NOTCH3 cysteine correction:

Developing a rational therapeutic approach for CADASIL

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The cover displays a family affected by CADASIL, as reflected in the lake.

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Voor CADASIL patiënten en hun families

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INTRODUCTION

CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy) is a hereditary dementia and stroke syndrome caused by mutations in the *NOTCH3* gene.[1] The disorder was first described as a separate disease entity in the 1990's[2-4] and is now acknowledged as the most prevalent hereditary cerebral small vessel disease worldwide. Currently, there is no therapy that can delay or prevent CADASIL disease progression.

This introductory chapter will give an overview of CADASIL clinical symptoms and pathophysiology, and will provide background to the various studies included in this thesis, which are focussed on pre-clinical therapeutic development for CADASIL.

SCOPE OF THIS THESIS

The aim of this thesis was to obtain pre-clinical proof of concept for a rational therapeutic approach for CADASIL, called NOTCH3 cysteine correction. To this end, we performed a detailed characterization of the type of *NOTCH3* mutations that cause CADASIL, and identified *NOTCH3* mutations that do not cause the disease. Furthermore, we performed *in vitro* proof of concept studies for NOTCH3 cysteine correction, and demonstrate the technical feasibility of this approach in CADASIL patient derived cell models. To enable future *in vivo* testing of this therapeutic approach, we generated a novel CADASIL mouse model and developed a biomarker in this model. Finally, we investigated the prevalence of CADASIL-causing *NOTCH3* mutations in a large public exome database and identified a novel genotype- phenotype correlation in CADASIL.

CADASIL: A HEREDITARY CEREBRAL SMALL VESSEL DISEASE

Chapter 2 of this thesis provides a detailed description of clinical symptoms in CADASIL and the role of brain imaging in the diagnostic setting. Briefly, the main clinical symptoms in CADASIL are ischemic strokes and cognitive decline leading to dementia. Ischemic strokes typically occur in watershed areas of the brain, and are recurrent in most patients. Mean age at first stroke is 45-50 years, but onset is variable, ranging from the 3rd to the 8th decade.[5-8] Mild cognitive decline is detectable prior to the onset of first stroke,[9,10] and progresses rapidly as the number of lacunar infarcts accumulates, ultimately resulting in severe vascular dementia. Up to 50% of CADASIL patients have migraine, typically with aura.[11] If present, this is often the first clinical symptom. Psychiatric disturbances are found in one third of CADASIL patients, and are most frequently mood disorders.[5,8]

Brain MRI abnormalities are present in all CADASIL patients, and can be an important clue to making the clinical diagnosis. Progressive white matter

hyperintensities with a recognisable distribution are detectable from the early adult, pre-symptomatic phase onwards and are found in nearly all CADASIL patients by the age of 35.[12] Brain MRI abnormalities become more pronounced and more extensive as the disease progresses with age, with lacunar infarcts, microbleeds, dilated perivascular spaces and brain atrophy.[13-17]

CADASIL: A MONOGENIC DISORDER WITH DISTINCTIVE *NOTCH3* GENE MUTATIONS

In 1996, *NOTCH3*, located on the short arm of chromosome 19, was identified as the causative gene in CADASIL.[1] *NOTCH3* contains 33 exons encoding the NOTCH3 protein; a transmembrane protein which, in adults, is predominantly expressed in vascular smooth muscle cells and pericytes.[18] NOTCH3 is synthesized as a 280 kDa precursor protein, is cleaved in the Golgi (S1 cleavage, mediated by Furin) after which it is transported to the cell surface as a heterodimer composed of an extracellular domain (NOTCH3^{ECD}), which is non-covalently attached to an intracellular domain.[19] The NOTCH3^{ECD} contains 34 tandemly arranged epidermal growth factor like repeat (EGFr) domains. An EGFr domain is a modular protein subunit composed of approximately 40 amino acids, including a fixed number of six cysteine residues. In pairs, these six cysteines form three disulphide bridges which are important for correct folding of the EGFr domain.[20] *NOTCH3* mutations in CADASIL are very distinctive, as they are almost exclusively missense mutations that lead to a cysteine amino acid change in an EGFr domain.[21] There are some reports of rare small *NOTCH3* deletions and insertions, which also lead to the typical numerical cysteine amino acid change in an EGFr domain. Some authors have reported non-cysteine altering *NOTCH3* mutations in purported CADASIL patients, but their pathogenicity is still subject of debate.[22] Chapter 2 of this thesis provides an overview of *NOTCH3* mutations in CADASIL, and discusses both indisputable CADASIL causing mutations and those of which the association with CADASIL is uncertain.

CADASIL PATHOPHYSIOLOGY: AN UNEVEN NUMBER OF CYSTEINES AND NOTCH3 AGGREGATION

As a result of the cysteine altering *NOTCH3* mutation, the mutated NOTCH3 EGFr domain contains an uneven number of five or seven cysteine residues. This disrupts normal disulphide bridge formation and leads to increased multimerization and aggregation of the NOTCH3^{ECD}.[20,23,24]

CADASIL is characterized by accumulation of the NOTCH3^{ECD} in close vicinity of vascular smooth muscle cells (VSMCs) in the small- to medium sized arteries,[18] degeneration of VSMCs and thickening of the vessel wall with increased deposition

of extracellular matrix proteins.[25] Electron microscopy of e.g. a skin biopsy, typically reveals electron- dense deposits of granular osmiophilic material (GOM) in the vessel wall, which is considered pathognomonic for CADASIL.[26]

The NOTCH3 aggregation and accumulation process is believed to play a central role in CADASIL pathophysiology, via incompletely understood mechanisms. Recent studies have shown that functionally important proteins are recruited into the aggregates,[27-29] thereby contributing to the pathological process, which ultimately leads to a reduced cerebrovascular reactivity and disturbed cerebral blood flow.[30-32] This is turns leads to recurrent stroke, vascular cognitive decline and ultimately pseudobulbar palsy, disability and vascular dementia (Figure 1).

NOTCH3 plays an important role in VSMC differentiation and maturation,[33] and there is an on-going debate whether, next to toxic protein aggregation, loss of NOTCH3 signalling function also contributes to CADASIL pathogenesis. NOTCH3 signalling is activated when a ligand, Delta or Jagged, binds to the NOTCH3 ligand binding site (EGFr 10-11). This initiates two sequential cleavage steps (S2 and S3, mediated by TNFα-converting enzyme and presenilin-dependent γ-secretase, respectively), which enables the intracellular domain to translocate to the nucleus and regulate the transcription of target genes.[19] It has been shown that most CADASIL mutated NOTCH3 proteins retain normal NOTCH3 signalling function,[34-39] and knocking out the *NOTCH3* gene in mice does not lead to the development of a CADASIL-like phenotype.[33] Rather, CADASIL- causing *NOTCH3* mutations lead to toxic NOTCH3 aggregation (gain of toxic function) and CADASIL can therefore be classified as a protein aggregation disorder. Chapter 3 of this thesis describes the identification of individuals with a loss-of-function *NOTCH3* mutation who do not have CADASIL, providing a crucial additional piece of evidence that loss of NOTCH3 function is not the primary CADASIL disease instigator.

NOTCH3 cysteine correction: a rational therapeutic approach for CADASIL

There is no therapy available which can slow CADASIL disease progression, and there are no clinical trials ongoing for CADASIL (clinicaltrials.gov). The distinctive nature of *NOTCH3* mutations in CADASIL, and the central role of a numerical cysteine alteration in disease aetiology, inspired the hypothesis that correction of the number of cysteines within a mutated NOTCH3 EGFr domain may counteract toxic NOTCH3 aggregation. This hypothesis prompted the development of the NOTCH3 cysteine correction approach as a rational therapeutic strategy for CADASIL, as described in chapter 4 of this thesis. In NOTCH3 cysteine correction, a minimal amount of EGFr domains, including the mutated one, are removed and an EGFr fusion domain with the correct number of cysteines is formed.

NOTCH3 cysteine correction can be achieved by using antisense oligonucleotides (AONs). AONs are short strands of nucleic acids which can bind to a specific target (pre-)mRNA via Watson-Crick base pairing. In NOTCH3 cysteine correction, AONs are designed to interfere with splicing, in order to induce exon

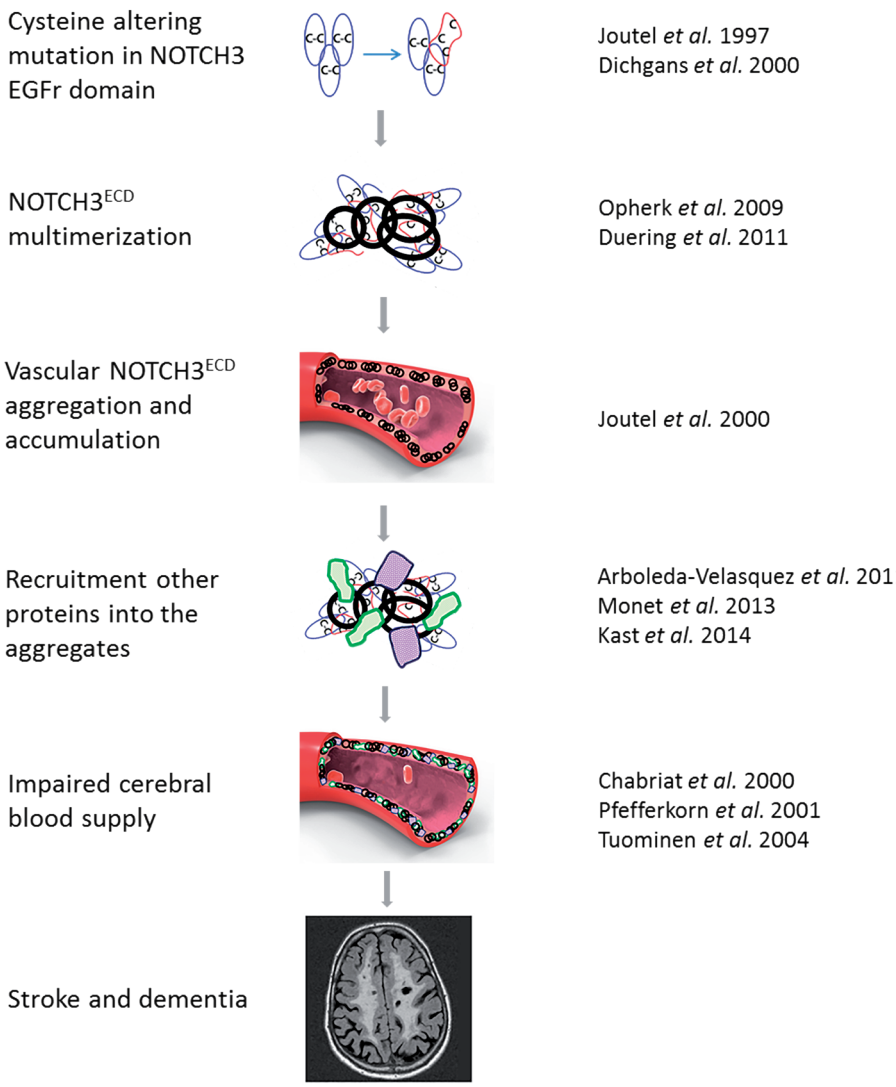

Figure 1. CADASIL pathophysiology. CADASIL-causing *NOTCH3* mutations alter the number of cysteines in one of the 34 EGFr domains of the NOTCH3^{ECD}. This causes multimerization and vascular aggregation of mutant NOTCH3^{ECD}, and recruitment of other proteins into the aggregates. This pathological process results in disturbed cerebrovascular reactivity and impaired cerebral blood supply, ultimately leading to stroke and dementia.

skipping of pre-selected *NOTCH3* exons. The AON chemistry used in this thesis is 2'O-methyl phosphorothioate (2OMePS). This is one of the most widely studied AON chemistries, which has been shown to be safe in humans.[40]

Developing a mouse model for pre-clinical testing of NOTCH3 cysteine correction

Pre-clinical therapeutic development usually requires testing of the therapy in an animal model of the disease. None of the currently available CADASIL mouse models is suitable for testing therapeutic strategies that target human *NOTCH3* specifically at the genomic or (pre-) mRNA level (Table 1). An ideal model for this purpose would be a transgenic model which contains the genomic human *NOTCH3* gene. Chapter 5 of this thesis describes the generation of such a novel CADASIL mouse model.

Year	Approach	Species N3	Mutation	Reference
2003;2009	transgenic cDNA	human	p.Arg90Cys; p.Cys428Ser	[41,42]
2005	knock-in	mouse	p.Arg142Cys	[43]
2010	transgenic genomic	rat	p.Arg169Cys	[44]
2011	knock-in	mouse	p.Arg170Cys	[45]
2011	transgenic cDNA	human	p.Cys455Arg; p.Arg1031Cys	[29]
2015	transgenic genomic	human	p.Arg182Cys	chapter 5, [46]

Table 1. CADASIL mouse models

CADASIL biomarker development

Disease variability in CADASIL complicates the development of feasible clinical end-points for future clinical trials. For example, using stroke as the primary end-point in a clinical trial of 2 years, would require thousands of patients to be included.[47] Therefore, there is a need for surrogate end-points and biomarkers that enable monitoring of disease progression in a sensitive manner within a limited time-frame. Chapter 5 of this thesis describes the development of a pre-clinical biomarker in the novel human *NOTCH3* transgenic mouse model.

Causes of disease variability in CADASIL

The causes of disease variability in CADASIL are not well understood. To date, no convincing or strong genotype-phenotype correlations have been found,[8] and GWAS studies have not identified any major genetic modifiers.[6,48] Environmental factors must play a role, as the disease is also variable within

families and even between monozygotic twins.[49] Smoking and hypertension have been found to aggravate disease severity,[8, 50] but only exert a relatively minor effect. Chapter 6 of this thesis concerns the discovery of a hitherto unrecognised genotype-phenotype correlation, which may contribute significantly to CADASIL disease variability.

CADASIL prevalence

To date, more than 200 distinct *NOTCH3* mutations have been described in CADASIL patients worldwide. CADASIL prevalence estimations have been performed only in relatively small populations in the United Kingdom, which has led to an estimated minimum prevalence of 2-5/100,000.[51-53] This number is widely held to be an underestimation. Chapter 6 of this thesis describes the finding of an unexpectedly high frequency of CADASIL- causing *NOTCH3* mutations in the public exome database ExAC, showing that these mutations are 100-fold more prevalent than what would be expected based on current CADASIL prevalence estimations and suggesting that they much more frequently cause a milder phenotype.

OVERVIEW OF THE THESIS CHAPTERS

Chapter 2 provides recommendations for CADASIL diagnostics, especially for the interpretation of the results of molecular genetic testing. Specifically, it aims to separate the indisputable CADASIL causing mutations from those of which the causative association with CADASIL is uncertain.

Chapter 3 describes the identification of individuals with a *NOTCH3* loss-of-function mutation, who do not have a CADASIL phenotype, providing important additional evidence that CADASIL pathogenesis is not driven by a loss of NOTCH3 function.

Chapter 4 describes the *in vitro* proof-of-concept for NOTCH3 cysteine correction, a rational therapeutic approach for CADASIL using antisense oligonucleotides, aimed at reducing toxic NOTCH3 protein aggregation.

Chapter 5 describes the generation of a genomic, human *NOTCH3* transgenic mouse model and the development of the NOTCH3 score as a biomarker in this model.

Chapter 6 shows that CADASIL causing *NOTCH3* mutations are much more prevalent than currently estimated, suggesting not only that CADASIL is hugely underdiagnosed, but also that *NOTCH3* mutations more frequently cause a relatively mild phenotype, partially explained by a newly discovered genotype-phenotype correlation

Chapter 7 discusses the implications of the findings in this thesis and directions for future studies.

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INTERPRETATION OF NOTCH3 MUTATIONS IN THE DIAGNOSIS OF CADASIL

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ABSTRACT

CADASIL is an autosomal dominantly inherited disease, characterized by mid-adult onset of cerebrovascular disease and dementia. CADASIL is caused by mutations in the *NOTCH3* gene which encodes the NOTCH3 protein. Pathogenic mutations in CADASIL are highly distinctive in the sense that they typically lead to the loss or gain of a cysteine residue in one of the 34 epidermal growth factor-like repeat (EGFr) domains of the NOTCH3 protein. The majority are missense mutations, but small deletions, insertions and splice-site mutations have been reported, which typically also lead to a numerical cysteine alteration. Whether numerical cysteine altering mutations are a rule in CADASIL remains subject of debate, as there are reports suggesting pathogenicity of other types of mutations. However, for most of these the association with CADASIL was later revoked or is questionable. Here, we discuss and provide recommendations for the interpretation of *NOTCH3* mutations in the diagnosis of CADASIL.

INTRODUCTION

CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy) is a hereditary small vessel disease, caused by mutations in the *NOTCH3* gene. CADASIL patients can present with a history of migraine with aura, young to mid adult onset of cerebrovascular disease, mood disturbance, apathy, cognitive decline progressing to dementia, and diffuse white matter lesions and subcortical infarcts on neuroimaging.

Compared to other inherited brain disorders with a comparable impact, such as Huntington's chorea or inherited early-onset Alzheimer's dementia, CADASIL is still relatively unknown in the medical community. This is not so much due to the fact that it is a rare disease, but more to the fact that there is only a short history of recognition of the disease. CADASIL was first described as a clearly defined and separate disease entity in the early 1990's,[1-3] followed by the identification of mutations in the *NOTCH3* gene in 1996.[4] Since then, *NOTCH3* mutation screening has allowed for the identification of CADASIL families all over the globe, with an ever increasing prevalence due to increased awareness of the disease, diagnosis in clinically less severe or atypical cases and improved diagnostic tools. The minimum prevalence of CADASIL has been estimated to be between 2 and 4 per 100.000,[5-7] but the actual prevalence will probably prove to be higher. In the Netherlands, for example, the number of diagnosed CADASIL families started out with seven in 1998 and has increased to more than 150 Dutch families in 2013, with new families still being identified at a steady rate. (Lesnik Oberstein and Boon, unpublished results)

The clinical diagnosis CADASIL is made based on a combination of the following: otherwise unexplained cerebral ischemic events and/or cognitive decline at a relatively young age, distinctive brain MRI abnormalities and a family history with a dominant pattern of inheritance of stroke or dementia. The clinical diagnosis can be confirmed by the detection of a characteristic cysteine-altering mutation in *NOTCH3*. If the results of molecular testing are unclear or comprehensive *NOTCH3* screening is not available, the diagnosis can be confirmed by taking a skin biopsy. This shows pathognomonic vessel wall abnormalities, including positive NOTCH3 immunostaining and the presence of electron dense deposits called granular osmiophilic material (GOM).[8,9] The correct diagnostic interpretation of *NOTCH3* variants other than the typical cysteine-altering missense mutations requires expertise in both the clinical features and the distinguishing molecular aspects of CADASIL. Incorrect interpretation of mutations can lead to an erroneous CADASIL diagnosis, with far-reaching implications for the patient and his or her family members. Here, we provide recommendations for the clinical diagnosis and interpretation of *NOTCH3* mutations in CADASIL.

NOTCH3 GENE, NOTCH3 PROTEIN AND CADASIL PATHOGENESIS

The *NOTCH3* gene is one of the four mammalian NOTCH homologues. *NOTCH3* contains 33 exons encoding the NOTCH3 protein, a single pass transmembrane protein of 2321 amino acids predominantly expressed in vascular smooth muscle cells (VSMC).[10] Here, it plays an important role in VSMC maturation and differentiation.[11] The NOTCH3 protein is composed of an extracellular domain (NOTCH3^{ECD}), non-covalently bound to an intracellular domain. (Figure 1) After binding of a ligand (Delta-like or Jagged) to the NOTCH3^{ECD} , the protein undergoes two proteolytic cleavage steps, leading to translocation of the intracellular domain to the nucleus where it functions as a nuclear transcription factor.[12] The NOTCH3^{ECD} consists of 34 epidermal growth factor- like repeat (EGFr) domains. These are modular protein subunits of approximately 40 amino acids which, by definition, each contain a fixed

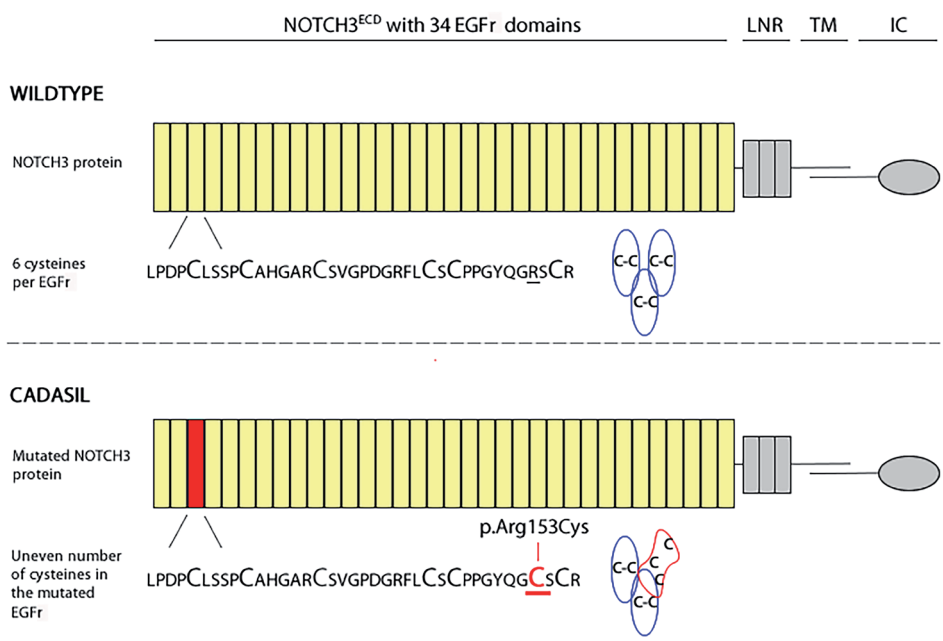

Figure 1. Schematic representation of the NOTCH3 protein. The NOTCH3 protein is a transmembrane protein, composed of an extracellular domain (NOTCH3^{ECD}), a transmembrane (TM) and an intracellular domain (IC). The NOTCH3^{ECD} contains 34 EGFr and three NOTCH/Lin repeats(LNR). The EGFr, each composed of approximately 40 amino acids, all contain 6 cysteine amino acids which form 3 disulphide bridges. In this example of CADASIL mutated NOTCH3 (with the representative p.Arg153Cys mutation), the number of cysteine residues in EGFr 3 is changed from 6 to 7, leaving one cysteine unpaired and disrupting normal disulphide bridge formation.

number of 6 cysteine residues. In pairs, these cysteines form three disulphide bridges which are important for EGFr secondary structure. *NOTCH3* mutations in CADASIL invariably lead to an uneven number of cysteines (typically 5 or 7) in the mutated EGFr.[13] This results in an unpaired cysteine, which is predicted to disrupt normal disulphide bridge formation, causing misfolding of EGFr and increased NOTCH3 multimerization.[14,15]

In patients, $NOTCH3^{ECD}$ is seen to accumulate in the vessel wall, in close proximity to VSMC.[10] This $NOTCH3^{ECD}$ accumulation has a direct or indirect toxic effect on VSMC, leading to VSMC degeneration.[9,16,17] The arteriopathy in CADASIL is systemic,[9] as is also illustrated by the presence of vascular pathology in for example skin arterioles,[8,17] but the small penetrating cerebral and leptomeningeal arteries are most severely affected.[18] Next to $NOTCH3^{ECD}$ accumulation and the presence of GOM, affected arteries show a thickened vessel wall with lumen stenosis, abundance of extracellular matrix proteins and destruction of vascular smooth muscle cells.[19,20] The vessel wall changes result in an impaired cerebrovascular reactivity and decreased cerebral blood flow, believed to cause both chronic cerebral ischemia and acute ischemic events.[21-23]

CLINICAL DIAGNOSIS BASED ON SYMPTOMS, BRAIN MRI AND FAMILY HISTORY

The main symptoms in CADASIL, affecting the majority of patients, are recurrent ischemic events (transient ischemic attacks and strokes) and cognitive decline leading to vascular dementia. Migraine with aura, psychiatric disturbances and apathy are other frequent symptoms. Most patients experience their first stroke around 45-50 years of age,[24-27] but the range of age at onset is broad, varying from as early as the third decade to as late as the eighth decade. In the majority of patients, ischemic events are recurrent,[24,26,27] eventually resulting in severe disability.[25] Cognitive decline initially manifests as a decreased executive function,[28-30] followed by a slowly progressive and/or stepwise deterioration in cognitive function which becomes apparent in daily activities around the age of 50, leading to vascular dementia in about 80% of patients.[25] Migraine is diagnosed in approximately 35% of CADASIL patients. The migraine attacks are often the presenting clinical symptom with a mean age at onset of around 26 years, and are remarkable by the high prevalence of auras (90% of patients).[24,27] Often, the migraine attacks are atypical, with prolonged, brainstem or hemiplegic auras and confusion, fever, meningeal signs or even coma. Also, during gestation and shortly after childbirth there appears to be an increased risk of transient neurological symptoms,[31] mostly resembling migraine auras. Approximately one third of CADASIL patients have psychiatric problems, usually depressions.[24,27] Apathy is a common symptom in

later stages.[32] Less frequent symptoms include epileptic seizures (5-10%), usually secondary to stroke,[3,24,26,33] and acute encephalopathy.[3,27,34-36]

The suspicion of CADASIL often arises when brain MRI imaging reveals symmetrically distributed white matter hyperintensities (WMH), which are much too extensive for the patient's age. (Figure 2) MRI abnormalities in CADASIL precede clinical symptoms of ischemic events and cognitive decline by as much as 10-15 years, increase with age and have a recognizable, but not pathognomonic, distribution. In the early stages the WMH, best seen on T2- weighted images or fluid –attenuated inversion recovery (FLAIR) sequences, are small and punctuate and are often seen to initiate in the anterior temporal lobes, periventricular frontal white matter, and external capsules,[37-39] eventually affecting all of the white matter. Lacunar infarcts, most readily detected on T1- weighted images, can be seen from the age of 30 onwards, and are found in the basal ganglia, thalamus, brainstem and subcortical white matter.[37] Microbleeds, predominantly seen in the subcortical white matter and thalamus, and dilated perivascular spaces are frequently

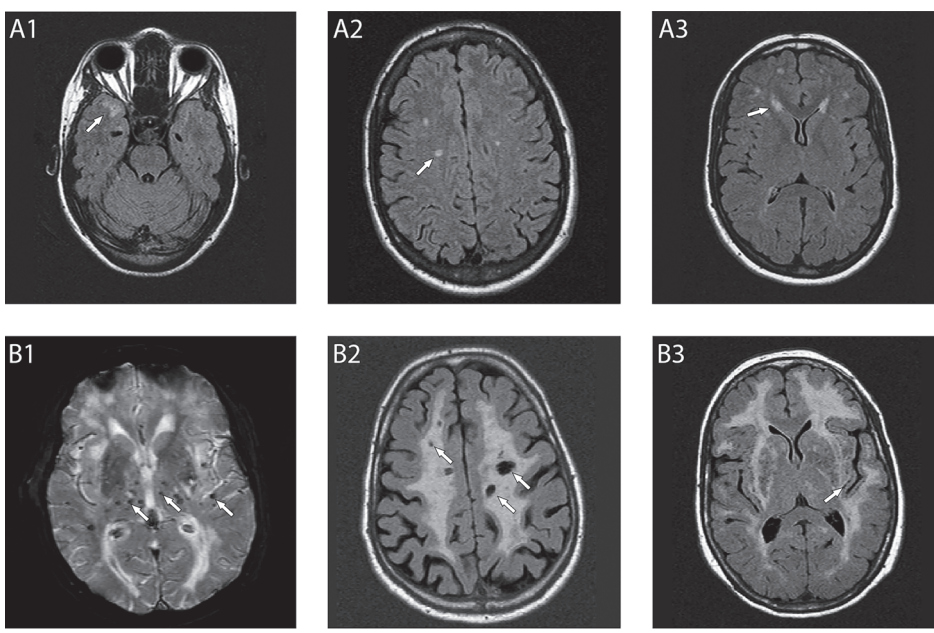

Figure 2. Brain MRI abnormalities in CADASIL patients. In the early stages of CADASIL, brain MRI abnormalities consist of small circumscript white matter hyperintensities (WMH) (indicated by arrows), as shown here in the anterior temporal lobes (A1), in the semioval center (A2) and periventricularly (A3). (FLAIR images) Brain MRI images of patients with advanced stages of CADASIL showing confluent WMH (B1-B3), microbleeds (three marked with an arrow) (B1), lacunar infarcts (three marked with an arrow) (B2) and dilated perivascular spaces (B3). (B1: T2*- weighted gradient echo images, B2, B3: FLAIR images)

encountered.[40-42] The MRI lesion load and pattern can vary quite significantly, such that some patients hardly show any lacunar infarcts even at later stages, or lack WMH in the temporal lobes.[43] A consistent factor is the presence of symmetrical WMH, often visible from the early twenties onward, but present in most if not all patients from 35 years of age. MRI abnormalities in CADASIL can resemble those seen in other diseases, such as other types of small vessel disease or multiple sclerosis. In fact, a significant number of CADASIL patients are first erroneously diagnosed as having multiple sclerosis.[44]

The family history of a CADASIL patient typically shows an autosomal dominant inheritance pattern of stroke and dementia, and family members often have migraine with aura or mood disturbances. However, the severity of the disease is variable, also within families. It has been noted, that CADASIL patients frequently report a negative family history,[45] which in many cases can be attributed to the fact that affected family members, especially in older generations, have received other diagnoses such as Alzheimer's dementia or multiple sclerosis. Taking this into account, a thorough family history will usually reveal affected family members. However, a small number of CADASIL patients with *de novo NOTCH3* mutations have been reported, so a negative family history does not rule out CADASIL.[46,47]

CADASIL can be diagnosed in a plethora of clinical presentations or settings, where the early onset of stroke, white matter hyperintensities on brain MRI and a family history of stroke, dementia or migraine with aura, may each contribute more or less strongly in leading the clinician to consider CADASIL. However, CADASIL should always, be considered in an individual presenting with otherwise unexplained ischemic events or cognitive decline and symmetrical white matter hyperintensities on brain MRI.

