diagnostic CADASIL testing in the Diagnostic DNA laboratory of the Department of Medical Genetics at the University of Turku. After this, four new mutations (p.Arg141Cys, p.Glu434_Leu436dup, p.Cys1015Arg and p.Arg1076Cys) have been identified in Finnish patients, including the first duplication mutation of three amino acids (containing one cysteine). The presently-known mutational spectrum of pathogenic *NOTCH3* mutations in CADASIL families in Sweden is presented in Table 6 and in Finland in Table 7.

Table 6: Verified CADASIL families in Sweden.

Family	Number of verified patients	Number of suspected patients	Mutation	*NOTCH3* exon	Origin
1	1	1	p.Cys67Ser	2	Värmland
2	1	3	p.Arg90Cys	3	Värmland
3	2	3	p.Arg133Cys	4	Västerbotten
4	5	5	p.Arg133Cys	4	Norrland
5	2	2	p.Arg133Cys	4	Denmark
6	1	-	p.Arg133Cys	4	Finland
7	2	2	p.Arg141Cys	4	Västerbotten
8	1	1	p.Arg169Cys	4	Mälardalen
9	3	4	p.Cys174Arg	4	Västergötland
10	1	1	p.Arg182Cys	4	Mälardalen
11	3	3	p.Arg182Cys	4	Mälardalen
12	2	2	p.Cys206Tyr	4	Värmland
13	2	2	p.Cys251Tyr	5	Småland
14	2	4	p.Arg332Cys	6	Mälardalen
15	1	1	p.Arg558Cys	11	Norway
	n=29	n=33			

Verified CADASIL patients according to positive mutational analysis or detected GOM. Number of suspected patients according to pedigree information, symptoms and pattern of inheritance. Abbreviations: p, protein; Arg, Arginine; Cys, Cysteine; Tyr, Tyrosine; Ser, Serine.

In Sweden, 15 CADASIL families, in which the causative *NOTCH3* mutation has been identified, have been diagnosed. Three of the families originate from neighbouring Nordic countries. Eleven different mutations have been detected and no founder or major mutation has been identified. All except for two of the mutations are located in exons 3-6, and analysis of these exons would be the rational procedure to begin the diagnostic work. This would require two different amplification reactions and three sequence reactions. After these analyses (if negative), the EM examination of a skin biopsy would likely prove the most efficient and cost effective solution for CADASIL diagnosis. If GOM is detected and identification of the mutation would benefit the patient's family members, the genetic analysis could be continued by sequencing exons each of in an order of the mutational hot spot regions in turn (see the Supplement Table 1 in original article IV).

Table 7: Verified CADASIL families in Finland.

Family	Number of verified patients	Number of suspected patients	Mutation	*NOTCH3* exon	Origin
1	8	19	p.Arg133Cys	4	South Savo
2	3	4	p.Arg133Cys	4	South Savo
3	1	3	p.Arg133Cys	4	South Savo
4	14	30	p.Arg133Cys	4	North Carelia
5	2	3	p.Arg133Cys	4	North Carelia
6	1	2	p.Arg133Cys	4	North Carelia
7	1	1	p.Arg133Cys	4	North Carelia
8	2	6	p.Arg133Cys	4	Central Finland
9	6	10	p.Arg133Cys	4	Central Ostrobothnia
10	4	5	p.Arg133Cys	4	Central Ostrobothnia
11	3	5	p.Arg133Cys	4	Central Ostrobothnia
12	10	27	p.Arg133Cys	4	Central Ostrobothnia
13	25	47	p.Arg133Cys	4	Central Ostrobothnia
14	10	17	p.Arg133Cys	4	Central Ostrobothnia
15	2	6	p.Arg133Cys	4	Ostrobothnia
16	3	8	p.Arg133Cys	4	Ostrobothnia
17	3	5	p.Arg133Cys	4	Ostrobothnia
18	3	7	p.Arg133Cys	4	Ostrobothnia
19	5	10	p.Arg133Cys	4	South Ostrobothnia
20	5	16	p.Arg133Cys	4	Satakunta
21	1	1	p.Arg133Cys	4	Satakunta
22	1	1	p.Arg133Cys	4	Satakunta
23	2	2	p.Arg133Cys	4	Pirkanmaa
24	4	6	p.Arg133Cys	4	Uusimaa
25	1	1	p.Arg133Cys	4	-
26	1	1	p.Arg133Cys	4	-
27	1	1	p.Arg141Cys	4	Pirkanmaa
28	1	1	p.Arg182Cys	4	Satakunta
29	1	4	p.Glu434_Leu436dup	8	Uusimaa
30	2	3	p.Gly528Cys	10	Uusimaa
31	2	4	p.Cys1015Arg	19	Central Ostrobothnia
32	1	1	p.Tyr1069Cys	20	Ostrobothnia
33	3	6	p.Tyr1069Cys	20	Ostrobothnia
34	1	1	p.Tyr1069Cys	20	North Savo
35	2	6	p.Tyr1069Cys	20	Central Ostrobothnia
36	1	1	p.Tyr1069Cys	20	-
37	1	1	p.Tyr1069Cys	20	-
38	1	1	p.Tyr1069Cys	20	-
39	1	1	p.Arg1076Cys	20	North Savo
	n=139	n=274			