WHEN TO PERFORM A SKIN BIOPSY IN CADASIL

If *NOTCH3* screening is unavailable or does not reveal a typical cysteine altering missense mutation in a patient otherwise presenting with a convincing CADASIL phenotype, then taking a skin biopsy for NOTCH3 immunostaining and electron microscopy (EM) is recommended. (figure 3) The sensitivity of NOTCH3 immunostaining of a skin biopsy has been reported to be between 85-100%, and the specificity between 90-100%.[8,48] The detection of GOM in the vessel wall using electron microscopy is considered pathognomonic for CADASIL. Most studies report the presence of GOM deposits in all CADASIL patients,[49-52] with only two studies reporting low GOM detection rate.[53,54] Skin biopsy analysis using both NOTCH3 immunostaining and EM should therefore allow for a conclusive confirmation or rejection of the diagnosis when performed by an experienced (neuro)pathologist.

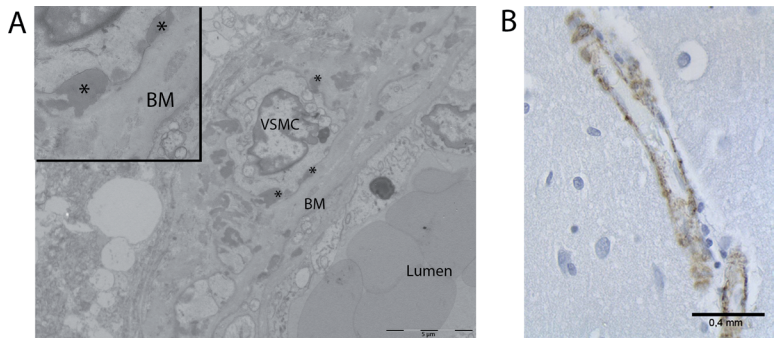

Figure 3. Vessel wall abnormalities in CADASIL. (A) Electron microscopy on brain tissue from a deceased CADASIL patient. Pathognomic deposits of granular osmiophillic material (GOM) are seen, surrounding the vascular smooth muscle cell (VSMC). (B) NOTCH3 immunohistochemistry of a skin biopsy showing the typical granular positive NOTCH3 staining of the vessel wall. BM= basement membrane, *=GOM, VSMC=vascular smooth muscle cell.

NOTCH3 MUTATIONS IN CADASIL

A typical CADASIL causing mutation is a heterozygous *NOTCH3* missense mutation that leads to a numerical cysteine amino acid change in one of the 34 EGFr of the NOTCH3 protein.(see supplementary table 1 for a list of known CADASIL- causing missense mutations) Pathogenic mutations have been found throughout exons 2-24, which are the exons that encode the EGFr, but the majority of mutations are found in exon 4. The prevalence of mutations in other exons varies between countries; in the French, German and English CADASIL population exon 3 is the second most frequently mutated exon,[13,53,55] whereas in Dutch CADASIL patients, exon 11 is the second most frequently mutated exon.[56] These geographic differences probably are due to the presence of founder mutations.

When a clinical diagnosis of CADASIL is suspected, sequencing of exons 2-24 of the *NOTCH3* gene, including flanking intronic sequences, is the most reliable method to confirm the diagnosis. In patients with a typical clinical and radiological presentation and family history, the mutation detection rate exceeds 95%. (Lesnik Oberstein, unpublished observations) Detection of the disease- causing mutation then allows for (predictive) DNA-testing of family members. However, sequencing exons 2-24 is costly and can be time-consuming, and is not available in all countries. Targeted sequencing strategies are used in centres that do not offer complete *NOTCH3* sequencing analysis, usually sequencing analysis of only the most frequently mutated exons. In cases with a confirmed clinical diagnosis by skin biopsy but no mutation detected by sequencing analysis, additional molecular techniques can be performed to detect larger deletions or duplications, or to assess the potential effect of novel variants on splicing.

Although it has been suggested that some mutations may be associated with a milder or more severe phenotype,[25,57] so far no clear genotype- phenotype correlations have been found.[24,25,58,59] As disease severity can vary not only between, but also within families, and even between monozygotic twins,[60] it is likely that modifying factors other than the *NOTCH3* mutation play a role in determining the course of the disease.

Homozygous and compound heterozygous NOTCH3 mutations

A small number of CADASIL patients with homozygous *NOTCH3* mutations have been reported.[36,61-64] (Table 1) In some of these patients, the phenotype was found to be more severe, whereas others reported clinical severity similar to heterozygous mutation carriers. Overall, patients with homozygous mutations described so far have a phenotype within the normal CADASIL spectrum, and variation in severity may as readily be attributed to the natural variability seen in CADASIL.

Reference	Homozygous mutation	Exon	Amino acid change	Age patient	Age at first stroke
Tuominen et al.[62]	c.397C>T	4	p.Arg133Cys	52	28
Vinciguerra et al.[64]	c.547T>A	4	p.Cys183Ser	44	40
Ragno et al.[36]	c.1582G>T	10	p.Gly528Cys	54	No stroke at age 54
Liem et al.[61]	c.1732C>T	11	p.Arg578Cys	65	64
Soong et al.[63]	c.1630C>T c.1630C>T	11 11	p.Arg544Cys p.Arg544Cys	63 68	63 58

Table 1. Homozygous mutations in CADASIL patients. A small number of CADASIL-causing mutations in a homozygous state have been reported. The severity of symptoms in these patients seems to be within the CADASIL spectrum. Mutations are described according to HGVS nomenclature,[93] and may therefore differ from the mutation description in the original research article.

One CADASIL patient has been reported who has a typical cysteine-altering missense mutation on one of his *NOTCH3* alleles, but also a large intragenic *NOTCH3* deletion, leading to a premature stop codon, on his other allele.[65] In effect, this means he is functioning on only one *NOTCH3* allele which, moreover, harbors a CADASIL-causing mutation. The phenotype of this patient was within the normal CADASIL spectrum. In patients with a *NOTCH3* mutation on one allele and a large *NOTCH3* deletion on the other allele, the mutation may be interpreted to be a homozygous mutation if only sequencing analysis is performed. Therefore, in mutations appearing to be homozygous, the presence of a *NOTCH3* deletion on

one of the alleles should be excluded, for example by performing MLPA analysis. Although this has no clinical consequences for the patient, because the disease severity in these patients is within the normal CADASIL spectrum, it is of importance for the counseling of family members.

Small NOTCH3 deletions, duplications, splice site mutations and a deletion/insertion

Although the vast majority of CADASIL causing mutations are missense mutations, a few other rare types of *NOTCH3* mutations have been reported, which also lead to the typical CADASIL-associated uneven number of cysteine residues in EGFr. These include some small intragenic deletions, duplications, splice site mutations and a deletion/insertion.[14,25,43,51,66-71] (Table 2) As these mutations lead to a typical numerical cysteine alteration in NOTCH3 EGFr, they are likely to be pathogenic, although the clinical diagnosis was not confirmed by skin biopsy in all cases and molecular analysis was not always comprehensive.

Deletions leading to altered spacing of cysteine residues

In one study, a 12 base pair deletion not involving a cysteine residue was reported to be associated with CADASIL.[72] The diagnosis was confirmed by skin biopsy, segregated with the clinical phenotype and all EGFr encoding exons had been sequenced. Although not resulting in a numerical cysteine alteration, this deletion does result in altered spacing between two cysteines in an EGFr, thereby possibly disrupting normal disulphide bridge formation. As no RNA analysis was performed, it cannot be excluded that the deletion results in abnormal splicing and thereby potentially alters the number of cysteine residues.

A second study describes a small intronic deletion leading to retention of intron 3.[73] This results in an insertion of 25 amino acids between the 5th and 6th cysteine of EGFr 2, thereby likely disturbing disulphide bridge formation between these two cysteines.

NOTCH3 VARIANTS NOT ALTERING CYSTEINE RESIDUES IN EGFR

NOTCH3 mutations not leading to a numerical cysteine amino acid change in NOTCH3 EGFr have also been described to be associated with CADASIL. Upon closer scrutiny, however, these associations often remain uncertain, or pathogenicity was later disproven.

Ref.	Mutation	Exon / intron	RNA analysis	Amino acid change	Numerical cysteine alteration	Sequencing analysis all EGFr encoding exons	Diagnosis confirmed by skin biopsy
Wang et al.[43]	c.226_234del	exon 3	-	p.Cys76_ Leu78del	deletion 1 cysteine EGFr 1	yes	yes
Opherk et al.[25]	c.231_248del	exon 3	-	p.Gln77_ Cys82del	deletion 1 cysteine EGFr 2	not reported	not reported
Mazzei et al.[66]	c.277_279dup	exon 3	-	p.Cys93dup	insertion 1 cysteine EGFr 2	not reported	yes
Dichgans et al.[14]	c.239_253del c.459_467del	exon 3 exon 4	- -	p.Asp80_ Ser84del p.Arg153_ Cys155del	deletion 1 cysteine EGFr 2 deletion 1 cysteine EGFr 3	no† no†	yes yes
Joutel et al.[67]	c.341-2A>G	intron 3	r.341_361del	p.Gly114_ Pro120del	deletion 1 cysteine EGFr 2	yes	yes
Dichgans et al.[68]	c.714_758del	exon 5	-	p.Asp239_ Asp253del	deletion 3 cysteines EGFr 6	no‡	no
Lackovic et al.[71]	c.955_956delGCinsTG	exon 6	-	p.Ala319Cys	Replacement alanine with cysteine	no±	yes
Lee et al.[69]	c.1057_1071dup	exon 7	-	p.Asp353_ Ser357dup	insertion 1 cysteine EGFr 9	yes	yes
Tikka et al.[51]	c.1300_1308dup	exon 8	-	p.Glu434_ Leu436dup	insertion 1 cysteine EGFr 11	not reported	yes
Saiki et al.[70]	c.2411-1G>T	intron 15	r.2411_2566del	p.Gly804_ Asn856delinsAsp	deletion 1 cysteine EGFr 20 deletion complete EGFr 21 deletion 1 cysteine EGFr 22	not reported	yes

Table 2. Small *NOTCH3* deletions, duplications, splice site mutations and a deletion/insertion leading to a numerical cysteine alteration. Pathogenic mutations other than missense mutations are rare in CADASIL. The reported small deletions, duplications, splice site mutations and deletion/insertion lead to a numerical cysteine amino acid change in one of the EGFr of NOTCH3. Mutations are described according to HGVS nomenclature,[93] and may therefore differ from the mutation description in the original research article.

† exons 2 and 3 screened; ‡ exons 2-5 screened; ± exons 2-6 screened

NOTCH3 missense mutations not involving a cysteine residue

The first group of mutations with an uncertain association with CADASIL are missense mutations in *NOTCH3* which do not involve a cysteine residue. Various studies have reported such variants to be causative of CADASIL.[43,74-81] (Table 3) In the majority of these reports however, the association is not proven, because either *NOTCH3* was incompletely screened and thus a typical cysteine altering mutation in another exon was not excluded, or the clinical diagnosis was not confirmed by skin biopsy.[75,76,78-80] In four studies, describing the p.Ala1020Pro, p.Arg213Lys, p.Tyr1098Ser, and p.Arg75Pro variants respectively, sequencing of exons 2-24 was comprehensive and the diagnosis was confirmed by skin biopsy.[43,74,77,81] However, the p.Ala1020Pro, and also the p.His170Arg mutation have been reported to be polymorphisms by others.[82] (Supplementary table 2) That polymorphisms are mistakenly interpreted as causative mutations is further suggested by the fact that these variants have been found to co-segregate with a typical cysteine altering missense mutation.[83] (Lesnik Oberstein and Boon, unpublished findings)

The p.Arg213Lys and p.Tyr1098Ser mutations were found in a Japanese and a Chinese patient, respectively, but segregation analysis was not performed. Also for these mutations it remains possible they are polymorphisms, as a coinciding cysteine altering mutation, such as a large deletion or intronic mutation, might have been missed due to technical reasons. This may also apply to the p.Arg61Trp mutation, of which the pathogenicity was described as uncertain.[84] A possible exception may be the p.Arg75Pro mutation, which has been frequently reported in Japanese, Chinese and Korean patients.[43,74,85,86] In two of these families, comprehensive sequencing of *NOTCH3* was performed, the mutation segregated with affected family members and GOM were present in skin biopsy.[74] However, MRI abnormalities were not typical for CADASIL and NOTCH3 immunostaining was not performed. Therefore, drawing definite conclusions regarding the relation between this mutation and CADASIL remains precarious, but it cannot be excluded that this mutation does cause CADASIL or a CADASIL-like phenotype. In summary, with one possible exception, there is no compelling evidence that missense mutations that do not involve the loss or gain of a cysteine residue are associated with CADASIL. Therefore, if such a non-cysteine altering mutation is detected, the clinical diagnosis should be critically re-evaluated and, if the clinical symptoms are persuasive, be confirmed by skin biopsy. If the skin biopsy shows the typical vessel wall abnormalities seen in CADASIL, molecular testing should be elaborated by analysis of larger deletions or duplications using for example MLPA, and the potential effect on splicing of variants with unknown pathogenicity should be ascertained. Finally, if possible, segregation of the mutation with affected family members should be determined.

Reference	Mutation	Exon	Amino acid change	Sequencing analysis all EGFr encoding exons	Diagnosis confirmed by skin biopsy or autopsy
Brass et al.[84]	c.259C>T	2	p.Arg61Trp	yes	yes
Mizuno et al.[74]	c.224G>C	3	p.Arg75Pro	yes	yes
Ampuero et al.[75]	c.451C>G** c.509A>G*	4 4	p.Gln151Glu p.His170Arg	no†	not reported
Roy et al.[76]	c.605C>T	4	p.Ala202Val	no‡	not reported
Kotorii et al.[77]	c.638G>A	4	p.Arg213Lys	yes	yes
Uchino et al.[78]	c.709G>A*	5	p.Val237Met	not reported	not reported
Ferreira et al.[79]	c.1729A>G	11	p.Thr577Ala	not reported	not reported
Ferreira et al.[80]	c.2932A>C	18	p.Ser978Arg	not reported	not reported
Scheid et al.[81]	c.3058G>C*	19	p.Ala1020Pro	yes	yes
Wang et al.[43]	c.3292A>T	20	p.Tyr1098Ser	yes	yes

Table 3. *NOTCH3* missense variants not involving a cysteine residue which have been suggested to be causative of CADASIL. Missense variants not involving cysteine residues have been reported in association with CADASIL in several studies. Most of these were later described to be polymorphisms, or their pathogenicity is questionable due to incomplete molecular analysis or uncertain clinical diagnosis. One possible exception is the p.Arg75Pro mutation, which was found to segregate with the clinical phenotype in two thoroughly studies families.[74] Mutations are described according to HGVS nomenclature,[93] and may therefore differ from the mutation description in the original research article. †exons 2-6,8,11,14,18-19, 22-23 screened; ‡ exons 2-4, 11, 18-19 screened. * Also described as a polymorphism [94,95] (Supplementary table 2) ** Also described as a variant with unknown pathogenicity [82]

NOTCH3 mutations leading to loss-of-function

A second group of mutations about which there is debate as to their pathogenicity in CADASIL, are those leading to loss of NOTCH3 function (out-of-frame deletions or stop mutations). These mutations have been only rarely reported and there is no convincing evidence that such loss-of-function mutations cause CADASIL. As in the case of the non- cysteine altering missense mutations, the reports mentioning an association between CADASIL phenotype and loss-of-function mutations either lack comprehensive *NOTCH3* molecular analysis, or the clinical diagnosis has not been confirmed.[87,88] The hypothesis that loss-of-function mutations may also cause CADASIL is further discredited by the fact that *NOTCH3* loss-of-function mutations have been found in thoroughly studied individuals in whom CADASIL was clinically excluded,[65] as well as by the fact that Notch3 knock-out mice do not develop a CADASIL phenotype.[89] In summary, loss of *NOTCH3* function mutations appear not to cause CADASIL, and should be considered a coincidental finding until proven otherwise.

NOTCH3 MUTATIONS OUTSIDE OF THE EGFR ENCODING EXONS

In the initial report on the identification of *NOTCH3* mutations in CADASIL, one mutation in exon 25 was described.[4] Later reports have shown that mutations located outside EGFr encoding exons 2-24 do not lead to a CADASIL phenotype.[90-92]

EXPERT COMMENTARY

Upon the discovery of *NOTCH3* as the causative gene for CADASIL in 1996, it was clear that mutations were highly distinctive, namely all were missense mutations leading to a cysteine alteration in one of the 34 EGFr of NOTCH3. In the past two decades, this has remained a consistent finding in CADASIL patients reported from all over the world. However, there have also been a substantial number of reports suggesting that other types of mutations, mostly missense mutations that do not involve a cysteine, can also cause CADASIL. To date there is no convincing evidence that these mutations indeed cause CADASIL and they should be considered to be coincidental findings, until proven otherwise. Incorrect interpretation of mutations leads to erroneous diagnosis, with far reaching implications for both the patient and his or her family members. In order to be certain of the diagnosis and the pathogenicity of the mutation, the analysis of patients with mutations that do not alter cysteines should always include comprehensive molecular *NOTCH3* screening to exclude a coinciding cysteine-altering mutation, as well as a thorough clinical (re-)evaluation, including skin biopsy. This should preferably be performed by an experienced team, including a clinical laboratory geneticist, clinical geneticist, neurologist, neuroradiologist and neuropathologist.

FIVE-YEAR VIEW

Nearly two decades after the identification of *NOTCH3* as the causative gene for CADASIL, it has become clear that the disease is much more prevalent than initially assumed and it is now considered the most prevalent type of hereditary vascular dementia. However, there is likely still a substantial number of undiagnosed patients, due to lack of awareness of the disease and the non-specificity of many of the signs and symptoms. However, once the diagnosis has been considered, it is readily confirmed by the presence of a cysteine altering missense mutation in *NOTCH3*. As molecular testing is becoming more widely available, we expect that in the coming 5 years patients will be even more frequently diagnosed and a clearer picture will emerge of both the prevalence and the clinical spectrum of the disease. Thus far, there is no therapy to delay the onset of the disease. Future rational therapeutic strategies may be those modifying, down- regulating

or capturing the toxic mutated protein. In order to determine the effect of such therapeutic strategies in the future, a thorough and complete knowledge of both the mutational spectrum and the natural history of CADASIL is of vital importance.

KEY ISSUES

- CADASIL is caused by gain-of-function mutations in *NOTCH3*, leading to toxic NOTCH3 accumulation in the vessel wall.
- Only *NOTCH3* mutations that alter the number of cysteines in one of the 34 epidermal growth factor repeat (EGFr) domains of the NOTCH3 protein have been proven to cause CADASIL:
  - The great majority are missense mutations
  - Mutations are found only in EGFr-encoding exons (2-24)
  - Most mutations are located in exon 4
- Some CADASIL patients with homozygous and compound heterozygous mutations have been described. The symptoms of these patients seem to be within the normal CADASIL spectrum.
- Small *NOTCH3* in-frame deletions, insertions or splice-site mutations can also cause CADASIL. These typically also alter the number of cysteines in one of the 34 EGFr of NOTCH3.
- Mutations in *NOTCH3* leading to loss of NOTCH3 function do not cause CADASIL. These rare mutations include mutations leading to a frame-shift and stop mutations.
- Missense mutations in *NOTCH3* not altering a cysteine residue are unlikely to be pathogenic, and should be considered coincidental findings until proven otherwise. If such a mutation is detected, the following should be considered:
  - A coinciding cysteine altering mutation may have been missed due to technical reasons or incomplete sequencing analysis.
  - The clinical diagnosis of CADASIL may be incorrect and should be confirmed by electron microscopy and NOTCH3 immunohistochemistry on a skin biopsy.

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No	Nucleotide change	Amino acid change	Exon	EGFR	Reference
1	c.127T>G	p.Cys43Gly	2	1	Peters et al.[3], Opherk et al.[4]
2	c.128G>T	p.Cys43Phe	2	1	Lesnik Oberstein [5]
3	c.145T>G	p.Cys49Gly	2	1	Oki et al.[6]
4	c.145T>C	p.Cys49Arg	2	1	Wang et al.[7]
5	c.146G>A	p.Cys49Tyr	2	1	Joutel et al.[8]
6	c.146G>T	p.Cys49Phe	2	1	Peters et al.[3], Opherk et al.[4]
7	c.157G>T	p.Gly53Cys	2	1	Wang et al.[7]
8	c.160C>T	p.Arg54Cys	2	1	Escary et al.[9]
9	c.179C>G	p.Ser60Cys	2	1	Peters et al.[3], Opherk et al.[4]
10	c.193T>G	p.Cys65Gly	2	1	Cleves et al.[10], HGMD
11	c.194G>C	p.Cys65Ser	2	1	Peters et al.[3], Opherk et al.[4]
12	c.194G>A	p.Cys65Tyr	2	1	Bianchi et al.[11]
13	c.199T>A	p.Cys67Ser	3	1	Tikka et al.[1]
14	c.200G>A	p.Cys67Tyr	3	1	Moon et al.[12]
15	c.213G>T	p.Trp71Cys	3	1	Joutel et al.[13]
16	c.226T>C	p.Cys76Arg	3	1	Markus et al.[14]
17	c.228T>G	p.Cys76Trp	3	1	Peters et al.[3], Opherk et al.[4]
18	c.259T>C	p.Cys87Arg	3	2	Peters et al.[3], Opherk et al.[4]
19	c.260G>A	p.Cys87Tyr	3	2	Opherk et al.[4]
20	c.265G>T	p.Gly89Cys	3	2	Pavlovic et al.[15], Lackovic et al.[16]
21	c.268C>T	p.Arg90Cys	3	2	Joutel et al.[8]
22	c.278G>A	p.Cys93Tyr	3	2	Kalimo et al.[17]
23	c.278G>T	p.Cys93Phe	3	2	Dichgans et al.[18]
24	c.316T>C	p.Cys106Arg	3	2	Yamada et al.[19]
25	c.318C>G	p.Cys106Trp	3	2	Opherk et al.[4]
26	c.322T>C	p.Cys108Arg	3	2	Wang et al.[20]
27	c.323G>C	p.Cys108Ser	3	2	Testi et al.[21]
28	c.323G>A	p.Cys108Tyr	3	2	Peters et al.[3], Opherk et al.[4]
29	c.324C>G	p.Cys108Trp	3	2	Rojas-Marcos et al.[22]
30	c.328C>T	p.Arg110Cys	3	2	Joutel et al.[8]
31	c.349T>C	p.Cys117Arg	4	2	Wang et al.[7]
32	c.350G>T	p.Cys117Phe	4	2	Dichgans et al.[23]
33	c.350G>C	p.Cys117Ser	4	2	Spinicci et al.[24]
34	c.350G>A	p.Cys117Tyr	4	2	Ampuero et al.[25]
35	c.353C>G	p.Ser118Cys	4	3	Lee et al.[26]
36	c.368G>A	p.Cys123Tyr	4	3	Escary et al.[9]
37	c.368G>T	p.Cys123Phe	4	3	Dichgans et al.[18]

No	Nucleotide change	Amino acid change	Exon	EGFR	Reference
38	c.382T>G	p.Cys128Gly	4	3	Coto et al.[27]
39	c.383G>A	p.Cys128Tyr	4	3	Kalimo et al.[17]
40	c.383G>T	p.Cys128Phe	4	3	LOVD
41	c.391G>T	p.Gly131Cys	4	3	Ungaro et al.[28]
42	c.397C>T	p.Arg133Cys	4	3	Joutel et al.[8]
43	c.402C>G	p.Cys134Trp	4	3	Joutel et al.[29]
44	c.421C>T	p.Arg141Cys	4	3	Joutel et al.[8]
45	c.425T>G	p.Phe142Cys	4	3	Kalimo et al.[17]
46	c.431G>A	p.Cys144Tyr	4	3	Dichgans et al.[18]
47	c.431G>C	p.Cys144Ser	4	3	Dichgans et al.[18]
48	c.431G>T	p.Cys144Phe	4	3	Grigg et al.[30]
49	c.434C>G	p.Ser145Cys	4	3	Opherk et al.[4]
50	c.436T>C	p.Cys146Arg	4	3	Joutel et al.[8]
51	c.437G>A	p.Cys146Tyr	4	3	Malandrini et al.[31]
52	c.445G>T	p.Gly149Cys	4	3	Peters et al.[3], Opherk et al.[4]
53	c.449A>G	p.Tyr150Cys	4	3	Dichgans et al.[18]
54	c.457C>T	p.Arg153Cys	4	3	Joutel et al.[8]
55	c.463T>A	p.Cys155Ser	4	3	Ampuero et al.[25]
56	c.464G>A	p.Cys155Tyr	4	3	LOVD
57	c.464G>C	p.Cys155Ser	4	3	Opherk et al.[4]
58	c.484T>A	p.Cys162Ser	4	4	Escary et al.[9]
59	c.484T>C	p.Cys162Arg	4	4	Andreadou et al.[32]
60	c.486C>G	p.Cys162Trp	4	4	Lesnik Oberstein et al.[33]
61	c.493G>T	p.Gly165Cys	4	4	Ampuero et al.[25]
62	c.505C>T	p.Arg169Cys	4	4	Joutel et al.[34]
63	c.511G>T	p.Gly171Cys	4	4	Joutel et al.[8]
64	c.520T>C	p.Cys174Arg	4	4	Santa el al.[35]
65	c.520T>A	p.Cys174Ser	4	4	Tikka et al.[1]
66	c.521G>A	p.Cys174Tyr	4	4	Dichgans et al.[23]
67	c.521G>T	p.Cys174Phe	4	4	Kotorii et al.[36]
68	c.539C>G	p.Ser180Cys	4	4	Escary et al.[9]
69	c.542T>G	p.Phe181Cys	4	4	Granild-Jensen et al.[37]
70	c.544C>T	p.Arg182Cys	4	4	Joutel et al.[34]
71	c.547T>A	p.Cys183Ser	4	4	Dichgans et al.[18]
72	c.547T>C	p.Cys183Arg	4	4	Dichgans et al.[23]
73	c.548G>T	p.Cys183Phe	4	4	Peters et al.[3], Opherk et al.[4]
74	c.553T>C	p.Cys185Arg	4	4	Joutel et al.[8]

No	Nucleotide change	Amino acid change	Exon	EGFR	Reference
75	c.553T>G	p.Cys185Gly	4	4	Joutel et al.[29]
76	c.553T>A	p.Cys185Ser	4	4	Adib-Samii et al.[38], HGMD
77	c.566A>G	p.Tyr189Cys	4	4	Lesnik Oberstein et al.[5]
78	c.580T>A	p.Cys194Ser	4	4	Markus et al.[14]
79	c.580T>C	p.Cys194Arg	4	4	Kalimo et al.[17]
80	c.581G>A	p.Cys194Tyr	4	4	Escary et al.[9]
81	c.581G>T	p.Cys194Phe	4	4	Dichgans et al.[18]
82	c.581G>C	p.Cys194Ser	4	4	Adib-Samii et al.[38], HGMD
83	c.601T>C	p.Cys201Arg	4	5	Uyguner et al.[39]
84	c.602G>A	p.Cys201Tyr	4	5	Opherk et al.[4]
85	c.616T>C	p.Cys206Arg	4	5	Matsumoto et al.[40]
86	c.617G>A	p.Cys206Tyr	4	5	Escary et al.[9]
87	c.619C>T	p.Arg207Cys	4	5	Lesnik Oberstein et al.[41]
88	c.634T>A	p.Cys212Ser	4	5	Joutel et al.[8]
89	c.635G>A	p.Cys212Tyr	4	5	Bentley et al.[42]
90	c.636C>G	p.Cys212Trp	4	5	Spinnici et al.[24]
91	c.659A>G	p.Tyr220Cys	4	5	Rojas-Marcos et al.[43]
92	c.664T>G	p.Cys222Gly	4	5	Joutel et al.[8]
93	c.665G>A	p.Cys222Tyr	4	5	Kalimo et al.[17]
94	c.665G>C	p.Cys222Ser	4	5	Wang et al.[7]
95	c.671G>A	p.Cys224Tyr	4	5	Joutel et al.[8]
96	c.697T>A	p.Cys233Ser	5	5	Joutel et al.[29]
97	c.697T>C	p.Cys233Arg	5	5	Adib-Samii et al.[38], HGMD
98	c.698G>A	p.Cys233Tyr	5	5	Peters et al.[3], Opherk et al.[4]
99	c.699T>G	p.Cys233Trp	5	5	Lesnik Oberstein [5]
100	c.719G>C	p.Cys240Ser	5	6	Opherk et al.[4]
101	c.733T>C	p.Cys245Arg	5	6	Peters et al.[3], Opherk et al.[4]
102	c.733T>A	p.Cys245Ser	5	6	Razvi et al.[44]
103	c.751T>C	p.Cys251Arg	5	6	Markus et al.[14]
104	c.751T>A	p.Cys251Ser	5	6	Lesnik Oberstein [5]
105	c.751T>G	p.Cys251Gly	5	6	Viskelis et al.[45]
106	c.752G>A	p.Cys251Tyr	5	6	Tikka et al.[1]
107	c.773A>G	p.Tyr258Cys	5	6	Joutel et al.[8]
108	c.778T>G	p.Cys260Gly	5	6	De Silva et al.[46]
109	c.779G>A	p.Cys260Tyr	5	6	Peters et al.[3], Opherk et al.[4]
110	c.812G>T	p.Cys271Phe	6	6	Kam-Ming Au et al.[47]
111	c.886G>T	p.Gly296Cys	6	7	Garcia-Estevez et al.[48]

No	Nucleotide change	Amino acid change	Exon	EGFR	Reference
112	c.895A>T	p.Ser299Cys	6	7	Golomb et al.[49]
113	c.994C>T	p.Arg332Cys	6	8	Oliveri et al.[50]
114	c.1004C>G	p.Ser335Cys	6	8	Peters et al.[3], Opherk et al.[4]
115	c.1010A>G	p.Tyr337Cys	6	8	Lesnik Oberstein [5]
116	c.1012T>C	p.Cys338Arg	6	8	Dotti et al.[51]
117	c.1078T>C	p.Cys360Arg	7	9	Kim et al.[52]
118	c.1096T>C	p.Cys366Arg	7	9	HGMD
119	c.1098T>G	p.Cys366Trp	7	9	Pradotto et al.[53]
120	c.1135T>C	p.Cys379Arg	7	9	Del Rio-Espínola et al.[54]
121	c.1136G>C	p.Cys379Ser	7	9	Peters et al.[3], Opherk et al.[4]
122	c.1144G>T	p.Gly382Cys	7	9	Lesnik Oberstein [5]
123	c.1163G>A	p.Cys388Tyr	7	9	Ishida et al.[55]
124	c.1183T>C	p.Cys395Arg	7	10	Peters et al.[3], Opherk et al.[4]
125	c.1187C>G	p.Ser396Cys	7	10	Testi et al.[21]
126	c.1241C>G	p.Ser414Cys	8	10	Kim et al.[52]
127	c.1258G>T	p.Gly420Cys	8	10	Joutel et al.[29]
128	c.1261C>T	p.Arg421Cys	8	10	Peters et al.[3], Opherk et al.[4]
129	c.1279C>T	p.Arg427Cys	8	10	HGMD
130	c.1282T>C	p.Cys428Arg	8	10	Dotti et al.[51]
131	c.1283G>A	p.Cys428Tyr	8	10	Peters et al.[3], Opherk et al.[4]
132	c.1283G>C	p.Cys428Ser	8	10	Joutel et al.[29]
133	c.1303T>C	p.Cys435Arg	8	11	Lesnik Oberstein [5]
134	c.1318T>A	p.Cys440Ser	8	11	Federico et al.[56]
135	c.1318T>G	p.Cys440Gly	8	11	Markus et al.[14]
136	c.1318T>C	p.Cys440Arg	8	11	Peters et al.[3], Opherk et al.[4]
137	c.1337G>C	p.Cys446Ser	8	11	Opherk et al.[4]
138	c.1337G>T	p.Cys446Phe	8	11	Lesnik Oberstein et al.[33]
139	c.1345C>T	p.Arg449Cys	8	11	Thomas et al.[57]
140	c.1363T>C	p.Cys455Arg	8	11	Arboleda-Velasquez et al.[58]
141	c.1364G<A	p.Cys455Tyr	8	11	Kim et al.[52]
142	c.1370G>C	p.Cys457Ser	8	11	Adib-Samii et al.[38], HGMD
143	c.1394A>G	p.Tyr465Cys	9	11	Lesnik Oberstein [5]
144	c.1450T>G	p.Cys484Gly	9	12	LOVD
145	c.1451G>A	p.Cys484Tyr	9	12	Opherk et al.[4]
146	c.1451G>T	p.Cys484Phe	9	12	Peters et al.[3]
147	c.1484G>A	p.Cys495Tyr	9	12	Peters et al.[3], Opherk et al.[4]
148	c.1510T>C	p.Cys504Arg	10	12	Lee et al.[59]

No	Nucleotide change	Amino acid change	Exon	EGFR	Reference
149	c.1531T>C	p.Cys511Arg	10	13	Peters et al.[3], Opherk et al.[4]
150	c.1532G>T	p.Cys511Phe	10	13	Mosca et al.[60]
151	c.1532G>A	p.Cys511Tyr	10	13	Bianchi et al.[61]
152	c.1582G>T	p.Gly528Cys	10	13	Dotti et al.[51]
153	c.1592G>C	p.Cys531Ser	10	13	Mazzei et al.[62]
154	c.1594C>T	p.Arg532Cys	10	13	Bianchi et al.[63]
155	c.1624T>C	p.Cys542Arg	11	13	Kim et al.[52]
156	c.1625G>A	p.Cys542Tyr	11	13	Joutel et al.[8]
157	c.1630C>T	p.Arg544Cys	11	14	Lesnik Oberstein et al.[41]
158	c.1645T>C	p.Cys549Arg	11	14	Lesnik Oberstein [5]
159	c.1646G>A	p.Cys549Tyr	11	14	Peters et al.[3], Opherk et al.[4]
160	c.1672C>T	p.Arg558Cys	11	14	Joutel et al.[8]
161	c.1703G>A	p.Cys568Tyr	11	14	Ferreira et al.[64]
162	c.1721A>G	p.Tyr574Cys	11	14	Mazzei et al.[65]
163	c.1732C>T	p.Arg578Cys	11	14	Joutel et al.[34]
164	c.1735T>C	p.Cys579Arg	11	14	Roy et al.[66]
165	c.1759C>T	p.Arg587Cys	11	15	Kim et al.[67]
166	c.1771T>C	p.Cys591Arg	11	15	LOVD
167	c.1774C>T	p.Arg592Cys	11	15	Praline et al.[68], HGMD
168	c.1790G>C	p.Cys597Ser	11	15	Bohlega et al.[69]
169	c.1816T>C	p.Cys606Arg	11	15	Testi et al.[21]
170	c.1819C>T	p.Arg607Cys	11	15	Escary et al.[9]
171	c.1918C>T	p.Arg640Cys	12	16	LOVD
172	c.1999G>T	p.Gly667Cys	13	17	LOVD
173	c.2038C>T	p.Arg680Cys	13	17	Pradotto et al.[70]
174	c.2129A>G	p.Tyr710Cys	13	18	Rutten et al.[71]
175	c.2149C>T	p.Arg717Cys	14	18	HGMD
176	c.2182C>T	p.Arg728Cys	14	18	Joutel et al.[8]
177	c.2324G>C	p.Cys775Ser	15	20	Peters et al.[3]
178	c.2815T>C	p.Cys939Arg	18	24	Testi et al.[21]
179	c.2857G>T	p.Gly953Cys	18	24	Markus et al.[14]
180	c.2923G>T	p.Gly975Cys	18	25	Kotorii et al.[72]
181	c.2929T>A	p.Cys977Ser	18	25	Lee et al.[26]
182	c.2951T>G	p.Phe984Cys	18	25	Escary et al.[9]
183	c.2953C>T	p.Arg985Cys	18	25	Joutel et al.[8]
184	c.2963G>A	p.Cys988Tyr	18	25	Kim et al.[67]
185	c.2989T>G	p.Cys997Gly	18	25	Ungaro et al.[28]

No	Nucleotide change	Amino acid change	Exon	EGFR	Reference
186	c.3011G>A	p.Cys1004Tyr	19	26	Guerrot et al.[73]
187	c.3016C>T	p.Arg1006Cys	19	26	Joutel et al.[8]
188	c.3037G>T	p.Gly1013Cys	19	26	Testi et al.[21]
189	c.3043T>C	p.Cys1015Arg	19	26	Lesnik Oberstein et al.[41]
190	c.3062A>G	p.Tyr1021Cys	19	26	Kalimo et al.[17]
191	c.3065G>T	p.Cys1022Phe	19	26	Vedeler et al.[74]
192	c.3084G>T	p.Trp1028Cys	19	26	Viana-Babtista et al.[75]
193	c.3091C>T	p.Arg1031Cys	19	26	Joutel et al.[8]
194	c.3172G>T	p.Gly1058Cys	20	27	Kalimo et al.[17]
195	c.3182G>A	p.Cys1061Tyr	20	27	LOVD
196	c.3206A>G	p.Tyr1069Cys	20	27	Tikka et al.[1]
197	c.3226C>T	p.Arg1076Cys	20	27	Lesnik Oberstein et al.[33]
198	c.3296G>A	p.Cys1099Tyr	20	28	Ferreira et al.[76]
199	c.3393C>G	p.Cys1131Trp	21	29	Pescini et al.[77]
200	c.3427C>T	p.Arg1143Cys	21	29	HGMD
201	c.3691C>T	p.Arg1231Cys	22	31	Joutel et al.[8]
202	c.3750C>G	p.Cys1250Trp	23	32	Del Rio-Espínola et al.[78]
203	c.3781T>C	p.Cys1261Arg	23	32	Joutel et al.[13]
204	c.3782G>A	p.Cys1261Tyr	23	32	Peters et al.[3], Opherk et al.[4]
205	c.3893G>T	p.Cys1298Phe	24	33	Rinnoci et al.[79]
206	c.3944G>A	p.Cys1315Tyr	24	33	Valenti et al.[80]

Supplementary table 1. Currently known cysteine altering missense mutations in *NOTCH3* causing CADASIL. This list is compiled data from a list of CADASIL causing mutations described by Tikka et al.[1] in 2009, supplemented with mutations described thereafter. All mutations are described according to HGVS nomenclature, and may therefore differ from the mutation description in the original research article. LOVD= leiden open variation database (www.lovd.nl) [2], HGMD= human gene mutation database

No	Amino acid change	Exon	EGFR	Reference
1	p.Arg103Gln	3	2	Schmidt et al.[81]
2	p.His170Arg	4	4	Joutel et al.[8]
3	p.Pro496Leu	9	12	Joutel et al.[8]
4	p.Ser497Leu	9	12	Schmidt et al.[81]
5	p.Ser502Phe	9	12	Schmidt et al.[81]
6	p.Val764Ala	14	19	Schmidt et al.[81]
7	p.His981Tyr	18	25	Ross et al.[82]
8	p.Ala1020Pro	19	26	Schmidt et al.[81]
9	p.His1133Gln	21	29	Joutel et al.[8]
10	p.Val1183Met	22	30	Joutel et al.[8]
11	p.His1235Leu	22	31	Schmidt et al.[81]
12	p.Arg1262Leu	23	32	Schmidt et al.[81]

Supplementary table 2. *NOTCH3* missense variants not involving a cysteine residue described as non- pathogenic. In healthy individuals, variants have been detected which lead to an amino acid change in the EGFR encoding exons of NOTCH3, but which do not involve a cysteine residue. Some of these polymorphisms, namely pHis170Arg and p.Ala1020Pro have also erroneously been reported to be pathogenic. Note: this list results from polymorphisms described in larger studies in healthy individuals, and is not a complete list of all *NOTCH3* polymorphisms described.

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HYPOMORPHIC NOTCH3 ALLELES DO NOT CAUSE CADASIL IN HUMANS

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ABSTRACT

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is caused by stereotyped missense mutations in *NOTCH3*. Whether these mutations lead to the CADASIL phenotype via a neomorphic effect, or rather by a hypomorphic effect, is subject of debate. Here, we report two novel *NOTCH3* mutations, both leading to a premature stop codon with predicted loss of NOTCH3 function. The first mutation, c.307C>T, p.Arg103*, was detected in two brothers aged 50 and 55 years, with a brain MRI and skin biopsy incompatible with CADASIL. The other mutation was found in a 40-year-old CADASIL patient compound heterozygous for a pathogenic *NOTCH3* mutation (c.2129A>G, p.Tyr710Cys) and an intragenic frameshift deletion. The deletion was inherited from his father, who did not have the skin biopsy abnormalities seen in CADASIL patients. These individuals with rare *NOTCH3* mutations indicate that hypomorphic *NOTCH3* alleles do not cause CADASIL.

INTRODUCTION, RESULTS AND DISCUSSION

CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) is a monogenic disorder caused by mutations in the *NOTCH3* gene (MIM #600276).[1] CADASIL patients typically present with stroke and cognitive decline at a mean age of 45–50 years. Migraine with aura and mood disturbances occurs in up to 40% of patients. Disease severity and age of onset is variable, also within families, and the clinical presentation can be atypical. The invariable and preclinical presence of a recognizable pattern of white matter hyperintensities (WMH) on brain MRI plays a key role in clinical diagnosis, seen in all individuals with a CADASIL causing *NOTCH3* mutation from at least 35 years of age. Next to increasingly extensive WMH, subcortical infarcts are typically seen in symptomatic patients, whereas cortical and cerebellar lesions are rare.[2] The clinical diagnosis is confirmed by *NOTCH3*-sequencing analysis, with the detection of a typical cysteine-altering mutation.[3] If the clinical or molecular results are inconclusive, immunohistochemistry and electron microscopy (EM) can be performed on a skin biopsy, to determine the presence of pathognomonic vessel wall abnormalities. These consist of positive NOTCH3 staining, electron microscopic deposits of granular osmiophilic material (GOM), and vascular smooth muscle cell (VSMC) degeneration.[4,5] Next to a compatible clinical and family history, the trias of brain MRI, molecular *NOTCH3* analysis, and, if necessary, skin biopsy usually allows for conclusive confirmation or rejection of CADASIL diagnosis.

NOTCH3 encodes the NOTCH3 protein, a transmembrane protein that, in adults, is predominantly expressed in the vasculature by VSMC and pericytes.[6] The ectodomain of NOTCH3 (NOTCH3^{ECD}) consists of 34 epidermal growth factor-like repeat (EGFr) domains, which each contain exactly six highly conserved cysteine residues. *NOTCH3* mutations in CADASIL are located in the exons encoding the NOTCH3^{ECD} (exons 2–24),[2] and invariably lead to an uneven number of cysteine residues, typically five or seven, in the corresponding EGFr. This leaves one cysteine unpaired and has been shown to lead to increased multimerization of the mutated protein.[7] In more than 95% of cases, the cysteine amino acid change is caused by a missense mutation.[2] Despite the stereotyped nature of *NOTCH3* mutations in CADASIL, it is still unknown whether the primary determinant of the CADASIL phenotype is a hypomorphic or rather a neomorphic effect. The most adhered to hypothesis is that there is a neomorphic effect, with toxic accumulation of the mutated protein at the VSMC membrane.[6] However, in a recent study, the loss-of-function hypothesis is revived, as a reduced NOTCH3 signaling was found to be associated with CADASIL pathophysiology *in vitro* and *in vivo*.[8]

Here, we describe individuals from two families with *NOTCH3* mutations that are predicted to result in a premature stop codon. We combined clinical, molecular,

radiological, and pathological investigations to determine whether patients with these hypomorphic mutations have a CADASIL phenotype.

The index patient of family 1, a 55-year-old Caucasian male (index, II-2, Fig. 1A), was referred to our multidisciplinary CADASIL out-patient clinic, because a novel *NOTCH3* variant with unknown pathogenicity had been detected in a laboratory elsewhere. The variant, a *NOTCH3*nonsense mutation in exon 3 (c.307C>T, p.Arg103*) (NM_000435.2), was confirmed in the Leiden Laboratory for Diagnostic Genome Analysis in genomic DNA and in mRNA isolated from blood (Fig. 1B) (www.lovd.nl/NOTCH3). The case history reported a polyneuropathy, migraine with aura, and ischemic strokes at the age of 50 and 52 years. Neurological examination revealed mild motor weakness of the left hallux, absence of vibration sense in feet, difficulty in fine motor tasks of the left hand, and a bilateral action tremor. Mini-mental state examination (MMSE) was normal (30/30). Brain MRI (axial FLAIR and T2* gradient echo, sagittal T1 and coronal FLAIR, and T2 weighted images) was reviewed by a neuroradiologist specialized in CADASIL and showed three old infarctions, respectively, in the right and left occipital cortex and in the left parietal cortex, consistent with large vessel stroke. Furthermore, a few small circumscript WMH were seen. There were no lacunar infarctions and no WMH in a distribution or extent consistent with CADASIL (Fig. 1C). A skin biopsy was then taken from the inner side of the forearm, which showed a normal vessel wall structure, negative NOTCH3 staining (Novus Biologicals, Littleton, CO; dilution 1:2000) and no electron microscopic GOM deposits or VSMC degeneration (Supp. Fig. S1 A and B). Family history was negative for stroke and dementia. The index has three siblings, all older than 45 years of age. They all have a history of migraine but no other neurological symptoms. His mother (I-2) was tested negative for the *NOTCH3* mutation and his father (I-1) is deceased, but had no medical history of stroke or dementia. His 50-year-old brother (II-3) had received genetic counselling elsewhere and had opted for predictive *NOTCH3* mutation testing, showing the presence of the familial *NOTCH3* nonsense mutation. This brother was then also referred to our CADASIL clinic, where neurological examination and brain MRI (axial T1, T2, FLAIR weighted images) were found to be completely normal (Fig. 1D).

In the second family, the index patient is a 40-year-old male (index, II-1; Fig. 2A), with a medical history of migraine with aura, bipolar disorder, and sarcoidosis. There was no history of (transient) neurological deficits. Neurological examination was normal, his MMSE was 28/30. Brain MRI (axial T1, T2, proton density, and sagittal FLAIR weighted images) had been made at a hospital elsewhere and showed partly confluent WMH in a pattern consistent with CADASIL, including the anterior temporal lobes (Supp. Fig. S2). To confirm the clinical diagnosis, blood was sent to the Leiden Laboratory for Diagnostic Genome Analysis for *NOTCH3* mutation

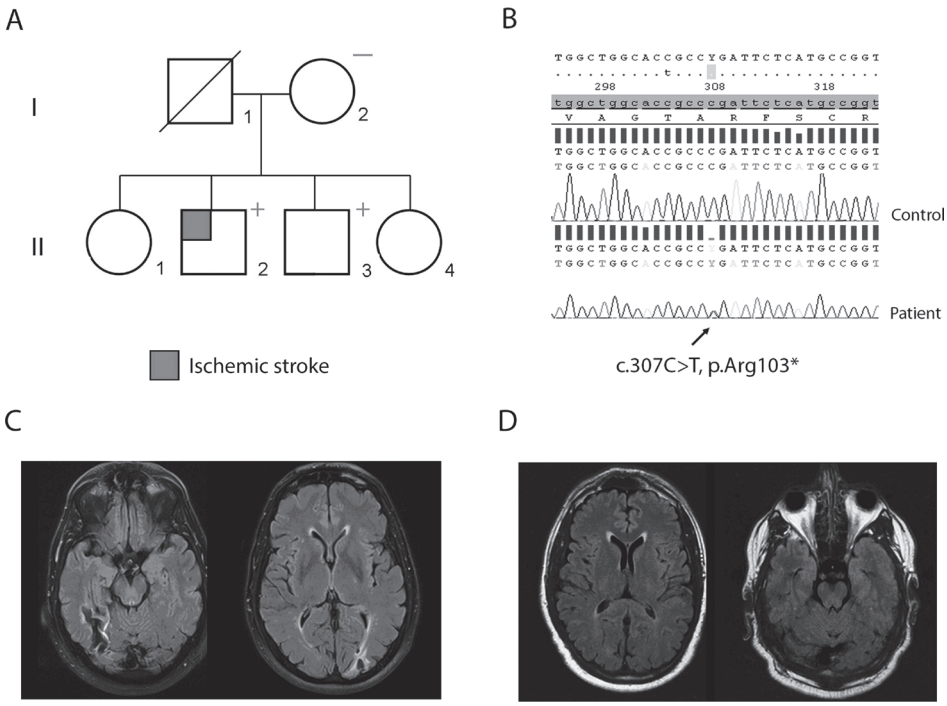

Figure 1. Family 1: A *NOTCH3* nonsense mutation in the absence of a CADASIL phenotype. **A:** Pedigree of family 1. The index patient (II-2) has a history of ischemic stroke but no abnormalities on skin biopsy. His brother (II-3) has no history of neurological deficits. +, presence of p.Arg103* mutation; –, absence of p.Arg103* mutation. **B:** Sequencing analysis of the index showing a *NOTCH3* nonsense mutation in exon 3 (c.307C>T, p.Arg103*). **C:** Brain MRI (FLAIR) of the 55-year-old index (individual II-2) showing cortical defects with surrounding gliosis, consistent with old ischemic infarcts (shown here in the occipital lobes). A few minor nonspecific subcortical WMH are present. No WMH are present in the anterior temporal lobes. **D:** Brain MRI (FLAIR) of the 50-year-old brother of the index (individual II-3) showing no abnormalities.

analysis. Sequence analysis was performed of exons 2–23, including intron/exon boundaries. A characteristic CADASIL causing *NOTCH3* mutation (c.2129A>G, p.Tyr710Cys) was detected in exon 13 (Fig. 2B), which appeared to be homozygous. To exclude the possibility of a *NOTCH3* deletion as the reason for the absence of the wild-type allele, multiplex ligation-dependent probe amplification (MLPA) analysis was performed using a homemade MLPA kit containing probes for all coding exons (2–33) of *NOTCH3*. This revealed the presence of a large intragenic heterozygous frameshift deletion of exons 3–16 (Fig. 2B). This patient is, therefore, compound heterozygous for a typical CADASIL-causing missense mutation and a large intragenic*NOTCH3* deletion. Although the clinical diagnosis of CADASIL was

confirmed by the detection of the *NOTCH3* missense mutation, a family study was performed to determine the pathogenicity of the intragenic *NOTCH3* deletion. *NOTCH3* mutation analysis of the patient's parents showed that the p.Tyr710Cys mutation was inherited from his mother (I-2) and the *NOTCH3* deletion was inherited from his father (I-1). A comprehensive medical assessment of the parents was complicated by the fact that they live in the former Dutch colonies. The medical history of the mother, however, was notable for a brain scan made around the age of 40 years after transient functional deficit of the arm. This scan was no longer available and she did not consent to further medical investigations. The 65-year-old father ostensibly has a history of psychiatric problems, but he has no history of stroke or dementia. He did not consent to brain imaging, but he did agree to have a skin biopsy taken, which was sent to us for pathological analysis and fibroblast culture. NOTCH3 staining of skin vasculature was negative and EM showed no GOM, VSMC degeneration, or other abnormalities (Supp. Fig. S1 C and D). To further determine the breakpoints of the *NOTCH3* deletion, we performed *NOTCH3* RT-PCR on RNA derived from the father's fibroblasts, using an exon 2 forward and an exon 18 reverse primer. Sequencing of the obtained RT-PCR product showed that exons 3–16 are completely missing as a result of the deletion (Fig. 2D). This is predicted to lead to a disruption of the *NOTCH3* reading frame and a premature stop codon in exon 17 (r.198_2566del, p.Cys67ProfsX34) (www.lovd.nl/NOTCH3).