Verified CADASIL patients according to positive mutational analysis or detected GOM by 1.1.2009. Number of suspected patients according to pedigree information, symptoms and pattern of inheritance. Abbreviations: p, protein; Arg, Arginine; Cys, Cysteine; Glu, Glutamic acid; Leu, Leusine; Gly, Glycine; Tyr, Tyrosine; Ser, Serine; dup, duplication.

In Finland, altogether 39 CADASIL families and eight *NOTCH3* mutations have been identified. From these, 26 families carry the p.Arg133Cys mutation. Two other mutations (p.Arg141Cys and p.Arg182Cys) have been detected in exon 4 and so far only one patient carrying each mutation has been identified. Five other mutations detected (p.Glu434_Leu436dup, p.Gly528Cys, p.Cys1015Arg, p.Tyr1069Cys and p.Arg1076Cys) are located in exons 8, 10, 19 and 20 (the last two mutations). The first step in confirming the CADASIL diagnosis should be an analysis for the founder mutation, especially if the patient's family originates from the area where the p.Arg133Cys families cluster. The mutation can be tested with a restriction analysis using the MspAI1 enzyme, which also reveals the p.Arg182Cys mutation, or by sequence analysis of exon 4, which would also detect other possible mutations in the exon. Currently, the second-most common mutation seems to be p.Tyr1069Cys in exon 20, analysis of which would be the next logical step. From the beginning of 2008, this mutation has been added to the list of those mutations routinely tested for by means of restriction analysis (p.Arg133Cys, p.Arg182Cys and p.Tyr1069Cys) in the Diagnostic DNA laboratory of the Department of Medical Genetics at the University of Turku. At the same time, or subsequently, analyses of the remainder of the mutations detected in Finland would also be justifiable, either with restriction or sequence analyses. If a *NOTCH3* mutation is not detected, but the clinical symptoms and findings support a CADASIL diagnosis, an EM examination of the skin biopsy could be the next logical method in confirming (or excluding) a CADASIL diagnosis. If GOM is detected, the comprehensive search for a mutation in the remaining exons should be continued. However, currently the sequencing of exons 2–24, the whole area coding for the EGF repeats, is not available as a diagnostic test in Finland, and is used only as a research method.

6. CONCLUSIONS

I The consensus haplotype shared by the Finnish CADASIL families is evidence for a common ancestor and a single founder effect among the families carrying the p.Arg133Cys mutation. The age of this ancestral mutation is over 12 generations, and the mutational event dates back to the late 1600s or early 1700s.

II Among 120 CADASIL patients, the *APOE* ε4 allele associated to earlier occurrence of the first stroke in young patients when analysis was adjusted for cardiovascular risk factors. The result shows that *APOE* ε4 can have a harmful influence on the clinical course of CADASIL, and genes other than *NOTCH3* can also influence the disease phenotype. The *APOE* ε4 allele seems to exert its influence primarily at the early stages of CADASIL.

III The monozygotic twins A and B have a markedly discordant clinical course of CADASIL despite their identical genetic background, which suggests the influence of environmental factors. Twin B's smoking may have aggravated the disease phenotype, whereas twin A's longer regular use of statins and more intense physical activity may have had a beneficial effect. All these aspects may influence the phenotypic variation in CADASIL.

IV Both the mutation analysis of the *NOTCH3* gene and the EM examination of skin biopsy are very sensitive and specific methods in diagnosing CADASIL if performed with care. If the mutation is not found in the preliminary genetic analyses, or genetic analyses are not available, an EM examination of a skin biopsy is a practical and reliable method to exclude or confirm the CADASIL diagnosis. It is a valuable guide in deciding whether to proceed with the extensive genetic analyses. The confirmation of a *NOTCH3* mutation provides a practical tool for genetic testing and counselling in the patient's family.

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