The novel *NOTCH3* mutations detected in the two families described above are a nonsense mutation and an intragenic frameshift deletion, respectively. Both mutations are predicted to lead to a truncation at the protein's *N*-terminus, which then lacks essential domains required for NOTCH3 processing and signaling, including the transmembrane and intracellular domains. Therefore, these mutations must result in hypomorphic alleles, that is, alleles that have reduced gene activity. One individual with the nonsense mutation (c.307C>T, p.Arg103*; family 1, II-2) has a history of stroke, but his brain imaging is inconsistent with CADASIL and, moreover, his skin biopsy was negative for CADASIL vessel wall pathology. His brother (II-3), with the same nonsense mutation, was healthy at the age of 50 years and had a normal brain MRI. Together, these findings indicate that the *NOTCH3* nonsense mutation is a coincidental finding and does not cause CADASIL. In family 2, the father of the index (I-1) has a *NOTCH3* deletion of exon 3 through 16 on one allele. He allegedly has a history of psychiatric problems, but at the age of 65 years he has no medical history of stroke or dementia. Although a brain MRI would have contributed to a confirmation or exclusion of the diagnosis CADASIL, a skin biopsy was made that did not show any of the abnormalities consistently seen in CADASIL patients. As both the *NOTCH3* nonsense mutation and the

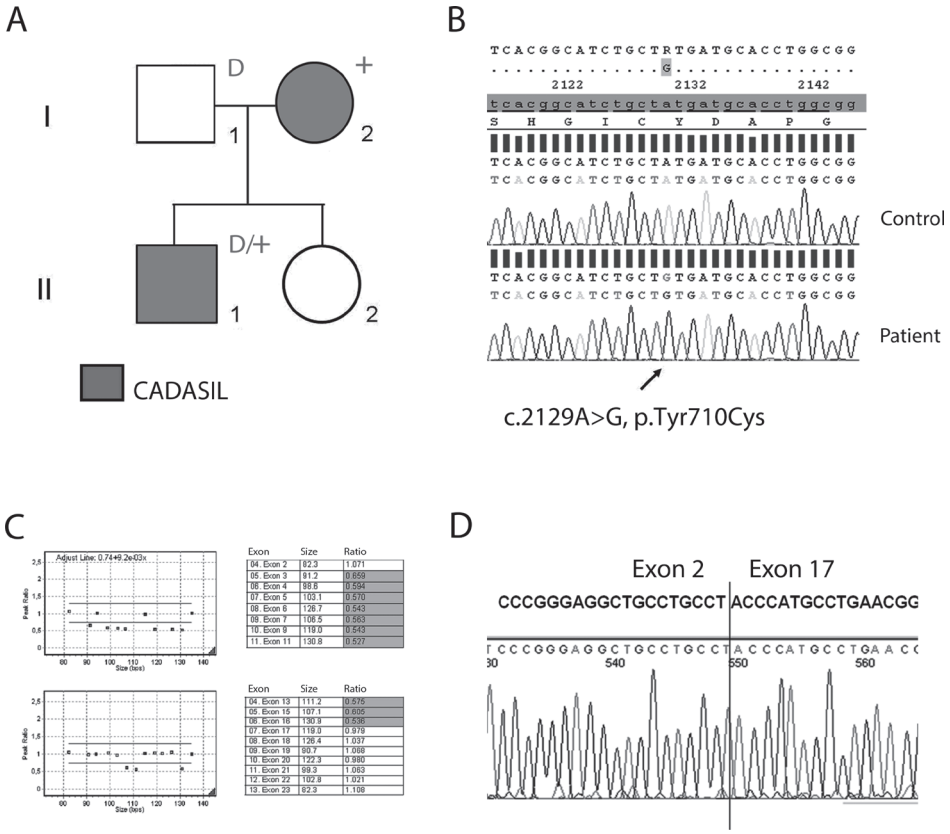

Figure 2. Family 2: A characteristic *NOTCH3* mutation and a *NOTCH3* deletion. A: Pedigree of family 2. The index patient is II-1. D, presence of del 3–16; +, presence of p.Tyr710Cys mutation. **B:** Sequencing analysis of the index (II-1) showing the presence of a characteristic *NOTCH3* missense mutation (c.2129A>G, p.Tyr710Cys), with absence of a wild-type allele. **C:** MLPA analysis of the index (II-1) revealing the presence of a large internal heterozygous deletion of exons 3–16. Exons with corresponding MLPA ratios are depicted graphically as well as summarized in a table. **D:** Sequencing analysis of cDNA derived from cultured fibroblast RNA of the father (I-1), showing a complete deletion of exons 3 up to and including exon 16.

intragenic *NOTCH3* deletion do not lead to a CADASIL phenotype, we conclude that hypomorphic *NOTCH3* alleles do not cause CADASIL. This conclusion is further strengthened by the fact that frameshift and nonsense*NOTCH3* variants have been reported previously in public databases and in an exome resequencing study on colorectal cancer patients (http://www.ncbi.nlm.nih.gov/snp/).[9,10] Whole *NOTCH3* gene deletions have also been reported in humans, but only in the context of contiguous gene deletions in patients with multiple congenital

anomalies (http://decipher.sanger.ac.uk). In contrast to our findings, small out-of-frame *NOTCH3* deletions, resulting in hypomorphic alleles, have been previously reported to be associated with CADASIL.[11,12] However, in these reports, the association of the hypomorphic allele with CADASIL is questionable; as the clinical diagnosis was not confirmed by skin biopsy, segregation analysis was not possible and in one study mutation analysis of the *NOTCH3* gene was incomplete. The few small *NOTCH3* deletions or insertions that have been proven to be the cause of CADASIL are all in-frame and typically affect cysteine residue number, or in one case, cysteine spacing.[5,13-16] The fact that only cysteine-altering mutations have been found to be unequivocally associated with CADASIL, strongly supports the concept that cysteine-altering *NOTCH3* mutations are a conditio sine qua non for CADASIL. In the clinical setting, mutations detected in *NOTCH3* should therefore be carefully evaluated for their effect on cysteine residues in the NOTCH3 protein. If the mutation, be it missense or otherwise, does not affect the number of cysteines in an EGFr domain, then this mutation should be interpreted with great caution and CADASIL diagnosis should only be confirmed based on additional investigations, such as a skin biopsy. Also, when there is an apparent homozygous mutation, the presence of a deletion on one of the alleles should be excluded. An experienced team is important for the correct interpretation of atypical *NOTCH3* mutations, to prevent an erroneous diagnosis of CADASIL. Such a team, in our opinion, should consist at least of a clinical geneticist, clinical laboratory geneticist, neurologist, genetic psychologist, (neuro)pathologist, and neuroradiologist.

Notably, we report herein the first CADASIL patient who is compound heterozygous for a typical cysteine-altering *NOTCH3* mutation on one allele and a large intragenic *NOTCH3* deletion on the other allele. Interestingly, this patient has a phenotype within the normal CADASIL spectrum.

In conclusion, our findings indicate that hypomorphic *NOTCH3* alleles do not cause CADASIL in humans, which has important implications for diagnostic interpretation of *NOTCH3* mutations. Furthermore, this can refine the focus of future studies on the effect of CADASIL-causing mutations on the NOTCH3 protein and the development of rational therapeutic approaches.

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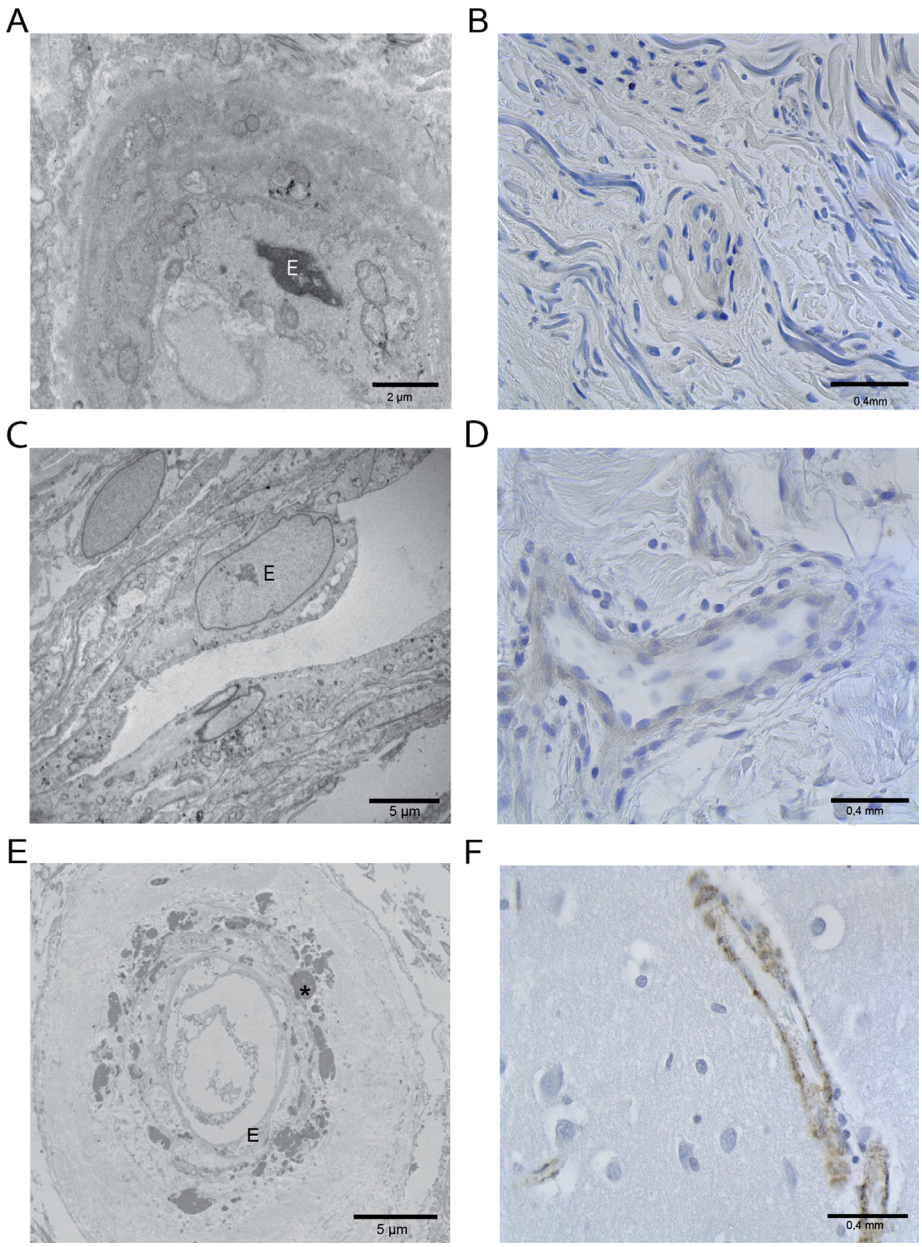
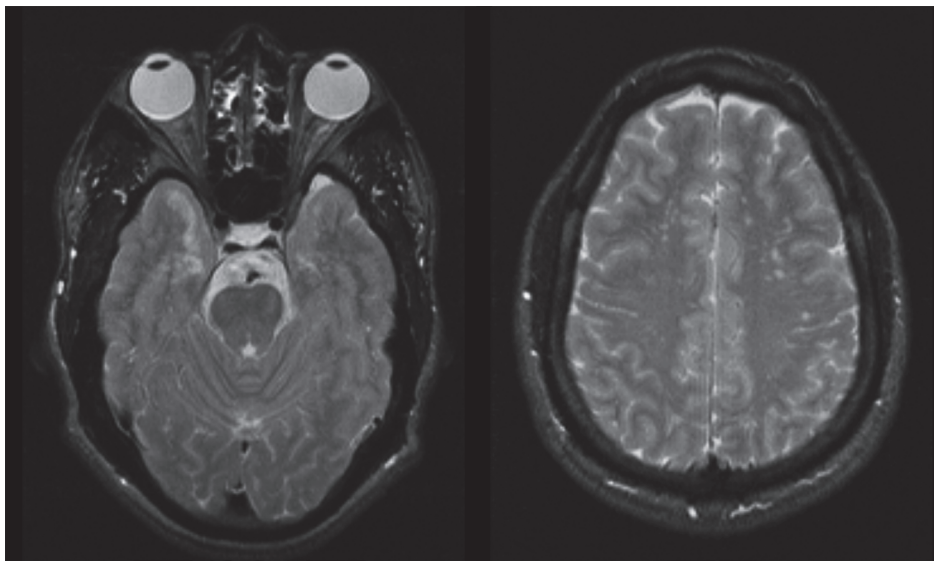


Supplementary figure 1. Pathological analysis of skin biopsies in family 1 and family 2. The skin biopsies of individual II-2 in family 1 (panel A and B) and individual I-1 in figure 3A (panel C and D) do not reveal the abnormalities typically seen in CADASIL. No GOM is seen on electron microscopy (panel A and C) and NOTCH3 staining is negative (panel B and D). Positive control skin biopsy of a CADASIL patient shows both GOM (panel E) and positive NOTCH3 staining (Panel F).
E=Endothelium *=GOM



Supplementary figure 2. Family 2: brain imaging.
Brain MRI (T2) of individual II-1 in family 2 at 37 years of age, showing subcortical WMH located in the semioval centre, with the highest load in the frontal lobes. WMH are also present in the anterior temporal lobes, as is typically seen in CADASIL (right more than left). No lacunar infarctions are seen.

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4

THERAPEUTIC NOTCH3 CYSTEINE CORRECTION IN CADASIL USING EXON SKIPPING: IN VITRO PROOF OF CONCEPT

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ABSTRACT

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy, or CADASIL, is a hereditary cerebral small vessel disease, caused by characteristic cysteine altering missense mutations in the *NOTCH3* gene. *NOTCH3* mutations in CADASIL result in an uneven number of cysteine residues in one of the 34 epidermal growth factor like- repeat (EGFr) domains of the NOTCH3 protein. The consequence of an unpaired cysteine residue in an EGFr domain is an increased multimerization tendency of mutant NOTCH3, leading to toxic accumulation of the protein in the (cerebro)vasculature, and ultimately reduced cerebral blood flow, recurrent stroke and vascular dementia. There is no therapy to delay or alleviate symptoms in CADASIL. We hypothesized that exclusion of the mutant EGFr domain from NOTCH3 would abolish the detrimental effect of the unpaired cysteine and thus prevent toxic NOTCH3 accumulation and the negative cascade of events leading to CADASIL. To accomplish this NOTCH3 cysteine correction by EGFr domain exclusion, we used pre-mRNA antisense-mediated skipping of specific *NOTCH3* exons. Selection of these exons was achieved using *in silico* studies and based on the criterion that skipping of a particular exon or exon pair would modulate the protein in such a way that the mutant EGFr domain is eliminated, without otherwise corrupting NOTCH3 structure and function. Remarkably, we found that this strategy closely mimics evolutionary events, where the elimination and fusion of NOTCH EGFr domains led to the generation of four functional NOTCH homologues. We modelled a selection of exon skip strategies using cDNA constructs and show that the skip proteins retain normal protein processing, can bind ligand and be activated by ligand. We then determined the technical feasibility of targeted *NOTCH3* exon skipping, by designing antisense oligonucleotides targeting exons 2-3, 4-5 and 6, which together harbor the majority of distinct CADASIL causing mutations. Transfection of these antisense oligonucleotides into CADASIL patient derived cerebral vascular smooth muscle cells resulted in successful exon skipping, without abrogating NOTCH3 signalling. Combined, these data provide proof of concept for this novel application of exon skipping, and are a first step towards the development of a rational therapeutic approach applicable to up to 94% of CADASIL causing mutations.

Key words: CADASIL, NOTCH3, exon skipping, therapy, cysteines

Abbreviations: CADASIL= cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy, VSMC= vascular smooth muscle cell, NOTCH3^{ECD}= NOTCH3 extracellular domain, EGFr = epidermal growth factor-like repeat, AON= antisense oligonucleotide

INTRODUCTION

CADASIL is a hereditary vascular dementia and stroke syndrome, caused by cysteine-altering missense mutations in the *NOTCH3* gene.[1, 2]

NOTCH3 is a single pass transmembrane receptor which is required for VSMC differentiation and maturation,[3] and integrity of especially resistance arterioles.[4, 5] The NOTCH3^{ECD} contains 34 EGFr domains, which are approximately 40 amino acid long modular protein subunits, characterized by a conserved position and number of six cysteine residues (Fig. 1A). In pairs, these cysteines form three disulphide bridges which stabilize EGFr domain structure. *NOTCH3* mutations in CADASIL are found throughout exons 2-24, which are the exons that encode the EGFr domains, with a strong clustering of mutations in exon 4.[2] As a result of the mutation, the number of cysteine residues in one of the 34 EGFr domains is typically altered to an uneven number of five or seven cysteines (Fig. 1B). This results in an unpaired cysteine residue and disrupted disulphide bridge formation in mutant NOTCH3^{ECD}, which has been shown to have increased multimerization properties compared to wildtype NOTCH3^{ECD}.[6] This increased multimerization leads to toxic aggregation of NOTCH3^{ECD} in and around VSMCs in small- to medium sized arteries of especially the brain, but also in arteries of other organs and tissues.[3, 7] NOTCH3^{ECD} aggregation is associated with pathological vessel wall changes including degeneration of VSMCs and deposition of granular osmiophilic material visualised by electron microscopy.[8] Clinical symptoms seem to be confined to the brain, where impaired cerebrovascular reactivity [9] and decreased cerebral blood flow [10] lead to chronic hypoxia, recurrent subcortical infarctions and vascular dementia. Typically, patients suffer from progressive cognitive and physical decline from a mean age of 45-50 years, preceded by at least a decade of progressive ischemic MRI abnormalities.[11] To date, there is no therapy available to alleviate or delay the onset of symptoms.

As a mutant NOTCH3 EGFr domain with an uneven number of five or seven cysteines appears to be the common CADASIL instigator, a rational therapeutic approach would be to eliminate this mutant EGFr domain. We hypothesized that exclusion of the mutant EGFr domain from NOTCH3 would abolish the detrimental effect of the unpaired cysteine and thus prevent toxic NOTCH3^{ECD} accumulation and the negative cascade of events leading to CADASIL. To accomplish this NOTCH3 cysteine correction by EGFr domain exclusion, we used pre-mRNA antisense-mediated skipping of specific *NOTCH3* exons. Selection of these exons was achieved using *in silico* studies and based on the criterion that skipping of a particular exon or exon pair would modulate the protein in such a way that the mutant EGFr domain is eliminated, without otherwise corrupting NOTCH3

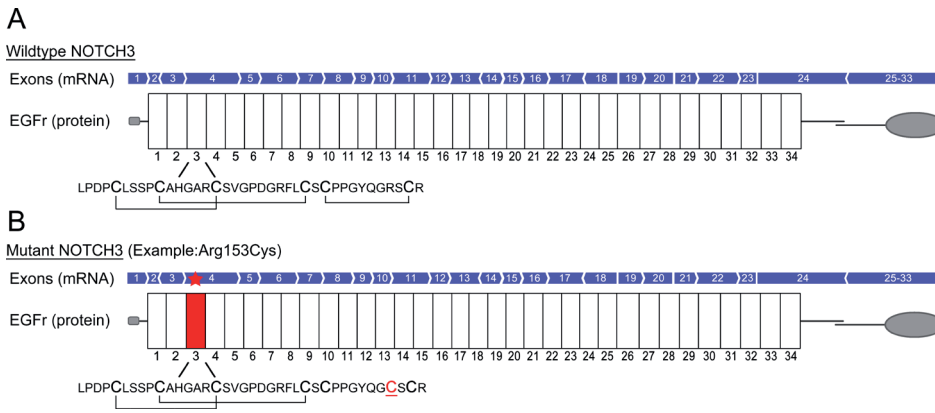


Figure 1. Schematic representation of wildtype and mutant NOTCH3. (A) The ectodomain of the wildtype NOTCH3 protein contains 34 EGFr domains, each with six cysteine residues forming three disulphide bridges between specific pairs of cysteine residues. (B) In mutant NOTCH3, there is an unpaired cysteine residue in the EGFr domain affected by the mutation. The example shown in this figure is the common p.Arg153Cys mutation in exon 4 (indicated in red). As a result of the mutation, EGFr 3 contains seven cysteine residues, resulting in an unpaired cysteine and disrupted disulphide bridge formation.

structure and function. Exon skipping is accomplished by using AONs, which are small synthetic RNA or DNA oligomers designed to hybridize to specific sequences of pre-mRNA. Using AONs, pre-mRNA splicing can be strategically manipulated, as targeted exons are hidden from the splicing machinery and thereby excluded from the mature mRNA. An antisense drug (Mipomersen) has been approved by the Food and Drug Administration for the treatment of familial hypercholesterolemia [12] and AON administration has shown promising results in clinical trials for multiple hereditary disorders, such as Duchenne Muscular Dystrophy and Spinal Muscular Atrophy.[13,14] *In silico* predictions show that the majority of CADASIL causing mutations are eligible for cysteine corrective exon skipping. As a proof of concept for this therapeutic approach, we modelled the NOTCH3 'skip' proteins using cDNA constructs and show that these modified proteins are expressed, undergo normal protein processing and retain both ligand binding capacity and ligand induced activation. Next, we designed AONs targeting frequently mutated *NOTCH3* exons, and show that CADASIL patient derived VSMCs transfected with the *NOTCH3* AONs produce the anticipated skipped *NOTCH3* RNA products. Taken together, these results provide a first proof of concept for a rational therapeutic strategy for CADASIL, in which the corruptive unpaired cysteine in mutant NOTCH3 is abolished by targeted EGFr exclusion, using exon skipping.

MATERIALS AND METHODS

In silico analysis to determine the effect of NOTCH3 exon exclusion on EGFr domain structure

In order to identify *NOTCH3* exons eligible for our cysteine correction approach, we determined the consequence of targeted *NOTCH3* exon exclusion on EGFr domain primary, secondary and tertiary structure. Specifically, we determined the effect of exon exclusion on the *NOTCH3* open reading frame, on cysteine number and spacing within EGFr domains, and on the formation of disulphide bridges within EGFr domains. As CADASIL causing mutations can be found throughout exons 2-24 of the *NOTCH3* gene, all these exons were considered as potential target exons. First, the effect of exon exclusion on EGFr domain primary and secondary structure was analysed using Ensembl (http://www.ensembl.org) and the online protein prediction program Prosite (http://prosite.expasy.org). Selection of exons or exon pairs was based on the following criteria: *a)* the exclusion of the exon(s) from pre-mRNA does not disrupt the open reading frame *b)* skipping of the exon(s) results in exclusion of the mutated EGFr and the formation of a novel EGFr (fusion) domain with six normally spaced cysteines and *c)* the EGFr fusion domain follows the EGFr domain consensus sequence. (http://smart.embl-heidelberg.de/smart/do_annotation.pl?DOMAIN=SM00181) Next, 3D homology modelling was performed to analyse the tertiary structure of predicted favourable EGFr fusion domains, using the YASARA & WHAT IF Twinset (www.YASARA.org).[15] This modelling was performed for the EGFr domains separately, as no continuous template was available.[16-18] The sequence identity between the NOTCH3 domains and modelling templates varied from 39% to 46%. Finally, we determined which exons would be eligible for cysteine corrective exon skipping, when formulating the selection criteria less stringently, i.e. exon skipping resulting in exclusion of a mutated EGFr domain and formation of an EGFr fusion domain containing six cysteines, but where the cysteine spacing within the EGFr fusion domain may differ to some degree from cysteine spacing in wildtype EGFr.

Expression and functional analysis of the NOTCH3 skip proteins

To study the expression, localization, ligand binding capacity and signalling function of the NOTCH3 proteins formed after targeted exon exclusion (NOTCH3 'skip' proteins), *NOTCH3* cDNA constructs were generated mimicking exon 2-3 skipping, exon 4-5 skipping and exon 6 skipping, respectively (for cloning details, see supplementary material). In addition, we generated cDNA constructs of wildtype *NOTCH3* and of *NOTCH3* lacking the ligand binding domain (EGFr 10-11).

Expression analysis using Western Blotting

To determine NOTCH3 skip protein expression, the *NOTCH3* wildtype and skip cDNA constructs were transfected into HEK293 cells, and protein lysates were analysed using Western Blot analysis. HEK293 cells were seeded in a poly-l-lysin coated 6-wells plate and transfected with 1 µg of *NOTCH3* cDNA using 2 µl of lipofectamine 2000 (Life Technologies, Bleiswijk, The Netherlands). Seventy-two hours after transfection, cells were scraped and pelleted in ice-cold PBS. Pellets were lysed in 100 µl TNT lysis buffer (50 mM Tris-HCl, 200 mM NaCl, 0.5% Nonidet P40) with protease inhibitor (Roche Diagnostics, Mannheim, Germany) for 20 minutes. Lysates were centrifuged at 16000g for 20 minutes and supernatants were used for protein concentration determination using the BCA assay following the manufacturer's instructions (Pierce, Rockford, USA). Protein isolates were electrophoresed on a 3-8% Tris acetate polyacrylamide gradient gel. Western blotting was performed on the transblot turbo system, using nitrocellulose membranes (all from Biorad, Veenendaal, the Netherlands). Membranes were blocked for 1 hour at room temperature using 25% seablock (Thermo Scientific, Rockford, USA) in PBS, and were incubated overnight with a primary antibody directed against the intracellular domain of NOTCH3 (anti NOTCH3^{ICD}, Cell Signaling, Danvers, USA, dilution 1:1000). The secondary antibody (IRDye 680 labelled goat anti mouse, Licor, Lincoln, USA, dilution 1:10000) was incubated for 1 hour at room temperature. Imaging was performed on the Odyssey scanner (Licor).

Localization analysis using immunocytochemistry

To determine cellular NOTCH3 skip protein localization, human fibroblasts were transfected with the *NOTCH3* skip constructs, and analysed using NOTCH3 immunocytochemistry. Fibroblasts were seeded onto gelatin (from porcine, Sigma-Aldrich, Zwijndrecht, The Netherlands) coated coverslips in a 24-wells plate, and transfected with 0.8 µg of the respective *NOTCH3* cDNA constructs using 2 µl of lipofectamine 2000. Twenty-four hours after transfection, cells were fixated for 15 minutes with 4% paraformaldehyde and 0.1% triton X-100 in PBS. For analysis of cell surface localization of the NOTCH3 skip proteins, cells were fixated without triton permeabilization, using 4% paraformaldehyde in PBS. Cells were blocked with 1% BSA in PBS during 20 minutes and were incubated overnight at 4°C with an antibody against the extracellular part of NOTCH3 (anti NOTCH3^{ECD}, Novus Biologicals, Littleton, USA, dilution 1:5000). The next day, the secondary antibody (Alexa488 goat anti rabbit, Life Technologies, dilution 1:1000) was incubated for 1 hour at room temperature. Cells were washed with PBS and mounted with vectashield (Vector Laboratories, Inc., Burlingame, USA) containing 0.2 µg/ml Dapi.

Immunofluoresence imaging was performed on a Leica DM5500 microscope (Leica, Wetzlar, Germany).

Ligand binding capacity of the NOTCH3 skip proteins

To determine the ability of the NOTCH3 skip proteins to bind to the canonical NOTCH3 ligand Jagged1, ligand binding assays were performed as described by Hicks et al.[19] Jagged1-Fc peptide (Life technologies) was preclustered to goat anti-human IgG Fc (cross adsorbed Dylight 594, ThermoFisher Scientific, Naarden, The Netherlands, dilution 1:100) in blocking mixture (DMEM/F12, 10% goat serum, 1% BSA), for 1 hour at room temperature. Fibroblasts were transfected with one of the respective *NOTCH3* cDNA constructs. To allow ligand binding, the *NOTCH3* cDNA transfected fibroblasts were incubated with the pre-clustered Jagged1-Fc peptide 24 hours after transfection for 1 hour at 37°C at a final concentration of 0.05 µg/ml. Cells were then washed in a HEPES-based buffer (10 mM HEPES, 1 mM $MgCl_2$, 5 mM $CaCl_2$, 150 mM NaCl), and subsequently in the same buffer without Ca^{2+}, and fixed for 15 minutes at room temperature with 4% paraformaldehyde in PBS and stained with the anti $NOTCH3^{ECD}$ antibody.

Ligand induced signalling capacity of the NOTCH3 skip proteins

To determine the ligand-induced signalling function of the NOTCH3 skip proteins, a luciferase assay was performed using a hexamerized CBF1 reporter plasmid. NIH3T3 cells were seeded in a 24-wells plate at a density of $8x10^4$ cells per well, and co-transfected with 0.4 µg of *NOTCH3* cDNA and 20 ng of pGL4.74 (hRluc/TK) Renilla luciferase expression vector. NOTCH3 signalling was induced twenty-four hours after transfection, by co-culturing the *NOTCH3* transfected cells with 3T3 cells expressing human Jagged1 (10^5 cells per well). As a negative control for Jagged1 induced signalling, the *NOTCH3* transfected cells were co-cultured with mock- transfected 3T3 cells not expressing Jagged 1 (10^5 cells per well). After 30 hours of co-culture, luciferase activity was measured using a dual luciferase assay kit (Promega, Madison, USA) following the manufacturer instructions. Experiments were performed in duplicate.

NOTCH3 AON design and multimerization analysis

NOTCH3 AON design was based on guidelines for dystrophin exon skipping,[20,21] using the splice site prediction program 'Human Splicing Finder' (http://www.umd.be/HSF/). AONs were designed against exons 2 to 6, which collectively harbour the majority of CADASIL causing mutations. All AONs used for exon skipping (Supplementary table 1) contain 2'-O-methyl modified ribose molecules and a full-length phosphorothioate backbone (2OMePS) (Eurogentec, Maastricht, The Netherlands; Prosensa, Leiden, The Netherlands). As a negative control for exon

skipping, an AON targeting the *DMD* gene was used (h56AON1).[20] A RNase H inducing gapmer AON, containing a 10 nucleotide stretch of DNA without a 2OMe modification (GUCGUCaatgttcactUCGCAG) was used as a control for downregulation of *NOTCH3* expression. The sequence of the gapmer AON was scrambled to generate a negative control AON for downregulation (control gapmer AON, CGUCGUaatgttcactCGACGU). To assess unfavourable AON multimerization properties, 2 µg of each AON was incubated at 37°C during 20 minutes, and subsequently run on a novex® TBE gel containing 20% acrylamide (Life Technologies) (Supplementary fig. 1).

Generation of CADASIL patient derived cerebral vascular smooth muscle cells

Informed consent was given by the patient and family members for the use of patient arteries to generate primary vascular smooth muscle cell cultures. Primary vascular smooth muscle cells were cultured from umbilical cord arteries ($VSMC^{Cys162Trp}$) and from arachnoid arteries ($VSMC^{Arg153Cys}$), both with a pathogenic cysteine altering missense mutation in exon 4 of *NOTCH3*. The umbilical cord arteries were obtained from a foetus with a *NOTCH3* mutation (c.486C>G, p.Cys162Trp) after elective termination of pregnancy. The arachnoid arteries were obtained from a deceased CADASIL patient (female aged 57, *NOTCH3* mutation c.457C>T, p.Arg153Cys). The arachnoid mater was dissected from the dorsal region of the frontal lobe by an experienced neuropathologist. Connective tissue and arachnoid mater surrounding the arteries were removed. Arteries were washed in sterile phosphate buffered saline (PBS) and dissected longitudinally. The endothelial layer was removed mechanically by scraping the luminal side of the vessels across the bottom of a Petri dish, and the arterial sections were fixed to the bottom of a sterile cell culture flask containing culture medium (DMEM-F12, glutaMAX), supplemented with 10% fetal calf serum (heat inactivated), 2 µM MEM sodium pyruvate, 0.5 U/ml penicillin, and 0.5 µg/ml streptomycin) (all from Life Technologies). First cellular outgrowth was seen within 2 weeks after vessel isolation. Cells were stained the anti $NOTCH3^{ECD}$ antibody (Novus Biologicals, dilution 1:1000) and with an anti-actin, α-smooth muscle antibody (Sigma- Aldrich, dilution 1:500) to confirm the smooth muscle cell phenotype (Supplementary fig. 2). Control arachnoid VSMCs ($VSMC^{control}$) were generated from a deceased individual without CADASIL (male aged 48), following the same protocol. For cell storage, cells were re-suspended in culture medium supplemented with 20% foetal calf serum and 10% DMSO, then stored in liquid nitrogen. Cell culturing was performed in the culture medium described above. All VSMCs used in the present study were between passage five to nine.

AON transfection and RT-PCR analysis of exon skipping

To determine the technical feasibility of *NOTCH3* exon skipping, the AONs were first transfected into primary human fibroblasts. The AONs that were able to induce the desired exon skip, were next transfected into the CADASIL patient derived vascular smooth muscle cells. Exon skipping was analysed using RT-PCR. For transfection, AONs were diluted in serum free DMEM and were complexed to lipofectamin 2000 (Life Technologies) at a lipofectamin:DNA ratio of 2.67:1, according to the manufacturer's instructions. Cultures of ~90% confluent cells were transfected with the *NOTCH3* AONs at a final concentration of 100 nM per AON. Cells were harvested for RNA isolation 24 and 48 hours after AON transfection. RNA isolation and cDNA synthesis were performed using the 'high pure RNA isolation kit' and the 'transcriptor first strand cDNA synthesis kit' (Roche Diagnostics) following the manufacturer's protocols. For cDNA synthesis, 300 ng of RNA was used in a 20 µl reaction with random hexamer primers at 55°C for 30 minutes. For exon 4-5 skip detection, PCR amplification was performed using an exon 3 forward (3F) and an exon 6 reverse (6R) primer at an annealing temperature of 63°C. For exon 6 skip detection, PCR amplification was performed using an exon 5 forward (5F) and an exon 7 reverse (7R) primer at an annealing temperature of 65°C. For exon 2-3 skip detection, PCR amplification was performed in the presence of 1:5 diluted GC-rich buffer solution (Roche Diagnostics) using an exon 1 forward (1F) and an exon 5 reverse (5R) primer at an annealing temperature of 62°C and an annealing time of 45 seconds instead of the 30 seconds used in exon 4-5 and exon 6 skip detection (primer sequences see Supplementary table 2). PCR products were analysed on 1.5% agarose gels. The PCR fragments that corresponded to the expected fragment lengths after exon skipping were excised and purified from gel using the zymoclean Gel DNA Recovery kit (Zymo research, Irvine, USA). Direct DNA sequencing was carried out by the Leiden Genome Technology Center using the BigDye Terminator Cycle Sequencing Ready Reaction kit and analysed on an ABI 3730 Sequencer (both PE Applied Biosystems, Carlsbad, USA).

Quantitative PCR analysis after exon skipping

To analyse the effect of *NOTCH3* AON transfection on the expression of *NOTCH3* and its downstream target genes, quantitative PCR analysis was performed using 2x Sensimix SYBR Hi-ROX (Bioline, Taunton, Massachusetts, USA) and primers at a final concentration of 1 pmol/µl (primer sequences see Supplementary table 3). Amplification was performed on the LightCycler® 480 II realtime PCR (Roche) using a thermal profile of 10 min at 95°C followed by 45 cycles of 10 s at 95°C, 10 s at 60°C and 10 s at 72°C. Single-product amplification was confirmed by post-

reaction dissociation analysis. Data were analysed using LinReg PCR,[22] and relative expression levels were determined based on N0 values normalized to *HPRT1*. Due to between-experiment variability in *NOTCH3* expression levels inherent to the use of the primary VSMCs, each experiment was normalized to the control AON individually.

Statistical analyses

The unpaired students *t*-test was used to compare expression levels of *NOTCH3* and NOTCH3 downstream target genes after exon skipping, and to determine the increase in luciferase activity after co-culture with Jagged1- expressing cells.

RESULTS

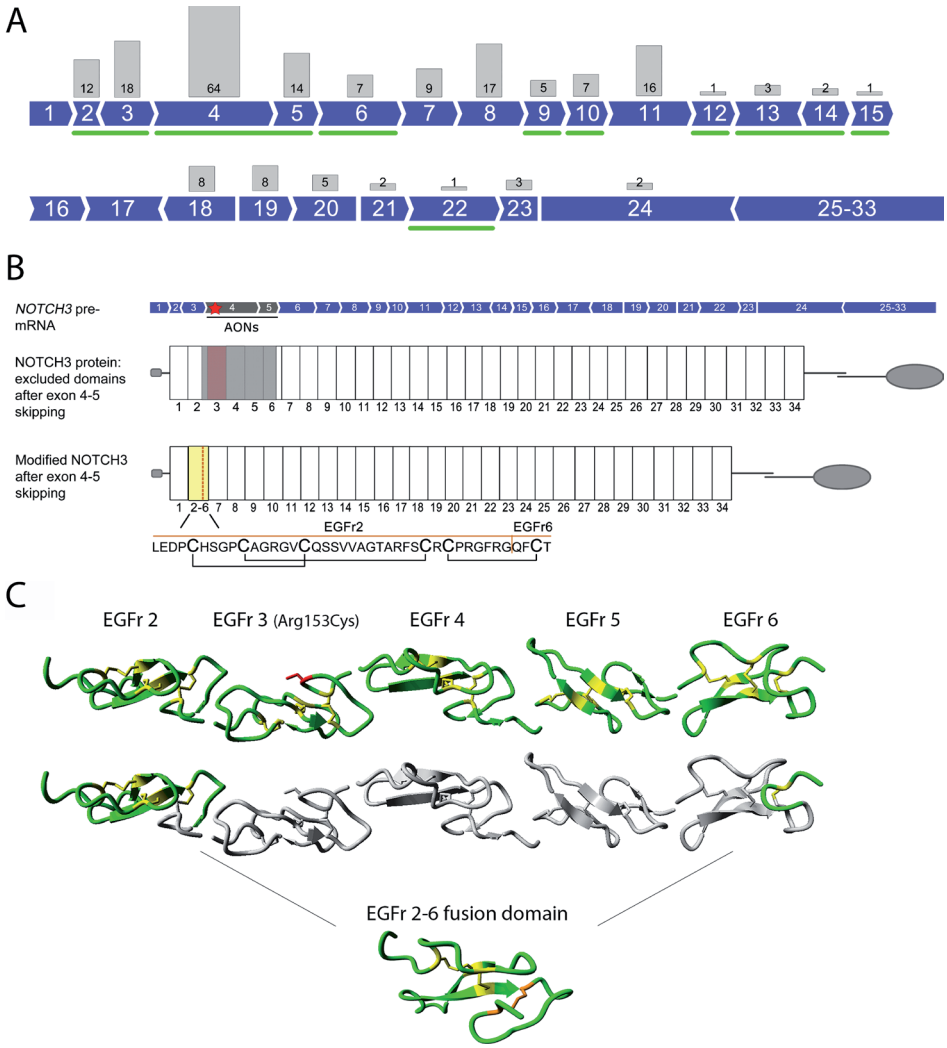
The majority of CADASIL causing mutations are eligible for cysteine corrective exon skipping

Based on the *NOTCH3* open reading frame and *in silico* protein predictions we identified nine exons or exon pairs, encompassing a total of 12 exons, which are eligible for cysteine corrective exon skipping (Fig. 2A). Skipping these exons or exon pairs is predicted to lead to the exclusion of the mutant EGFr and the formation of an EGFr fusion domain containing exactly six correctly spaced cysteines (Table 1). This panel of eligible exons includes exon 4, which harbours the majority of CADASIL- causing mutations. When applying less stringent selection criteria for cysteine spacing in the EGFr fusion domain, we identified another six eligible exons (Supplementary fig. 3A). Therefore, a total of 18 out of 23 mutation-harbouring exons are potential candidates for cysteine corrective exon skipping, covering 94% of known CADASIL causing mutations.

Formation of EGFr fusion domains after targeted NOTCH3 exon skipping

The most favourable exon skips are predicted to lead to the exclusion of the mutant EGFr domain and the concomitant formation of a novel 'EGFr fusion domain'

Figure 2. NOTCH3 cysteine correction using exon skipping. (A) Schematic overview of the open reading frame of exons 2-24 of *NOTCH3*. The vertical boxes indicate the number of reported distinct CADASIL causing mutations in each exon.[23] All CADASIL-causing mutations are located in exons 2- 24, the exons which encode the epidermal growth factor like repeat (EGFr) domains of NOTCH3. The nine exon skip strategies which are predicted to accomplish NOTCH3 cysteine correction with correct cysteine spacing, including a total of 12 exons skipped singly or in pairs, are underlined in green. (B) and (C) illustrate *NOTCH3* cysteine correction for exon 4-5 skipping, as the seminal model for this approach. (B) Exons 4 and 5 together encode part of EGFr 2, all of EGFr 3, 4 and 5, and part of ▶



▶ EGFr 6. Pre- mRNA *NOTCH3* exon 4-5 skipping using antisense oligonucleotides (AONs) thus leads to the exclusion of the mutated EGFr 3 (shown in red) from the protein, as well as the complete exclusion of EGFr domains 4 and 5, and a partial exclusion of EGFr domains 2 and 6. The remaining amino acids of EGFr domains 2 and 6 (encoded by exons 3 and 6 respectively), form an EGFr 2-6 fusion domain with six cysteine residues that are correctly spaced within the domain. The fusion site of this domain is located between the 5th cysteine derived from EGFr 2 and the 6th cysteine derived from EGFr 6. The number of amino acids between the 5th and 6th cysteine is exactly according to wildtype, namely eight. (C) 3D homology modelling of the EGFr 2-6 fusion domain predicts a stable conformation and normal disulphide bridge formation. Disulphide bridges formed between cysteines are shown in yellow. The unpaired cysteine originating from the Arg153Cys mutation is shown in red. The disulphide bridge formed between the 5th and 6th cysteine in the EGFr 2-6 fusion domain is shown in orange.

containing exactly six correctly spaced cysteines. The formation of the EGFr fusion domain is a result of the fact that each of the *NOTCH3* exons 2-24 does not encode a single EGFr domain, but rather one or more (parts of) EGFr domains. Therefore, cysteine spacing in the EGFr fusion domain is critically dependent on which (combination of) exon(s) is skipped. This can be illustrated by the example of skipping only exon 4. Although isolated exon 4 skipping maintains the open reading frame and results in an EGFr fusion domain with six cysteines (Supplementary Fig. 3B), the spacing of these cysteines differs from wildtype EGFr domain cysteine spacing. An EGFr fusion domain with correct cysteine spacing is attained, however, when exon 4 is skipped together with exon 5 (Fig. 2B). Joint skipping of exons 4 and 5 is predicted to lead to the fusion of the part of EGFr 2 encoded by exon 3 to the part of EGFr 6 encoded by exon 6, creating the novel EGFr 2-6 fusion domain. The fusion site within this novel EGFr 2-6 fusion domain is located between the 5th and 6th cysteine, with cysteine spacing exactly according to cysteine spacing in wildtype EGFr domains, i.e. there are eight amino acids between the 5th and 6th cysteine. The favourable prediction of joint exon 4-5 skipping is further supported by 3D homology modelling, which predicts normal disulphide bridge formation within the EGFr 2-6 fusion domain (Fig. 2C). For the other cysteine corrective exon skips, similar EGFr fusion domains are predicted to be formed (Table 1). One exception is exon 2-3 skipping, in which the first two EGFr domains are excluded, but skipping does not lead to the formation of an EGFr fusion domain (Supplementary fig. 4). Using this cysteine corrective exon skipping approach, slightly shorter NOTCH3 proteins are formed which lack the equivalent of one to four EGFr domains, but in which the pathogenic unpaired cysteine has been eliminated.

NOTCH3 skip proteins are expressed and localize at the cell membrane

To study the synthesis and processing of NOTCH3 skip proteins, we used cDNA constructs mimicking a selection of three exon skip strategies, namely skipping of exons 2-3, exons 4-5 and exon 6. Exon 4-5 skipping was selected because this would target the majority of CADASIL- causing mutations. Exon 6 was selected because in this case, skipping of a single exon leads to cysteine correction, which can have technical advantages over joint exon skipping. Joint exon 2-3 skipping was selected because this is the only skip strategy which does not lead to the formation of an EGFr fusion domain, but rather to the complete exclusion of EGFr 1 and all but four amino acids of EGFr 2, leaving EGFr 3 as the first domain in the skip protein (Supplementary fig. 4). To model these three skip strategies, *NOTCH3* cDNA constructs were generated lacking exons 2-3, exons 4-5 or exon 6, respectively. First, the cDNA constructs were transiently transfected into HEK293 cells. Western blot analysis of protein lysates prepared 72 hours after transfection

Target exon(s)	Resulting EGFr fusion domain	Amino acid sequence of the fusion domain
2+3	No fusion domain*	
4+5	2-6	LEDPCHSGPCAGRGVCQSSVVAGTARFSCRCPRGFRGQFCT
6	6-8	NVDDCPGHRCLNGGTCVDGVNTYNCQCPPEWTGLLCH
9**	11-12	DVNECLSGPCRNQATCLDRIGQFTCICMAGFSGSTCQ
10	12-13	DIDECQSSPCVNGGVCKDRVNGFSCTCPSGFEGTLCD
12	15-16	QVDECRSQPCRHGGKCLDLVDKYLCRCPSGTTGVNCE
13+14	16-19	NIDDCASNPCTFGVCRDGINRYDCVCQPGFTGRQCE
15	19-20	ARDACESQPCRAGGTCSSDGMGFHCTCPPGVQGPRCQ
22	29-31	DVDECASQPCQHGGSCIDLVARYLCSCPPGTLGPRCQ

Table 1. EGFr fusion domains predicted to form after cysteine corrective exon skipping

The EGFr fusion domains predicted to form after the respective cysteine corrective exon skips, each contain six correctly spaced cysteine residues. Cysteine corrective exon skipping is predicted to lead to the exclusion of the mutant EGFr domain, and the formation of a novel EGFr fusion domain. For each fusion domain, the fusion site is between the 5th cysteine of the N-terminal contributing EGFr and the 6th cysteine of the C-terminal contributing EGFr (e.g. in the EGFr 2-6 fusion domain formed after exon 4-5 skipping, the fusion site is between the 5th cysteine of EGFr 2 and the 6th cysteine of EGFr 6). Amino acids originating from the C-terminal contributing EGFr are underlined. The number of amino acids between the 5th and 6th cysteine in the fusion EGFr is according to canonical EGFr cysteine spacing, namely eight. * Skipping of exons 2 and 3 does not lead to the formation of an EGFr fusion domain, but rather to the exclusion of EGFr 1 and 2, leaving EGFr 3 as the first EGFr domain in the skip protein. One cysteine amino acid from EGFr 2 is retained, but is predicted not to be included in any EGFr domain. ** Skipping of exon 9 may interfere with the NOTCH3 ligand binding, as it results in exclusion of a part of the ligand binding domain (EGFr 10-11).

showed that the NOTCH3 skip proteins are expressed by the HEK293 cells (Fig. 3A). Both the full length precursor protein and the NOTCH3 intracellular domain were detected, indicating a correct S1 cleavage of the skip proteins. Next, we performed immunofluorescence analysis of fibroblasts transfected with the *NOTCH3* cDNA constructs, to analyze the cellular localization and processing of skip proteins. Staining of permeabilized cells with an antibody against the NOTCH3^{ECD} showed that all full length NOTCH3 skip proteins had a similar intracellular localisation to wildtype NOTCH3 i.e. predominantly perinuclear (Fig. 3B). This pattern of NOTCH3 intracellular staining, reflecting accumulation in the endoplasmatic reticulum or the Golgi network, has been previously described for both wildtype and mutant NOTCH3 in over-expression systems.[24,25] Next, we stained unpermeabilized cells to determine whether the NOTCH3 skip proteins are present at the cell surface, and found a positive NOTCH3 staining for all skip proteins at the cell surface, comparable to wildtype. Together, these results indicate that NOTCH3 skip proteins are expressed and correctly processed, with localization at the cell surface, comparable to wildtype NOTCH3.

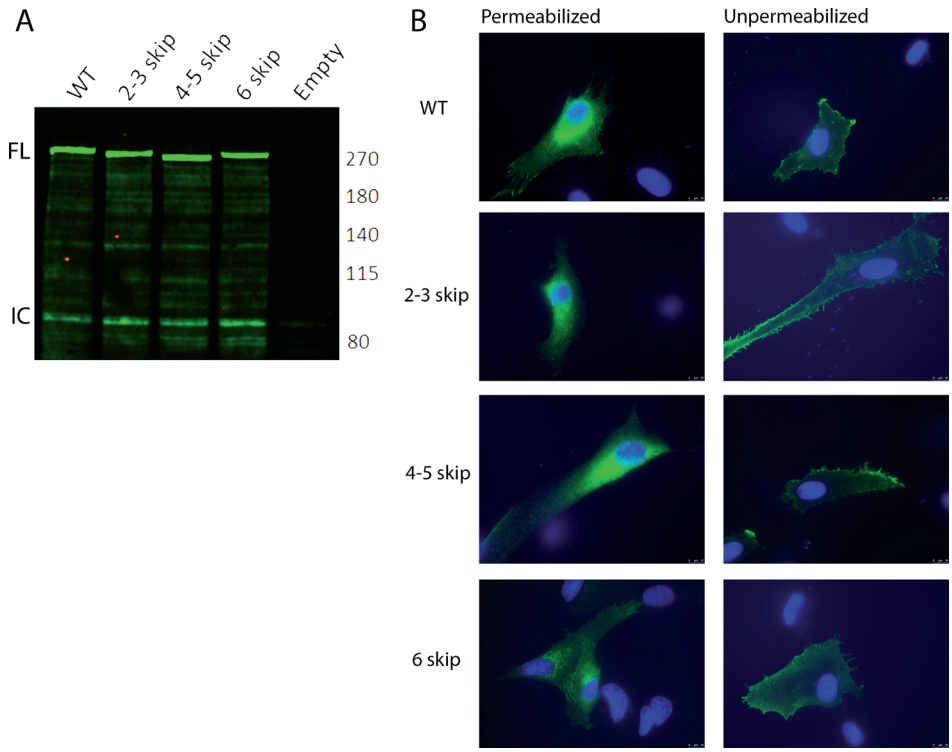


Figure 3. Expression and cell surface localization of NOTCH3 skip proteins
(A) Western blot analysis demonstrating expression of NOTCH3 skip proteins, after transient transfection of *NOTCH3* cDNA constructs in HEK293 cells. NOTCH3 detection with an antibody against the NOTCH3 intracellular domain reveals the presence of the unprocessed full length protein (FL), as well as the intracellular domain (IC). The full length skip proteins run slightly lower on the gel than the wildtype NOTCH3, as a result of the small size difference. (B) Immunocytochemical analysis of fibroblasts transfected with the same *NOTCH3* cDNA constructs. Staining of permeabilized cells with an antibody directed against the extracellular part of the protein shows a cytoplasmic NOTCH3 staining, which is predominantly perinuclear and is comparable between the wildtype NOTCH3 and NOTCH3 skip proteins. Staining of unpermeabilized cells reveals normal cell surface localization of NOTCH3 skip proteins.

NOTCH3 skip proteins show normal ligand binding and ligand induced signalling

Activation of the NOTCH3 protein is induced by the binding of a ligand (Jagged1/Delta) to the ligand binding domain of NOTCH3, comprised of EGFr 10-11. To determine whether excluding EGFr domains other than EGFr domain 10-11, interferes with normal ligand binding, *NOTCH3* cDNA transfected fibroblasts were incubated with soluble Jagged1 peptide. We found that all skip proteins co-localized with Jagged1 at the cell surface, comparable to wildtype NOTCH3

protein (Fig. 4A). In contrast, NOTCH3 lacking the ligand binding domain did not show this co-localization, and cells transfected with an empty vector only showed a weak Jagged1 signal (Supplementary fig. 5). Finally, we analysed whether the NOTCH3 skip proteins can be activated by ligand, using a luciferase reporter assay. We observed a significant increase in luciferase activity of skip-protein expressing cells upon co-culture with Jagged1- expressing cells, which was not seen in the cells expressing NOTCH3 protein lacking the ligand binding domain (Fig. 4B). The activation rate of the exon 2-3 skip and exon 4-5 skip proteins did not differ from wildtype NOTCH3, whereas the exon 6 skip protein showed a lower activation rate compared to wildtype. Taken together, these results indicate that NOTCH3 skip proteins retain ligand binding capacity and can be activated successfully by ligand.

Successful exon 2-3, 4-5 and 6 skipping in CADASIL patient derived vascular smooth muscle cells

Given the promising results of correct processing and functionality of NOTCH3 skip proteins, we next set out to determine the technical feasibility of targeted *NOTCH3* exon skipping. We started with testing exon 4-5 skipping, as this targets the majority of CADASIL causing mutations. Multiple AONs targeting exons 4 and 5 were designed (Supplementary table 1) and were tested in control fibroblasts (data not shown). The AONs with the most favourable exon skipping results were then transfected into VSMC^{Arg153Cys}. Joint exon 4-5 skipping was successfully achieved using a combination of three AONs: two AONs targeting exon 4 (h4cSD and h4c12) and one AON targeting exon 5 (h5c1) (Fig. 5A). The exon 4-5 skipping was also successful in patient derived VSMCs with another exon 4 mutation (VSMC^{Cys162Trp}) (Supplementary fig. 6). After establishing the feasibility of exon 4-5 skipping, we proceeded with skipping of exon 6 and joint skipping of exons 2 and 3. For each of these exons, a total of three potentially effective AONs were designed (Supplementary table 1). For exon 2-3 skipping, the most efficient skip was induced with the AON combination h2c1 and h3c3 (Fig. 5B). Exon 6 skipping was accomplished with all three tested exon 6 AONs individually, but was most efficient when using a combination of AONs h6c2 and h6c7 (Fig. 5C). For all skips, cell viability was comparable between cells transfected with control AON and cells transfected with the *NOTCH3* AONs. These results show that both single and joint *NOTCH3* exon skipping is feasible in CADASIL patient derived VSMCs.

AON- induced exon skipping does not reduce expression levels of NOTCH3 or NOTCH3 target genes

The full length 2'-O-methyl modification of the AONs we used, protects the RNA-AON hybrids from RNase H cleavage, which is essential here, because the aim of our approach is to modulate splicing rather than downregulating expression

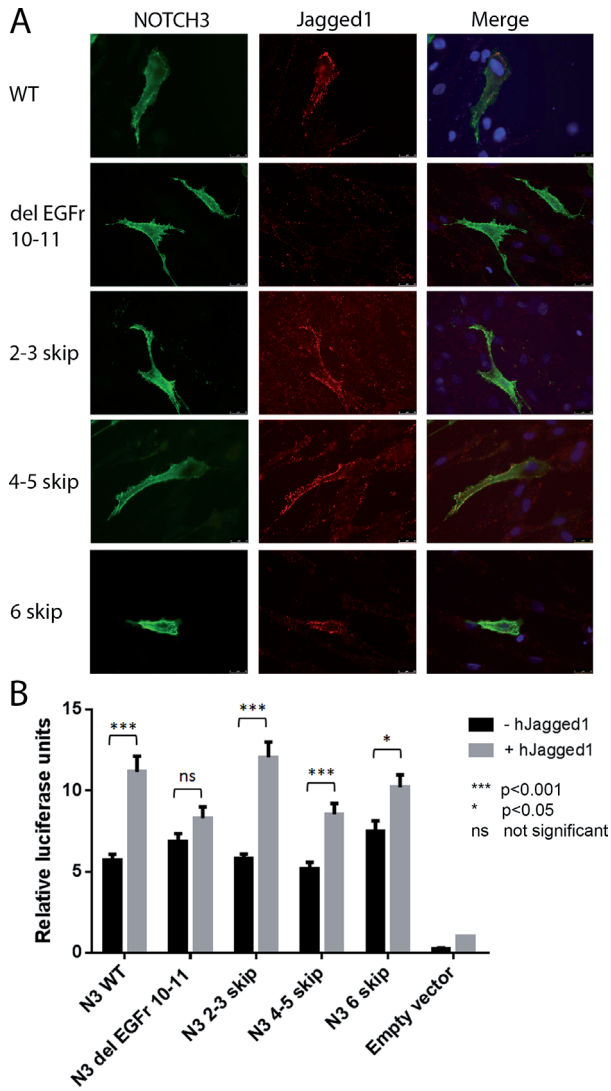


Figure 4. Ligand binding and ligand induced signalling of NOTCH3 skip proteins
(A) Fibroblasts were transfected with *NOTCH3* cDNA and incubated with a Jagged1-Fc peptide to allow for ligand binding. The Jagged1-Fc peptide was preclustered to an anti-Fc antibody (red fluorescence). The NOTCH3 expressing cells are visualized using an anti NOTCH3^{ECD} antibody (green fluorescence). Cells transfected with wildtype *NOTCH3* cDNA show an increased Jagged1 staining where the NOTCH3 protein is expressed, whereas this staining is absent in the cells transfected with NOTCH3 lacking the ligand binding domain (del EGFr 10-11). The cells expressing the NOTCH3 skip proteins all show an increased Jagged1 staining similar to wildtype NOTCH3, indicating normal ligand binding. Images are shown from one representative experiment at a 40x magnification. (B) NIH3T3 cells transiently transfected with *NOTCH3* cDNA, were co-cultured with Jagged1 expressing cells to induce NOTCH3 signalling, and assayed for luciferase activity using a hexamerized CBF1 ▶

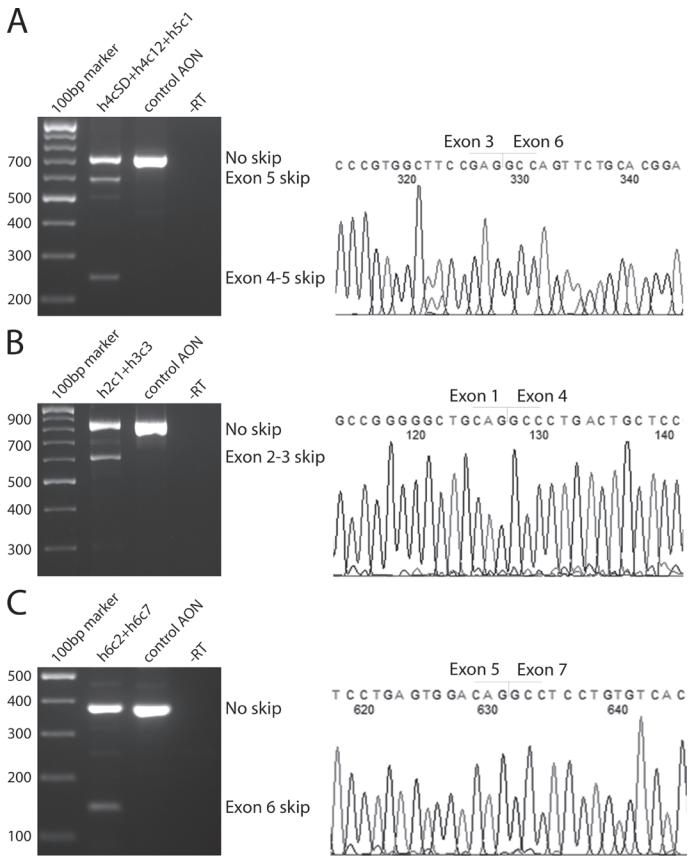


Figure 5. Successful AON-induced *NOTCH3* exon skipping in VSMC^{Arg153Cys}
RT-PCR and sequencing analysis of RNA from VSMC^{Arg153Cys} transfected with *NOTCH3* AONs or control AON. Depicted are results from (A) exon 4-5 skipping, (B) exon 2-3 skipping and (C) exon 6 skipping. The RT-PCR of the samples transfected with the *NOTCH3* AONs show that the intensity of the wildtype band is decreased and an additional, smaller product is present, which corresponds to the expected fragment length after exon skipping. Sequencing analysis of these smaller fragments confirmed that all 3 exon skips were successful. For the exon 4-5 skip, some isolated exon 5 skipping was seen in addition to joint exon 4-5 skipping.

- reporter plasmid. Cells transfected with wildtype *NOTCH3* cDNA show a significant increase in luciferase activity upon co-culture with 3T3 cells expressing human Jagged1, compared to co-culture with mock- transfected 3T3 cells not expressing human Jagged1. The cells transfected with the respective skip *NOTCH3* cDNA also showed a significant increase in luciferase activity. There was no significant increase in luciferase activity in cells expressing NOTCH3 lacking the ligand binding domain. The increase in luciferase activity for the exon 6 skip cDNA construct was slightly lower than for the other skips and wildtype. The activity is expressed as relative luciferase units, data are normalized for activity measured in empty vector transfected cells +hJagged1. Results are the mean ±SEM from eight independent experiments,***p<0.001, *p<0.05, ns= not significant.

of transcripts. Therefore, exon skipping should not alter total *NOTCH3* expression levels. Using qPCR, we show that transfection with the *NOTCH3* exon skipping AONs indeed did not reduce total *NOTCH3* expression levels, as opposed to transfection with RNaseH inducing *NOTCH3* gapmer AONs, which did reduce total *NOTCH3* expression levels (Fig. 6A). To determine whether transfection with exon skipping AONs had an effect on canonical NOTCH3 signalling, we also determined the expression levels of NOTCH3 downstream target genes *HES1*, *HEYL*, *PDGFR*-β and *Jagged1*. No significant changes were found in expression levels of these target genes after transfection with exon skipping AONs, as opposed to transfection with gapmer AONs, which did lead to reduced expression levels of NOTCH3 target genes *HES1* (p=0.009) and *HEYL* (p=0.09) (Fig. 6B and Supplementary fig. 7). These results show that transfection of patient derived VSMCs with *NOTCH3* exon skipping AONs achieves the anticipated exon skips, without reducing total *NOTCH3* expression levels and without affecting expression of NOTCH3 downstream target genes.

DISCUSSION

In this study, we introduce the novel approach of cysteine corrective *NOTCH3* exon skipping. We show that this targeted *NOTCH3* exon skipping is technically feasible and that NOTCH3 skip proteins undergo normal processing and retain ligand binding capacity and ligand induced activation.

Cysteine corrective exon skipping could in theory be applicable to 18 out of 23 EGFr domain encoding exons, covering 94% of CADASIL causing mutations. To determine the feasibility of our approach, we selected skipping of exons 2-3, 4-5 and 6, and tested AONs targeting these exons in patient derived VSMCs, which all showed effective exon skipping. Cysteine corrective exon skipping results in a modified NOTCH3 protein from which the mutant EGFr has been excluded and in which an EGFr fusion domain is created, with predicted normal disulphide bridge formation. We modeled the skip proteins of the three tested exon skips using cDNA constructs, and show that the skip proteins display normal processing, ligand binding and ligand induced activation, comparable to wildtype NOTCH3. Therefore, although the NOTCH3 skip proteins in this cysteine corrective model lack the equivalent of up to four EGFr repeats and contain a novel EGFr fusion domain, this does not seem to interfere with NOTCH3 processing and function. This finding is consistent with NOTCH data from various scientific fields, which also provide evidence for a redundancy in the number of NOTCH EGFr domains. A prime example is that, during evolution, duplication events and (partial) *NOTCH* deletions have occurred, which have resulted in four functional human NOTCH homologues with a varying number of EGFr domains, namely between 29 and 36 (www.ncbi.

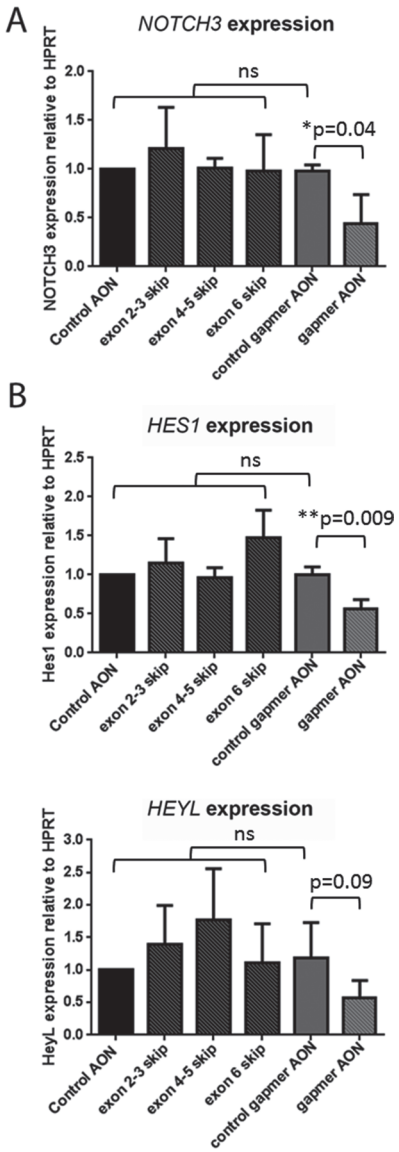


Figure 6. Expression of *NOTCH3* and NOTCH3 target genes after exon skipping
(A) *NOTCH3* qPCR analysis of VSMC^{control} transfected with *NOTCH3* AONs. After exon skipping, total *NOTCH3* expression levels are unaltered, whereas transfection with a downregulating *NOTCH3* gapmer AON results in decreased *NOTCH3* expression levels (Unpaired student's t-test, p=0.04). (B) qPCR analysis of NOTCH3 downstream target gene expression in VSMC^{control} transfected with *NOTCH3* AONs. Transfection with *NOTCH3* exon skipping AONs did not affect the expression levels of the canonical NOTCH3 downstream target genes *HES1* and *HEYL*, as opposed to transfection with downregulating *NOTCH3* gapmer AONs, which did lead to reduced expression of NOTCH3 target genes (Unpaired student's t-test, *HES1* p=0.009, *HEYL* p=0.09). Data represent the mean ±SD from three independent experiments, ns= not significant.

nlm.nih.gov).[26] In fact, EGFr 2 of NOTCH3 was generated during evolution by a deletion event in a genomic copy of NOTCH2, resulting in a fusion of part of NOTCH2-derived EGFr 2 and part of NOTCH2-derived EGFr 3.[27, 28] Interestingly, this evolutionarily formed EGFr 2-3 fusion domain is structurally almost identical to the EGFr 2-6 fusion domain, which is formed after exon 4-5 skipping (Supplementary fig. 8). Further support for redundancy in the number of EGFr domains in NOTCH3, is the report of a rare human *NOTCH3* splice site mutation leading to skipping of exon 16, showing compatibility of this protein, lacking EGFr domain 21 and parts of EGFr domains 20 and 22, with normal embryological development, growth and human reproduction.[29]

In this study we have not yet been able to address the effect of cysteine corrective exon skipping on vascular NOTCH3 aggregation. A reliable evaluation of this important aspect can only be provided by *in vivo* studies, as cell models do not recapitulate the CADASIL pathological hallmarks such as $NOTCH3^{ECD}$ aggregation, GOM formation and VSMC degeneration. For example, the CADASIL-patient derived VSMCs we use to test exon skipping do not show consistent differences in NOTCH3 staining between patient and control cells (Supplementary fig. 2). Likewise, the *NOTCH3* cDNA transfection approach we used is inherently associated with NOTCH3 overexpression and intracellular NOTCH3 aggregation, confounding any read-out of CADASIL-associated $NOTCH3^{ECD}$ aggregation.

To assess the feasibility and efficacy of *NOTCH3* exon skipping *in vivo* and to determine the effect on vascular NOTCH3 aggregation, we are in the process of characterizing a mouse model we generated. This mouse model is transgenic for the genomic full length human *NOTCH3* gene and shows the typical CADASIL-associated cerebrovascular NOTCH3 aggregation. We will first need to optimize *in vivo* AON administration by testing various AON chemistries and administration routes,[30,3132] and develop relevant therapeutic read-outs, before we can set out to assess a therapeutic effect of cysteine corrective exon skipping.

AONs are emerging as a promising treatment modality for genetic disorders, and can be used either to change the expression levels of mutant proteins or to restore the structure of deleterious proteins. An alternative AON-based approach to NOTCH3 cysteine correction could be NOTCH3 downregulation, as previous studies have shown that there is some redundancy in the amount of NOTCH3 signalling required for normal functioning.[33] In that case, AONs would need to be used that induce RNase H mediated degradation of RNA, thus leading to a reduction in the total load of mutant NOTCH3 protein expression and potentially therefore also NOTCH3 aggregation load. We used RNase H inducing gapmer AONs as a control for our exon skipping experiments, which showed an effective *NOTCH3* downregulation in VSMCs (Figure 6). Both AON-mediated exon skipping

and AON-mediated downregulation have been proven to be safe and effective upon both local and systemic AON administration in multiple clinical trials.[32]

In conclusion, we developed the concept of NOTCH3 cysteine correction as a potential therapeutic strategy for CADASIL. This concept, namely restorative protein modification through cysteine corrective exon skipping, may be added to the growing list of potential therapeutic applications of exon skipping.[34] These *in vitro* studies form the basis for planned *in vivo* studies in human *NOTCH3* transgenic mice, aimed at determining whether this approach leads to a delay or reduction of NOTCH3 aggregation in the cerebrovasculature, as a marker for a therapeutic effect.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

Cloning of NOTCH3 cDNA constructs

NOTCH3 cDNA constructs mimicking the various skip strategies were generated by excluding the respective exons from wild type human *NOTCH3* cDNA constructs. All the targeted exon exclusions were generated by performing inverse PCRs on smaller human *NOTCH3* subclones in pSE280 (Invitrogen). Inverse PCR's were performed with Phusion high fidelity DNA polymerase (Fermentas, Bleiswijk, The Netherlands), using phosphorylated primers to allow self-ligation of the PCR products. First, a cDNA construct lacking exons 4 and 5 was generated. For this construct, a fragment of the *NOTCH3* cDNA was synthesized using RT-PCR on mRNA obtained from cell line HCC2429 (a kind gift from Professor D.P. Carbone, USA), using an exon 1 forward primer with an EcoRI restriction site (5'-GGAATTCATGGGGCCGGGGG-3') and an exon 6 reverse primer (5'-GGCAGGCACAGTAGAAAGAA-3').This fragment was cloned into the pSE280 vector using EcoRI and ClaI. The exon 4-5 deletion was introduced in this fragment using primers at the 3' end of exon 3 (5'-CTCGGAAGCCACGGGGGCAC-3') and at the 5' end of exon 6 (5'phosphorylated -GCCAGTTCTGCACGGAGGACGTG-3'). A second fragment was generated using an exon 6 forward primer (3'- AGGTGAGAGCTGCAGTCAGAAT-5') and an exon 15 reverse primer (3'-AGGGGGAGAGGAGTTCACACT-5'). This was cloned into pSE280 using ClaI and AatII. Next, both fragments were cloned into the pET28a vector using EcoRI and ClaI for the first fragment, and ClaI and SalI for the second fragment. Sequence analysis revealed that the first 96 bp after the ATG start codon were missing. Therefore, the skip region was amplified from the large construct in pET28a using a forward primer containing a Nhe1 restriction site in exon 1 (5'-CTGCT-GCTAGC-GGGGCCGGGGGCTGCAG-3') and a reverse primer in exon 6 (5'-GGCCACCCAGCGTGTTGA-3'). The NheI and KpnI restriction sites within this fragment were used to replace the corresponding region in a pTT5-based eukaryotic expression vector containing NOTCH3 EGFr 1-9. Finally, the missing 3' sequence was added from a pcDNA3.1 construct containing full-length human *NOTCH3* [35] using restriction sites KpnI and BamHI. The complete coding sequence of both the wild type and the skip construct was sequenced.

To generate the cDNA construct lacking exons 2 and 3, an inverse PCR was performed on an EcoRI, KpnI subclone containing *NOTCH3* exons 1 to 5 from pTT5hN3FLwt, using primers at the 3' end of exon 1 (5'-CTGCAGCCCCCGGCCCCGCTA-3') and at the 5' end of exon 4 (5' phosphorylated-GCCCTGACTGCTCCCTGCCAGATCC CTG-3'). After verification of the skip clone by sequencing analysis, the missing 3' sequences was added by cloning in the 6309 bp KpnI fragment of pTT5hN3FLwt. Subsequently, the complete *NOTCH3* gene with the exon 2-3 deletion was cloned into pTT5 using EcoRI and BglII. The cDNA construct lacking exon 6 was made using inverse PCR on an EcoRI-AatII subclone containing exons 1 to 14, using primers

at the 3'end of exon 5 (5'-CTGTCCACTCAGGAGGGCA-3') and at the 5' end of exon 7 (5' phosphorylated GCCTCCTGTGTCACCTGGAT-3'). After verification of the skip clone by sequence analysis the 2344bp EcoRI-AatII fragment containing the exon 6 deletion was ligated together with the 4813bp AatII, BglII fragment of pTT5hN3FLwt, into the EcoRI and BglII digested pTT5 vector. Finally, as a negative control a cDNA construct lacking the exons which encode the ligand binding domain (EGFr 10-11) was generated. This deletion, encoded by a part of exon 7, complete exon 8 and a part of exon 9, was introduced in the EcoRI-AatII subclone containing exons 1 to 14, using primers in exon 7 (5'-CTGGTCACATGCCCCACC-3') and in exon 9 (5' phosphorylated GTGGACATTGACGAGTGTCAGAG-3'). For all cDNA constructs, plasmid mini preparations (Nucleospin, Macherey-Nagel, Düren, Germany) were used for transfections.

Target exon	AON sequence	Name AON
2	CUGGGUGCAACGACCUCCAUUU	h2c1
2	UCCAUUUGCACACGGGCUUCC	h2c2
2	CAACGACCUCCAUUUGCACAC	h2c3
3	UCCUCCAGCUGACACCGCUCA	h3c1
3	ACUGAACUCUGGCAGACACCA	h3c2
3	ACCACUGAACUCUGGCAGACA	h3c3
4	AGGGUGCACAGGGCACCGCGGG	h4c3
4	UGGGCACAAGGGCUGCUGAGGCAGG	h4c4
4	UCGCUUCGGCAGCUGCGGCC	h4c9
4	GGUUCUCACAUAGUGGCC	h4c10
4	UGAGUUUAGGACUGACCACA	h4c11
4	AGCAGAGGAAGCGUCCAUC	h4c12
4	CACAGUCGUAAGUGAGGUCG	h4c15
4	GGAAGACAGGCACAGUCGUA	h4c22
4	AUCUGGCAGGGAGCAGUCA	h4c23
4	UCUCCUGAGUAGGGCUCACU	h4c25
4	AGCAGUCAGGGCCUGGAGGGA	h4cSA
4	CACUCACCAGGAAGACAGG	h4cSD
5	CCCAUUGAGACAUCGGUGUCC	h5c1
5	UCCACUCAGGAGGGCACUG	h5c2
5	AGACAUCGGUGUCCUGGACAGU	h5c11
5	GACCCUCAAACCCUAGCAGG	h5cSA
6	ACUCAUCCACGUCCUCCGUG	h6c2
6	AAACGGCCACUCACCAGUCUU	h6c5
6	GCACAGUCAUCGAUAUUCUGA	h6c7

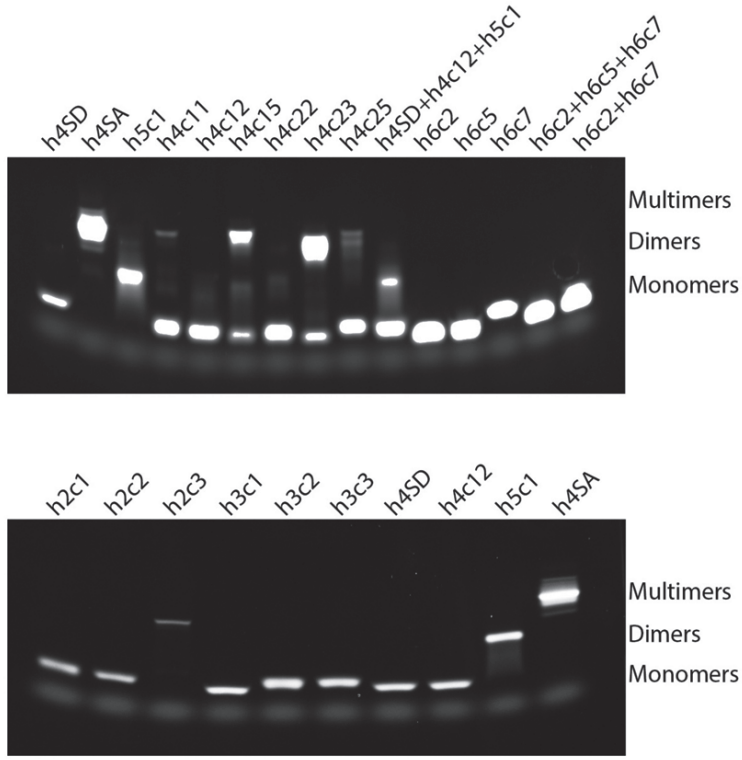
Supplementary table 1. *NOTCH3* antisense oligonucleotides (AONs) used for exon skipping

Primer name	Primer sequence	Detects
1F	CAGGGAAGGAGGGAGGA	Exon 2-3 skipping
5R	GCACTGGCAGTTATAGGTGTT	
3F	GAGGACCCCTGTCACTCAG	Exon 4-5 skipping
6R	GACTGCAGCTCTCACCTGTC	
5F	TGAGGGTCAGAATTGTGAAGTG	Exon 6 skipping
7R	GTCATCCAGGTGACACAGGAG	

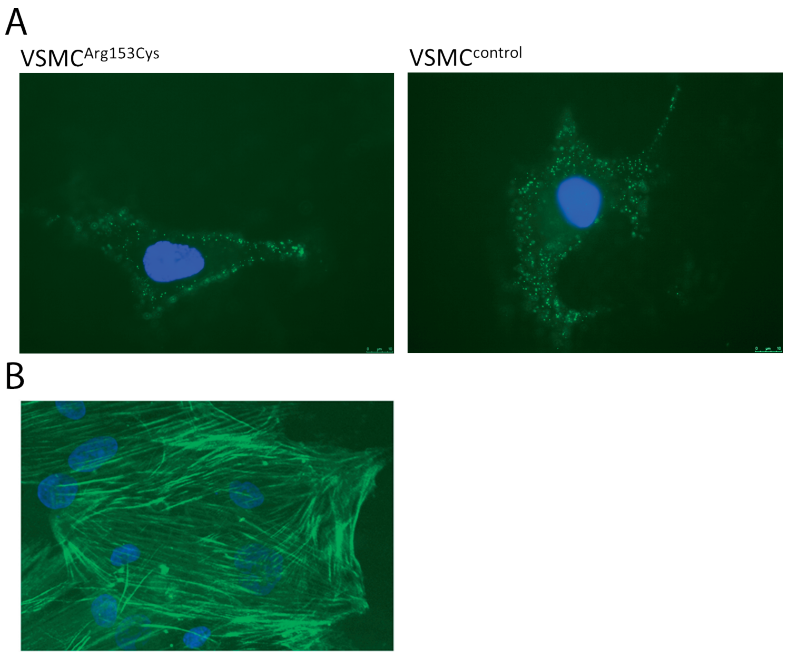
Supplementary table 2. *NOTCH3* primers used for RT-PCR analysis of exon skipping

Primer name	Target gene	Primer sequence
qpN3ex7_8-F	*NOTCH3*	ACGAGTGCTCTATCGGCGC
qpN3ex8-R	*NOTCH3*	CACAGCGAGGTCCAGTGTAGC
qpHEYL-F	*HEYL*	GGCTGCTTACGTGGCTGTT
qpHEYL-R	*HEYL*	GACCCAGGAGTGGTAGAGCAT
qpHES1-F	*HES1*	TACTTCCCCAGCACACTTGG
qpHES1-R	*HES1*	CGGACATTCTGGAAATGACA
qpJAG1-F	*Jagged1*	ACTGTCAGGTTGAACGGTGTC
qpJAG1-R	*Jagged1*	TCGTGCTGCCTTTCAGTTT
qpPDGRFB-F	*PDGFR-β*	CAGGAGAGACAGCAACAGCA
qpPDGFRB-R	*PDGFR-β*	TGTCCAGAGCCTGGAACTGT
HPRT1-F	*HPRT1*	GGGAGGCCATCACATTG
HPRT1-R	*HPRT1*	GTAATCCAGCAGGTCAGAAA

Supplementary table 3. Primers used for quantitative PCR analysis after exon skipping

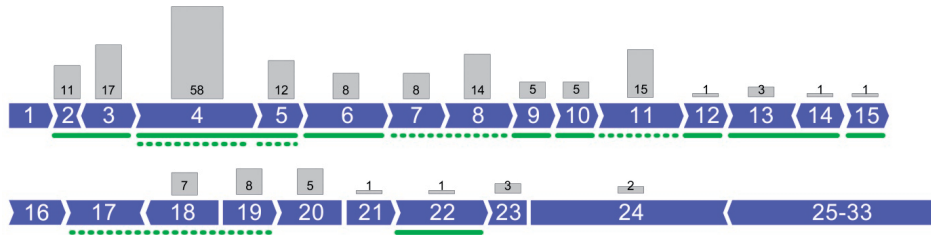


Supplementary figure 1. Multimerization analysis of the AONs used for exon skipping. All AONs that showed multimerization (h4cSA, h4c12, h4c23) were excluded from further analysis, as AON multimerization is known to diminish efficacy and may cause aggregation in vivo. AONs h5c1and h2c3 form dimers, but some dimerization of AONs has been shown to be beneficial for exon skipping (Aartsma-Rus 2012).



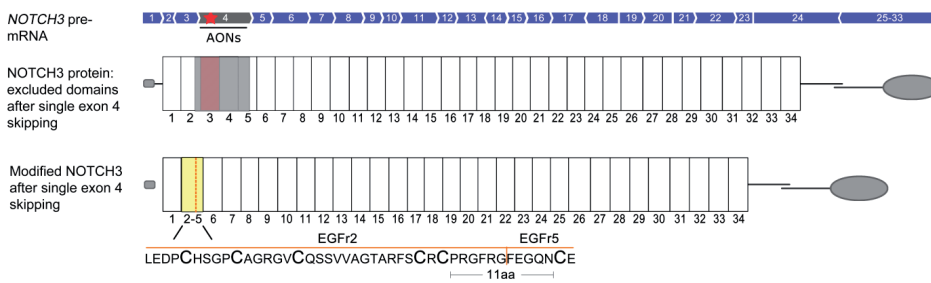
Supplementary figure 2. NOTCH3 and smooth muscle actin staining of CADASIL patient derived VSMCs. (A) NOTCH3 immunofluorescence analysis of VSMC^{Arg153Cys} and VMSC^{control}. Staining of unpermeabilized VSMC^{Arg153Cys} with an antibody against the extracellular part of NOTCH3 showed a granular staining pattern at the cell surface, which was comparable to that seen in VSMC^{control}. (B) Immunofluorescence analysis using a anti – smooth muscle actin antibody, confirming the smooth muscle cell phenotype of VSMC^{Arg153Cys}.

A



Target exon(s)	Resulting EGFr fusion domain	Amino acid sequence fusion domain	Altered cysteine spacing
4	2-5	LEDPCHSGPCAGRGVCQSSVVAGTARFSCRCPRGFRGFEGQNCE	+3 aa between the 5th and 6th cysteine
5	5-6	PAVPCAPSPCRNGGTCRQSGDLTYDCACLPGQFCT	-3 aa between the 5th and 6th cysteine
7+8**	8-11	NIDDCATAVCFHGATCHDRVASFYCACPMGKTGFTGTYCE	+3 aa between the 5th and 6th cysteine
11	13-15	DVDECASTPCRNGAKCVDQPDGYECRCAEGVNCE	-3 aa between the 5th and 6th cysteine
17-19	22-27	DINDCDPVRLEQLCQAGGQCVDEDSSHYCVCPEGRTGSHCE	+4 aa between the 1st and 2nd cysteine

B



Supplementary figure 3. Exons eligible for cysteine corrective exon skipping with altered cysteine spacing in the EGFr fusion domain.
(A) In addition to the 12 exons eligible for cysteine corrective exon skipping with correct cysteine spacing (underlined in green), there are also additional exons of which skipping leads to cysteine correction, but with altered cysteine spacing in the resultant EGFr fusion domain (dashed green underlining). Taken together, 18 out of 23 mutation-encoding exons can be targeted, covering 94% of known CADASIL –causing mutations. The vertical boxes indicate the number of reported distinct CADASIL causing mutations in the exon [23]. (B) As an example of exon skipping leading to cysteine correction with altered cysteine spacing, the predicted outcome of isolated exon 4 skipping is shown.

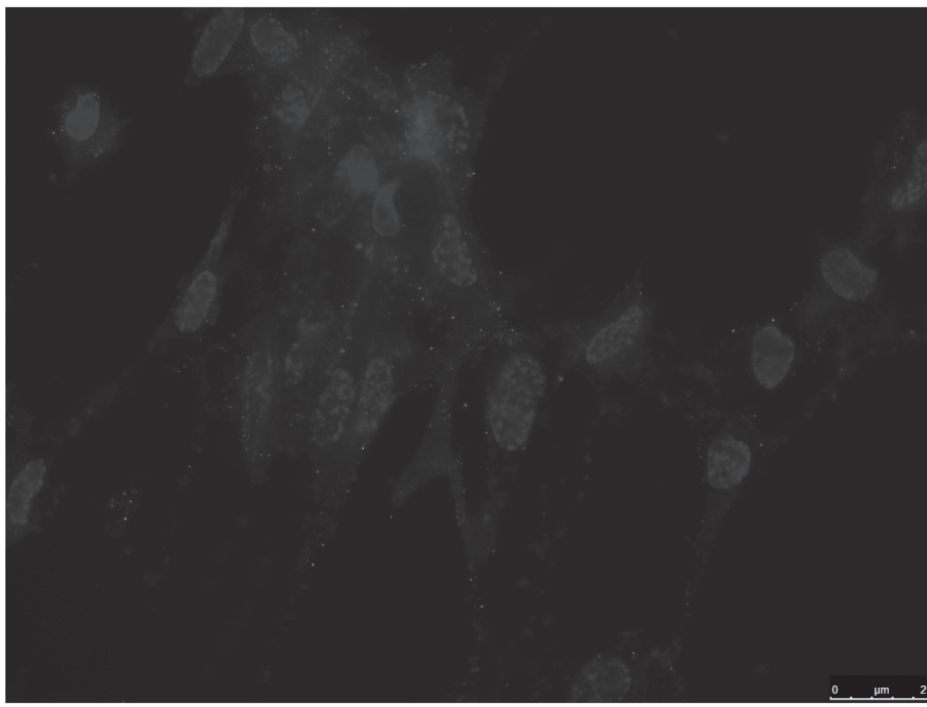
Signal peptide and EGFr domains 1-3 of mutant NOTCH3 (Arg90Cys)

SP	MGPGARGRRRRRRPMSPPPPPPPVRALPLLLLLAGPGAAG
EGFr1	APPCLDGSPCANGGRCTQLPSREAACLCPPGWVGERCQ
EGFr2	LEDPCHSGPCAGCGVCQSSVVAGTARFSCRCPRGFRGPDCS
EGFr3	LPDPCLSSPCAHGARCSVGPDGRFLCSCPPGYQGRSCR

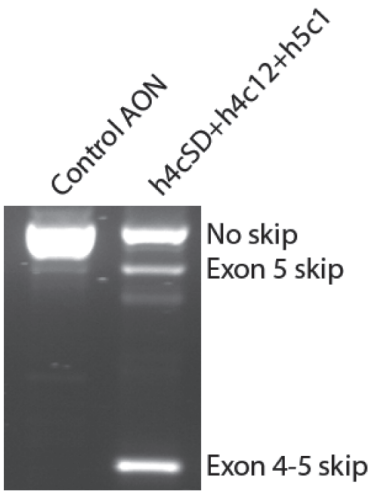
Signal peptide and excluded EGFr domains after exon 2-3 skipping

SP	MGPGARGRRRRRRPMSPPPPPPPVRALPLLLLLAGPGAAG
EGFr1	APPCLDGSPCANGGRCTQLPSREAACLCPPGWVGERCQ
EGFr2	LEDPCHSGPCAGCGVCQSSVVAGTARFSCRCPRGFRGPDCS
EGFr3	LPDPCLSSPCAHGARCSVGPDGRFLCSCPPGYQGRSCR

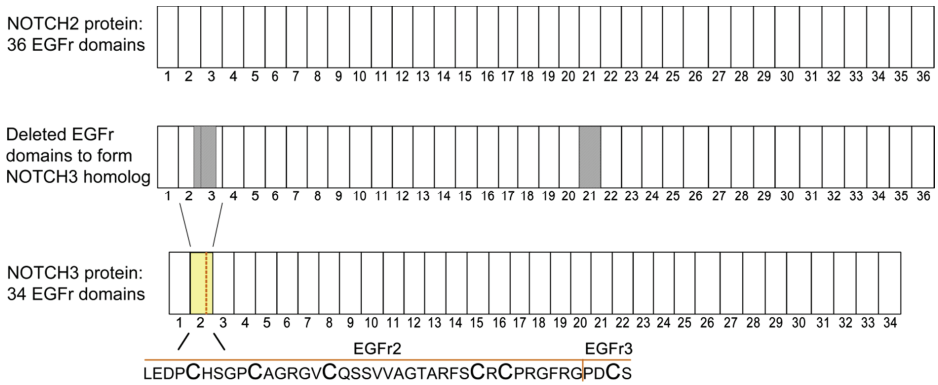
Supplementary figure 4. Predicted effect of *NOTCH3* exon 2-3 skipping.
The NOTCH3 protein contains a signal peptide (SP), located N-terminal to EGFr 1. Joint exon 2-3 skipping leads to the complete exclusion of EGFr1, and the exclusion of all except 4 amino acids of EGFr2. EGFr 3 is predicted to be the first EGFr domain in the skip protein. The remaining cysteine from EGFr 2 is predicted not to be included in any domain, but to reside N-terminal to EGFr 3.



Supplementary figure 5. Endogenous NOTCH3 expression and Jagged1 ligand binding in fibroblasts.
Fibroblasts transfected with an empty vector and incubated with a Jagged1-Fc show a weak NOTCH3 (green) and Jagged1 (red) signal.

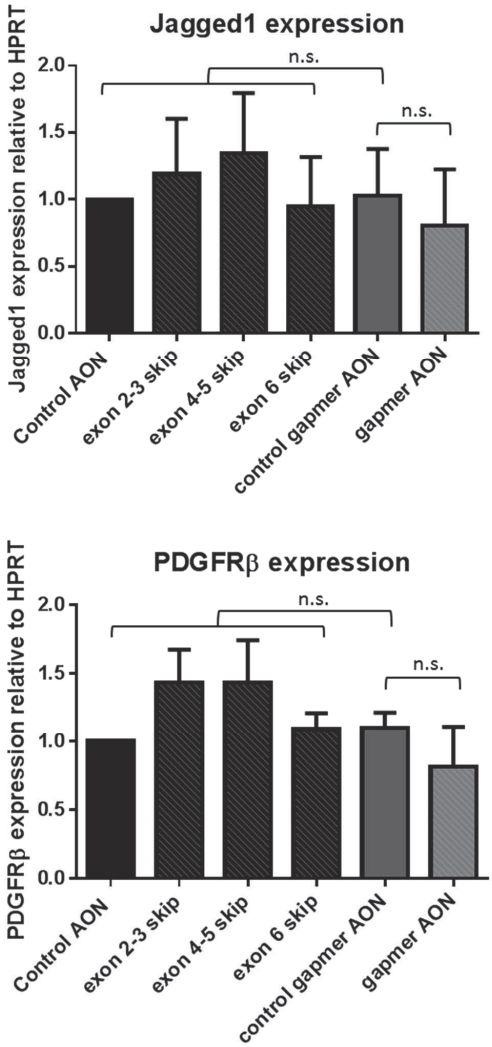


Supplementary figure 6. Successful exon 4-5 skipping in VSMC^{Cys162Trp}.
RT-PCR analysis, confirming effective exon 4-5 skipping in a second CADASIL cell model with another *NOTCH3* mutation (Cys162Trp).



Supplementary figure 7. EGFr fusion of NOTCH in evolution is similar to EGFr fusion after cysteine corrective *NOTCH3* exon skipping.

The ectodomain of the NOTCH2 protein consists of 36 EGFr domains. Evolutionarily, duplication of the NOTCH2 gene led to the emergence of NOTCH3 [26] The ectodomain of NOTCH3 has 34 instead of 36 EGFr domains, as a result of two internal deletions which occurred during evolution [27] One deletion included a part of EGFr 2 and a part of EGFr 3 of NOTCH2, resulting in an EGFr 2-3 fusion that is now NOTCH3 EGFr 2. The EGFr 2-3 domain is fused between the 5th and 6th cysteine, with preserved canonical spacing between the cysteines (mouse NOTCH3 EGFr 2 and 3 amino acid sequence shown). The second deletion is a complete deletion of EGFr 21.



Supplementary figure 8. Expression of NOTCH3 target genes after exon skipping. qPCR analysis of *VSMC*^{control} transfected with *NOTCH3* AONs. Exon skipping does not alter expression levels of NOTCH3 downstream target genes *Jagged1* and *PDGFR*-β. Transfection with a gapmer AON shows some reduction in expression of *Jagged1* and *PDGFR*-β, but this did not reach statistical significance compared to the control gapmer AON. (Unpaired student's t-test).

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5

THE NOTCH3 SCORE: A PRE-CLINICAL CADASIL BIOMARKER IN A NOVEL HUMAN GENOMIC NOTCH3 TRANSGENIC MOUSE MODEL WITH EARLY PROGRESSIVE VASCULAR NOTCH3 ACCUMULATION

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ABSTRACT

CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy) is a hereditary small vessel disease caused by mutations in the *NOTCH3* gene, leading to toxic NOTCH3 protein accumulation in the small- to medium sized arterioles. The accumulation is systemic but most pronounced in the brain vasculature where it leads to clinical symptoms of recurrent stroke and dementia. There is no therapy for CADASIL, and therapeutic development is hampered by a lack of feasible clinical outcome measures and biomarkers, both in mouse models and in CADASIL patients. To facilitate pre-clinical therapeutic interventions for CADASIL, we aimed to develop a novel, translational CADASIL mouse model. To this end, we generated transgenic mice in which we overexpressed the full length human *NOTCH3* gene from a genomic construct with the archetypal c.544C>T, p.Arg182Cys mutation. The four mutant strains we generated have respective human *NOTCH3* RNA expression levels of 100%, 150%, 200% and 350% relative to endogenous mouse *Notch3* RNA expression. Immunohistochemistry on brain sections shows characteristic vascular human NOTCH3 accumulation in all four mutant strains, with human *NOTCH3* RNA expression levels correlating with age at onset and progression of NOTCH3 accumulation. This finding was the basis for developing the 'NOTCH3 score', a quantitative measure for the NOTCH3 accumulation load. This score proved to be a robust and sensitive method to assess the progression of NOTCH3 accumulation, and a feasible biomarker for pre-clinical therapeutic testing. In conclusion, this novel, translational CADASIL mouse model is a suitable model for pre-clinical testing of therapeutic strategies aimed at delaying or reversing NOTCH3 accumulation, using the NOTCH3 score as a biomarker.

INTRODUCTION

Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) is a hereditary small vessel disease caused by mutations in the *NOTCH3* gene, leading to mid-adult onset stroke and dementia.[1] CADASIL is characterized by accumulation of the extracellular domain of the NOTCH3 protein ($NOTCH3^{ECD}$) in the media of small- to medium-sized arterioles.[2] In addition, electron dense deposits (granular osmiophilic material, GOM) are seen in close vicinity to the vascular smooth muscle cells (VSMCs).[3] The arteriopathy is systemic but most pronounced in the brain where it leads to degeneration of VSMCs[3] and a disturbed cerebral blood flow regulation.[4] This causes recurrent ischemic strokes and cognitive decline, starting at a mean age of 45-50 years.[5] To date, there is no therapy to prevent or delay symptoms in CADASIL.

NOTCH3 targeting therapies are in the pre-clinical phase of development (Rutten et al., unpublished, patent no. WO 2010085151 A2). The hitherto available CADASIL mouse models have important limitations with respect to their feasibility for testing such therapeutic strategies. Available models include transgenic models overexpressing human *NOTCH3* from a cDNA construct[6-8] or rat *Notch3* from a genomic construct,[9] and models in which a mutation was introduced into the endogenous *Notch3* gene.[10,11] The first and often only sign of CADASIL in these models is the presence of NOTCH3 accumulation in the vasculature,[12] and in all human *NOTCH3* transgenic models, the NOTCH3 accumulation only becomes apparent at a high age.[6-8] Only the mouse model that expresses mutant rat Notch3 protein from a genomic DNA construct shows early onset vascular Notch3 accumulation with subsequent development of brain parenchymal lesions.[9] However, this model is less suitable as a translational CADASIL model due to the species difference, which creates an additional hurdle in bringing therapeutic compounds to clinical trials. For example, this would be the case for antisense therapeutic strategies targeting mutated pre-mRNA, a therapeutic approach which is being developed for increasing numbers of CNS disorders.[13]

For therapeutic development, feasible clinical outcome measures and biomarkers are imperative, both in mouse disease models and in patients. In CADASIL patients, the variability in age at onset and progression of clinical symptoms, including the major symptoms of stroke and cognitive decline, limits their use as an outcome measure in clinical trials, because of the large number of patients that would have to be included to detect a treatment effect within a typical trial-timeframe of 2 years.[14] White matter lesions, detected on T2 weighted brain MRI images, are present prior to the onset of clinical symptoms and correlate with disease severity,[15] but are not a reliable predictor of disease progression.[16] Changes in magnetic resonance diffusion histograms are a better predictor of disease progression, but have only

been studied in symptomatic patients.[16,17] Ideally, CADASIL therapies would be initiated in the pre-symptomatic disease phase, i.e. in young adults with a proven familial *NOTCH3* mutation. Vascular NOTCH3 protein accumulation could be an interesting therapeutic biomarker for CADASIL, as increased vascular NOTCH3 staining and GOM are consistently found in skin arterioles of pre-symptomatic patients, decades before onset of stroke and cognitive decline.[18,19]

In this study, we set out to generate a novel, translational CADASIL mouse model and to develop a relevant biomarker in this model. We generated a series of human *NOTCH3* transgenic mouse strains, with various expression levels of mutant *NOTCH3*. These mice develop cerebrovascular NOTCH3 accumulation characteristic of CADASIL at an early age, and the *NOTCH3* expression level correlates with both the age at onset and progression of vascular NOTCH3 accumulation. We developed a quantitative measure for the vascular NOTCH3 accumulation load, which we show to be a sensitive and robust biomarker for CADASIL in these mice.

MATERIALS AND METHODS

Generation of NOTCH3 transgenic mice

For transgenesis, a 142,63 kb BAC clone was used (RP11-456N16 BAC, Bacpac resources, Oakland, USA) (Ensemble release 59). The BAC contains the full-length human genomic *NOTCH3* gene and 44kb of upstream and 67kb of downstream sequence, including flanking genes *SYDE1*, *ILVBL*, *EPHX3* and a part of the *BRD4* gene (Figure 1A). The c.544C>T (p.Arg182Cys) mutation was introduced using two-step Red-mediated recombination as previously described [20]. BAC constructs were injected into fertilized C57BL/6J Ico oocytes. Positive transgenic founder mice were identified by PCR on DNA isolated from mouse ears using human specific primers (for primer sequences see Additional file 1: Table S1). The presence of the mutation was confirmed by direct Sanger sequencing analysis of PCR products (Figure 1B). Five transgenic mouse strains were generated: one carrying the wild-type *NOTCH3* transgene (tgN3^{WT}) and four carrying the mutant *NOTCH3* transgene (tgN3^{MUT}). In each strain, integration of the BAC was confirmed by PCR analysis of *NOTCH3* and the flanking genes *SYDE1*, *ILVBL* and *EPHX3* (for primer sequences see Additional file 1: Table S1). All transgenic mouse strains bred normally. All experiments described in this study were approved by the local ethical committee for animal experimentation.

NOTCH3 expression analysis in NOTCH3 transgenic mice

Total RNA was extracted from a brain hemisphere using RNA-Bee (Tel-test Inc., Friendswood, USA). For RT-PCR analysis, first-strand cDNA was synthesized using

oligo (dT) primers. RT-PCR analysis was performed with primers across the human *NOTCH3* transcript (exons 2-4, exons 14-16, exons 30-32, exons 32-33, and the 3'UTR). For qPCR analysis, cDNA synthesis was performed with random hexamer primers, using the Revert Aid H Minus first strand kit (Thermo Scientific, Waltham, USA). Quantitative PCR was performed in four 10-week-old male and female transgenic mice and non-transgenic littermates, using both human- (exons 7-9, 216 bps) and mouse-specific (exons 6-8, 220 bps) primers. Mouse *Gapdh* was used as a reference gene, and human *NOTCH3* expression levels were calculated relative to endogenous mouse *Notch3* expression levels. A possible effect of differences in primer efficiencies of human- and mouse-specific *NOTCH3/Notch3* primersets was excluded by LinregPCR.[21]

NOTCH3 immunohistochemistry and quantification in NOTCH3 transgenic mice

Vascular NOTCH3 accumulation in brain was analysed prospectively in groups of three mice, at age 4 weeks, 6 weeks, 12 weeks, 24 weeks, 52 weeks and 82 weeks. In addition, vascular NOTCH3 accumulation was assessed in heart, aorta, liver, kidney, skin and tail at age 20 months. NOTCH3 immunohistochemistry was performed on cryosections that were fixated in acetone and incubated overnight with an antibody directed against the human NOTCH3^{ECD} (Novus Biologicals, Littleton, USA; dilution 1:2000). The following day, sections were incubated with peroxidase labelled polymer conjugated to anti-rabbit immunoglobulins (Envision kit, Dako, Glostrup, Denmark), and developed using 3,3'-diaminobenzidine. Quantification of the vascular NOTCH3 accumulation load was performed on brain cryosections. Per time point (6 weeks, 24 weeks, 52 weeks and 82 weeks), three tgN3^{MUT}350 mice and three tgN3^{MUT}150 mice were analysed. For each mouse, four frontal lobe brain sections were NOTCH3 immunostained simultaneously. Sectionswere scanned using the Ultra Fast Scanner (Philips, Eindhoven, The Netherlands), from which 10 images representative for the NOTCH3 accumulation observed in that mouse were obtained. To exclude a possible bias in image selection, a second, blinded observer was asked to obtain images independently (Additional file 1: Figure S1). ImageJ analysis was performed as follows: the image was converted into an 8-bit image, and filtered using the unsharp mask filter (radius 1, mask 0.60). Next, a threshold was set to a signal intensity of 150 to determine the NOTCH3-positive area. Within the NOTCH3-positive area, individual NOTCH3 particles were identified based on size and circularity (size=0-30, circularity=0.50-1.00). Finally, the NOTCH3 score was determined by quantifying the total area of the NOTCH3 positive particles within the image. The average of the three mice per time point was plotted and used for further statistical analysis.

NOTCH3 immunohistochemistry and quantification in human material

We used paraffin embedded frontal lobe brain sections from three deceased CADASIL patients (I: female age 59, p.Arg153Cys; II: female age 57, p.Arg153Cys; III: male age 70, p.Cys446Phe) and three deceased controls with no known cerebrovascular disorders (I: male age 67, II: male age 58, III: male age 53). Sections were de-waxed, rinsed with ethanol and blocked with methanol/H_2O_2. After heat-induced antigen retrieval in 0,01M citrate buffer pH 6, slices were washed three times with PBS, and incubated overnight at room temperature with a 1:1 cocktail of anti-NOTCH3^{ECD} (dilution 1:500) and anti-CD31 (Dako, Glostrup, Denmark; dilution 1:50) The following day, sections were washed and incubated for 1 hour at room temperature with a 1:1 cocktail of anti-rabbit Envision/HRP (Dako) and goat anti-mouse alkaline phosphatase (Vector Laboratories, Burlingame, CA, USA; dilution 1:25). Finally, sections were sequentially developed with 3,3'-diaminobenzidine solution and Vector Blue (Vector laboratories). Per individual, four images were taken at a 400x magnification on a Leica IM 500 microscope and analysed using ImageJ software. The vessel area was selected manually based on a positive CD31 staining. Within the vessel area, the NOTCH3 score was calculated using an intensity threshold of 100.

Electron microscopy in NOTCH3 transgenic mice

Brain tissue was fixed in 1,5% glutaraldehyde and 1% paraformaldehyde in 0,1M cacodylate buffer, post-fixed in a solution of 2% osmium tetroxide and 2% potassium ferrocyanide, dehydrated and embedded in epon 812 (LX112). After selection of areas of interest on 1µm toluidine stained sections, ultrathin sections were cut, contrasted with 3% uranylacetate and Reynolds lead citrate and examined with a JEOL JEM-1011 electron microscope (Advanced Microscopy Techniques, Woburn, USA).

Statistical analysis

Statistical analyses were performed using Graphpad Prism. Differences in NOTCH3 score between the two mouse strains at a given time point were analysed using the unpaired student's *t*-test. Differences in NOTCH3 score between time points were analysed using One-Way ANOVA and Fishers least significant difference post-hoc analysis. Differences in slope (i.e. the rate of increase of the NOTCH3 score) over time were analysed using linear regression.

Brain MRI of NOTCH3 transgenic mice

Brain MRI was performed in 15 mice at 20 months of age; six TgN3^{MUT}350 mice, five tgN3^{WT} mice, and four non-transgenic littermates. Mice were anesthetised by inhalation of 2% isoflurane in a 1:1 mixture of oxygen and air. Respiration rate was monitored with a respiratory pad and kept between 50 and 80 respirations

per minute by adjusting the isoflurane concentration. T2 weighted imaging was performed on a 7 Tesla Bruker Pharmascan using a 23 mm quadrature coil with the following parameters: TE=12 ms, RARE factor=8, effective TE=48 ms, TR=4 s, 8 averages. Field-of-view= 19x19 mm, matrix= 196x196, resulting in an in-plane resolution of 97 µm. Slice thickness=0.5 mm, with 32 slices.

Analysis of brain parenchyma in NOTCH3 transgenic mice

After MRI, anesthetised mice were sacrificed using cardial perfusion with ice-cold phosphate buffered saline. One brain hemisphere was formalin fixated and paraffin embedded. Sections were stained with hematoxylin and eosin (H&E) to analyse the presence of infarctions, with Kluver Barrera Luxol fast blue to visualise myelin and with Perl's iron to assess the presence of microbleeds. Astrogliosis was analysed using an anti-glial fibrillary acidic protein (GFAP) antibody (rabbit anti-GFAP, Dako; dilution 1:1000), which was incubated overnight at room temperature. As a secondary antibody, biotin labelled swine-anti Rabbit (Dako; dilution 1:600) was used, this was incubated 1 hour at room temperature. Finally, sections were incubated with avidin-biotin complex (Vectastain ABC-Elite Kit, Vector Lab, Burlingame, USA) for 30 minutes at room temperature and developed in 3,3′-diaminobenzidine solution. The detection of macrophages and myelin was performed using the Animal Research Kit peroxidase (Dako). Biotinylated primary antibodies against CD68 (anti-CD68 clone KP-1, Dako; dilution 1:1000) and myelin proteolipid protein (anti-PLP, clone plpc-1, Serotec, Kidlington, UK; dilution 1:500) were incubated overnight after heat-induced antigen retrieval in 0.01M EDTA pH 8.0. The following day, sections were incubated with HRP- conjugated streptavidin for 30 minutes at room temperature and developed in 3,3′-diaminobenzidine solution.

RESULTS

Four mutant human NOTCH3 transgenic mouse strains with distinct NOTCH3 expression levels

Using qPCR analysis on RNA isolated from brain of 10-week old mice, we found that the four mutant *NOTCH3* p.Arg182Cys transgenic mouse strains (tgN3^{MUT}) had human *NOTCH3* expression levels of 100%, 150%, 200% and 350%, respectively, compared to endogenous mouse *Notch3* expression (Figure 1C). Human *NOTCH3* expression in the lowest expressing tgN3^{MUT} strain, was comparable to that in the wild-type strain (tgN3^{WT}), i.e. ~100%. RT-PCR analysis with multiple *NOTCH3* primer sets spanning the complete transcript, showed that the complete human *NOTCH3* cDNA was present in the transgenic transcript (Additional file 1: Figure S2). There was no difference in endogenous mouse *Notch3* expression between transgenic and non-transgenic mice (Additional file 1: Figure S3).

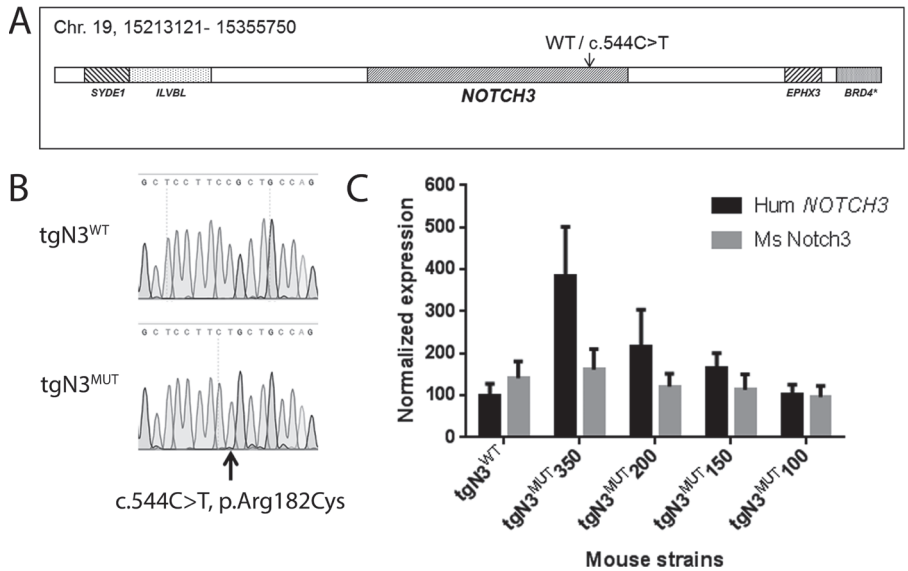

Figure 1. Generation of transgenic human *NOTCH3* mice. (A) Schematic representation of the BAC construct containing the human *NOTCH3* gene and flanking regions, used for generation of tgN3^{WT} and tgN3^{MUT} (c.544C>T, p.Arg182Cys) mice. (B) Sequencing analysis of PCR products of the human *NOTCH3* gene in transgenic mice confirmed the presence of the c.544C>T mutation in tgN3^{MUT} mice. (C) qPCR analysis of human and mouse *NOTCH3* expression in brain. In strain tgN3^{WT}, human *NOTCH3* expression was comparable to endogenous mouse *Notch3* expression. The four mutant strains showed human *NOTCH3* expression levels of 350%, 200%, 150% and 100%, as compared to endogenous mouse *Notch3* expression. Endogenous mouse *Notch3* expression was comparable between the transgenic mouse strains.

Age at onset of vascular NOTCH3 protein accumulation correlates with NOTCH3 expression levels

To analyse the presence and onset of a CADASIL vascular phenotype, NOTCH3 immunohistochemistry was performed on brain slices from mice between the ages of 4 weeks and 20 months. This showed that all tgN3^{MUT} strains developed cerebrovascular NOTCH3 accumulation, as seen by a positive, granular NOTCH3 staining of the vessel wall (Figure 2A), similar to that which is seen in CADASIL patients (Figure 2B). There was a considerable difference in age at onset of positive NOTCH3 staining per mouse strain, ranging from 6 weeks in tgN3^{MUT}350 mice to 12 months in tgN3^{MUT}100 mice. The age at onset directly correlated with the level of human *NOTCH3* RNA expression for all four tgN3^{MUT} strains i.e. the higher the *NOTCH3* RNA expression level, the earlier the onset of NOTCH3 accumulation (Table 1). Furthermore, in each mutant strain, the positive NOTCH3 immunostaining became progressively more intense and granular with age (Figure 2A). The

individual granular NOTCH3 deposits increased not only in number, but also in size. This was most prominent in mice with the highest *NOTCH3* RNA expression level (tgN3^{MUT}350), in which the NOTCH3 protein accumulation progressively evolved to a vessel wall packed with intense and big granular NOTCH3 deposits at age 20 months.

Mouse strain	***NOTCH3*** **expression level***	Age at onset NOTCH3 accumulation**
tgN3^{MUT} 350	350%	6 weeks
tgN3^{MUT} 200	200%	3 months
tgN3^{MUT} 150	150%	5 months
tgN3^{MUT} 100	100%	12 months

Table 1. The *NOTCH3* RNA expression level correlates with the age at onset of cerebrovascular NOTCH3 protein accumulation. * mRNA *NOTCH3* expression levels relative to endogenous mouse *Notch3* expression levels ** first sign of positive, granular NOTCH3 staining in brain vessels, as determined by an experienced neuropathologist (S.v.D).

Characteristic granular NOTCH3 staining was also present in arterioles of the heart, liver, kidney, skin and tail, but not in the aorta (Figure 2C, data not shown). Overall, the NOTCH3 accumulation observed in the extra-cerebral arterioles was less pronounced than in the brain. Electron microscopy of brain arterioles revealed characteristic electron dense deposits within the basement membrane (Figure 2D) reminiscent of GOM deposits seen in CADASIL patients (Figure 2E). Neither GOM nor increased cerebrovascular NOTCH3 staining was found in tgN3^{WT} mice at 20 months of age (Figure 2A). Taken together, these analyses show that transgenic human *NOTCH3* p.Arg182Cys mice develop an early and progressive systemic arteriopathy which closely resembles the vascular pathology seen in CADASIL patients, with age-at-onset correlating with the respective levels of mutant human *NOTCH3* expression.

Development of the NOTCH3 score, a quantitative biomarker for CADASIL

As we observed such an early and clear age- and *NOTCH3* expression level-dependent vascular NOTCH3 accumulation load, we set out to objectify this by developing a quantitative measure for NOTCH3 staining. This was accomplished by capturing and measuring the surface area of CADASIL specific granular NOTCH3 deposits within brain sections using ImageJ software (Figure 3A), which we called the 'NOTCH3 score'. This quantification was first performed in tgN3^{MUT}350 mice,

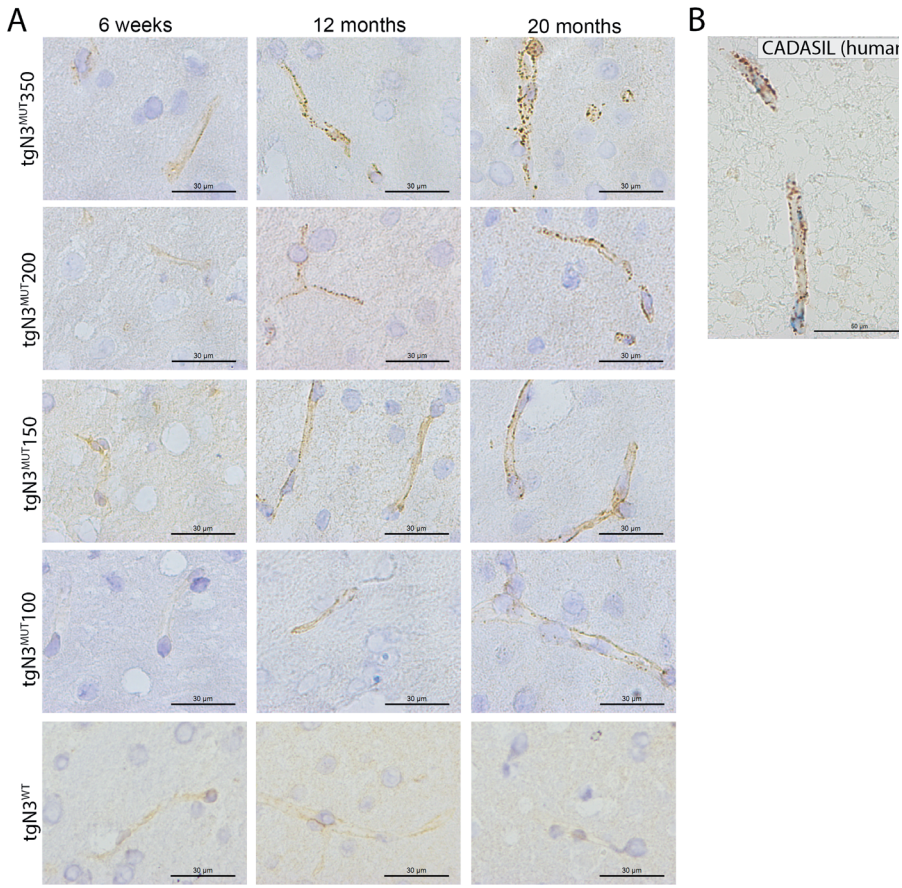
A
6 weeks
12 months
20 months
tgN3MUT350
tgN3MUT200
tgN3MUT150
tgN3MUT100
tgN3WT
B
CADASIL (human
C
heart
liver
skin
aorta
D
tgN3MUT350
BM
Vessel lumen
E
CADASIL (human
BM
VSMC
Vessel lumen

Figure 2. Vascular NOTCH3 protein accumulation and GOM deposits in transgenic human NOTCH3 p.Arg182Cys mice. (A) NOTCH3 immunostaining on brain sections of human *NOTCH3* transgenic mice. All four tgN3^{MUT} mouse strains developed a characteristic granular NOTCH3 staining pattern in the brain vasculature. TgN3^{WT} mice showed only a weak, diffuse NOTCH3 staining pattern, which did not increase with age (comparable to non-transgenic litter-mates, data not shown). The NOTCH3 accumulation load in the tgN3^{MUT} strains correlates well with the *NOTCH3* expression level and increases with age; in tgN3^{MUT}350 mice, first granular staining is already visible at 6 weeks of age; at 20 months of age nearly the whole vessel wall is packed with big granular NOTCH3 deposits. In the strains with a lower NOTCH3 expression level, the NOTCH3 accumulation starts at a later age and the granular deposits remain smaller. (B) Positive NOTCH3 staining in a brain vessel of a CADASIL patient. (C) NOTCH3 immunostaining of extra-cerebral arteries of 20-month-old tgN3^{MUT}350 mice showing clear granular NOTCH3 staining in vessels of the heart, liver and skin. The aortic wall shows a diffuse and faint NOTCH3 staining pattern comparable to that seen in non-transgenic littermates, whereas the smaller vessels around the aorta do show characteristic granular NOTCH3 staining. (D) Electron microscopy on brain vessels from 12-month-old tgN3^{MUT}350 mice shows characteristic electron dense deposits reminiscent of granular osmiophilic material (GOM). GOM deposits were first seen at 5-6 months of age. (E) Electron microscopy on brain tissue from a deceased CADASIL patient shows pathognomonic GOM deposits, adjacent to the basement membrane surrounding the VSMC. *=granular osmiophilic material (GOM), BM=basement membrane, VSMC=vascular smooth muscle cell.

which clearly showed that the NOTCH3 score increased with age, confirming our qualitative observations (Figure 3B). Next, we validated the NOTCH3 score in a second mouse strain, tgN3^{MUT}150, in which the same age-dependent increase in the NOTCH3 score was seen. At each time-point, the NOTCH3 score was lower for the tgN3^{MUT}150 mice compared to the tgN3^{MUT}350 mice, reflecting the correlation between *NOTCH3* expression level and NOTCH3 accumulation load (score at age 20 months: 659 ±51 vs. 1150 ±107, p=0.002) (Figure 3C). Furthermore, progression of NOTCH3 accumulation was slower in tgN3^{MUT}150 mice compared to tgN3^{MUT}350 mice, as shown by a significant difference in the slope of the NOTCH3 scores between the two mouse strains (11.1 ±0.5 vs. 6.2 ±0.3, p=0.002). Finally, we tested the approach in brain sections of three unrelated CADASIL patients. Measurement of the NOTCH3 accumulation load using the NOTCH3 score was technically feasible in human tissue (Figure 3D) and showed a significantly higher NOTCH3 score in patients than in controls (score 3.81 ±1.85 vs. 0.24 ±0.17, p=0.02) (Figure 3E).

No clear brain parenchyma phenotype in tgN3^{MUT} mice

Finally, to determine whether we could correlate the NOTCH3 score to a brain phenotype in tgN3^{MUT} mice, we performed brain MRI and histopathology in mice aged 20 months (Additional file 1, Figure S4, S5). In two of the six tgN3^{MUT}350 mice,

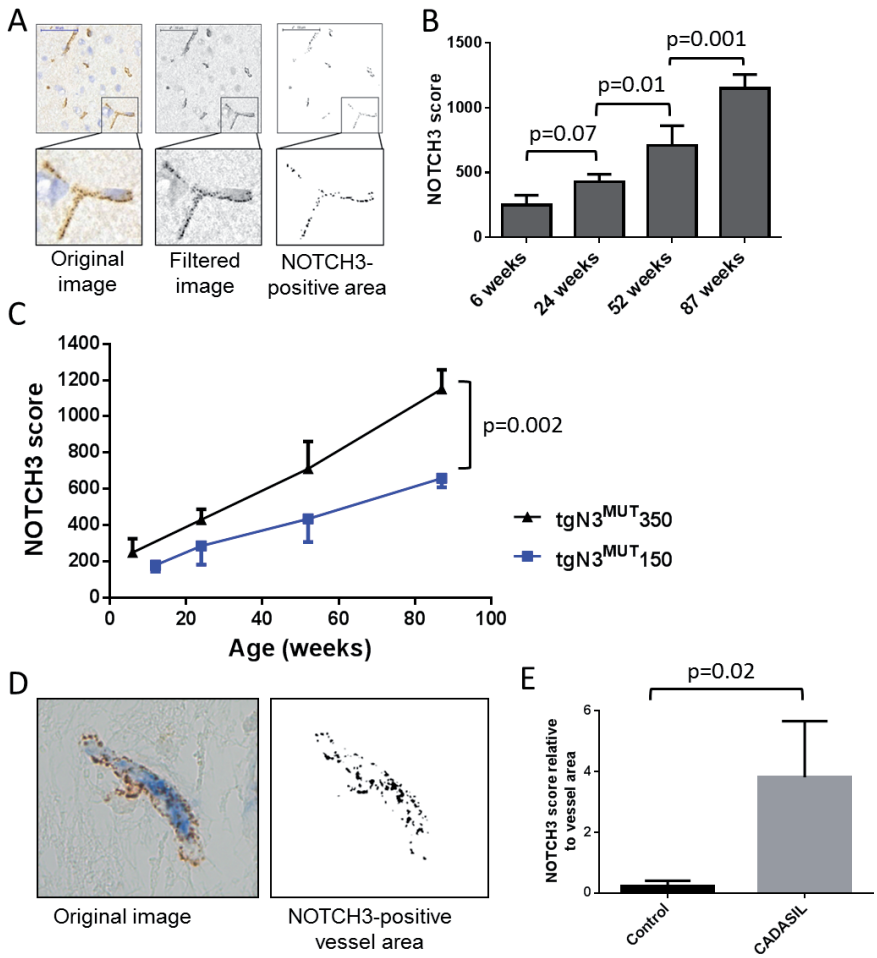

Figure 3. Quantitative analysis of vascular NOTCH3 protein accumulation in transgenic human NOTCH3 p.Arg182Cys mice and in brain tissue of CADASIL patients. (A) Image-J processing of NOTCH3-immunostained brain sections of tgN3^{MUT}350 mice. The images were filtered to reduce background signal and a standardised threshold was applied to determine the NOTCH3- positive area composed of individual granular NOTCH3 deposits, resulting in the NOTCH3 score. (B) Quantitative analysis of NOTCH3 accumulation in tgN3^{MUT}350 mice. The NOTCH3 score shows an age-dependent increase and allows for a sensitive discrimination between age groups (One-Way ANOVA, Fishers least significant difference). (C) Validation of the NOTCH3 score in tgN3^{MUT}150 mice, also showing an age-dependent increase. At each time point, the score is lower in tgN3^{MUT}150 than in tgN3^{MUT}350 mice (unpaired *t*-test), reflecting the correlation between *NOTCH3* RNA expression and NOTCH3 protein accumulation. Data represent the average +/- SD of the three mice analysed per time point. (D) ImageJ analysis of human brain sections double stained with NOTCH3 and CD31. The vessel area was selected based on the staining with the endothelial cell marker CD31, and within this area, the NOTCH3 score was determined. (E) CADASIL patients show a significantly higher NOTCH3 score than age-matched controls. (unpaired *t*-test) Data represent the average +/- SD of three CADASIL patients and three control individuals.

hyperintensities were seen on T2 weighted images, cranial to the corpus callosum and around the ventricles in the frontal lobe. However, similar hyperintensities were found in one non-transgenic littermate. Histopathological examination did not show any signs of astrogliosis or white matter lesions in the mutant mice. The absence of consistent and specific brain abnormalities in tgN3^{MUT} mice prohibited the testing of a potential correlation between NOTCH3 score and brain phenotype.

DISCUSSION

To facilitate the testing of pre-clinical therapeutic interventions for CADASIL, we generated a translational, human genomic *NOTCH3* transgenic mouse model with an early vascular phenotype, and developed a biomarker in this model. The mutant mice recapitulate the CADASIL vascular phenotype with early onset and progressive cerebrovascular NOTCH3 accumulation and GOM deposits in arterioles. The respective *NOTCH3* RNA expression levels in the four mouse strains correlate strongly and consistently with the age at onset and progression of NOTCH3 protein accumulation, with the highest expressing mouse strain developing vascular NOTCH3 accumulation as early as 6 weeks of age. The quantitative biomarker we developed, the NOTCH3 score, allows for a sensitive and objective measure of NOTCH3 accumulation, which can therefore be used for pre-clinical testing of therapeutic strategies aimed at delaying or reversing NOTCH3 accumulation.

Cerebrovascular NOTCH3 accumulation was selected as a potential biomarker because it was consistently and specifically found in tgN3^{MUT} mice, and showed an early age at onset and clear progression. Also, NOTCH3 accumulation is a plausible surrogate marker for CADASIL because it is universally present in the cerebrovasculature of CADASIL patients and is believed to play an important role in disease pathophysiology.[22] Because of the lack of consistent brain abnormalities in our mice, we were unable to correlate the NOTCH3 score to a brain phenotype. However, an early age at onset of NOTCH3 accumulation has previously been found to be associated with the development of brain parenchymal damage in mice.[9] Moreover, age is one of the most important predictors of CADASIL disease severity and progression,[14,23] implicating that the age-dependent increase in NOTCH3 score is a relevant surrogate marker for disease progression. Sample size calculations we performed show that the NOTCH3 score is a feasible biomarker for pre-clinical therapeutic studies, as an effect on NOTCH3 accumulation can be assessed in relatively small groups of mice. For example, treatment of 7 mice allows for the detection of a 50% effect on the progression of the NOTCH3 score, when treating from 6 to 24 weeks of age.

We found that the NOTCH3 score can also be measured in the cerebrovasculature of deceased CADASIL patients. Evidently, a NOTCH3 score in brain sections is not a feasible biomarker in clinical trials. However, vascular NOTCH3 accumulation has been extensively demonstrated in skin biopsies of CADASIL patients and is detectable decades before the onset of clinical symptoms.[18] In a single family study, an age-dependent increase in GOM deposits in skin biopsies was found up to 50 years of age.[24] Although previous studies did not find a correlation between skin biopsy NOTCH3 immunostaining and disease severity, these studies were limited by a qualitative assessment of the NOTCH3 staining intensity.[18,19] Whether such a correlation can be established using our quantitative NOTCH3 score, will have to be assessed in future prospective studies.

This novel CADASIL mouse model is especially suitable for testing therapeutic strategies for a number of reasons. The presence of the human *NOTCH3* gene in our mouse model allows for testing compounds specifically directed at human *NOTCH3*, thereby avoiding an additional hurdle in the translation from pre-clinical to clinical trials. The fact that we used a genomic *NOTCH3* construct allows for testing therapeutic interventions that target mutant *NOTCH3* at the genomic or (pre-) mRNA level. Such interventions, for example using antisense oligonucleotides to reduce or modify mutant NOTCH3 protein, are being developed in our lab (Rutten et al., unpublished, patent no. WO 2010085151 A2). Another practical advantage is that in this early onset model, treatment can be initiated at an early age.

CONCLUSIONS

In conclusion, we developed a novel, unique human *NOTCH3* transgenic mouse model and a NOTCH3 score which is a robust and sensitive biomarker for CADASIL. This translational model is ideally suited for pre-clinical testing of therapeutic strategies aimed at delaying or reversing NOTCH3 protein accumulation.

ABBREVIATIONS

CADASIL: Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy; $NOTCH3^{ECD}$: extracellular domain of the NOTCH3 protein; GOM: granular osmiophillic material; VSMCs: vascular smooth muscle cells; MRI: magnetic resonance imaging.

COMPETING INTERESTS

NOTCH3 antisense therapies have been patented by the Leiden University Medical Center. As co-inventors on this patent HD, AAR and SAJLO are entitled to a share of potential royalties.

AUTHORS' CONTRIBUTIONS

JWR, AMJMvdM and SAJLO designed the study. JWR, RRK, IMH, DSP, HGD, LAMB and CB performed experiments. Generation of transgenic mice: HGD, CB, AAR, JSV, AMJMvdM and SAJLO. Molecular characterization: JWR, RRK, HD, LAMB and AMJMvdM. Pathological analysis and quantification: JWR, RRK, IMH, SGvD and SAJLO. MRI analysis: JWR, DP, LvdW and SAJLO. JWR and SAJLO wrote the first draft of the manuscript. JWR, AMJMvdM and SAJLO revised the final draft of the manuscript. All authors read and approved the final manuscript.

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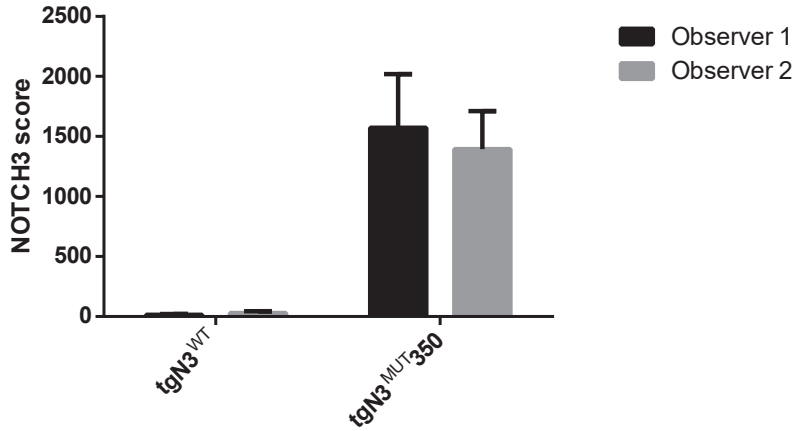
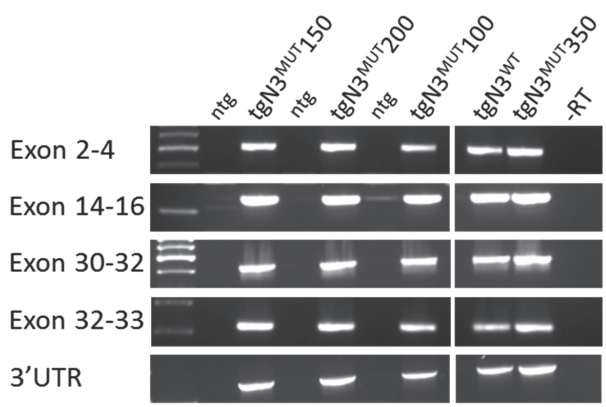
ADDITIONAL FILE

Genotyping primers	
Primer	Sequence
HuNOTCH3ex 4F	5′-CTGACTGCTCCCTGCCAGAT-3′
HuNOTCH3ex 4R	5′-TAAGTGAGGTCGCCACTCTG-3′
SYDE1Dex2 F	5′-AATTGCACAGGGCTGCTC-3′
SYDE1Dex2 R	5′-AGGTACCCCAGGCCCTAAA-3′
ILVBLex2 F	5′-AGGGGCGCGATTTCTTC-3′
ILVBLex2 R	5′-AGGCCCCTCCCTCTTTG-3′
EPHX3ex5 F	5′-GTAGGGGTGCAGGTGTATGG-3′
EPHX3ex5 F	5′-TGGCTTCAGTGCCCAGC-3′
RT-PCR primers	
Primer	Sequence
HuNOTCH3ex 2F	5′-GTGTGCAAATGGAGGTCGT-3′
HuNOTCH3ex 4R	5′-TAAGTGAGGTCGCCACTCTG-3′
HuNOTCH3ex 14F	5′-GCGATGGAATGGGTTTCCA-3′
HuNOTCH3ex 16R	5′-GCCAGGTTGGTGCAGATACCATGA-3′
HuNOTCH3ex 30F	5′-TCTCCGACCTGATCTGCCAG-3′
HuNOTCH3ex 32R	5′-TTGAGCAGGGCCAAAGTGGCT-3′
HuNOTCH3ex 32F	5′-AGCCACTTTGGCCCTGCTCAA-3′
HuNOTCH3ex 33R	5′-GTTGATCCAGCAAGCGCACG-3′
HuNOTCH3ex 32-33F	5′-GGACATGCAGGATAGCAAGGAG-3′
HuNOTCH3ex 33R_3UTR	5′-CGGTCACGCTGCAAGGCAAGG-3′
qPCR primers	
Primer	Sequence
HuNOTCH3ex 7F	5′-GGACGAGTGCTCTATCGGC-3′
HuNOTCH3ex 9R	5′-GTTCCTGTGAAGCCTGCCATA-3′
MsNotch3ex 6F	5′- TTCTACTGTGCCTGCCCT-3′
MsNotch3ex 8R	5′-CACACCGACCCAAATGTTCA-3′
MsGAPDH F	5′-TGCACCACCAACTGCTTAGC-3′
MsGAPDH R	5′-GGCATGGACTGTGGTCATGAG-3′

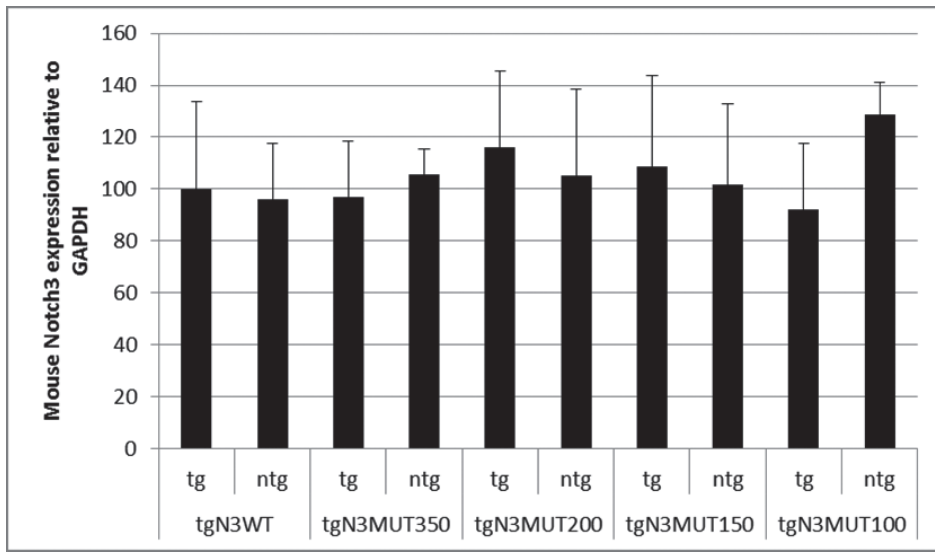
Table S1. Primers used for mouse genotyping and *NOTCH3* expression analysis.



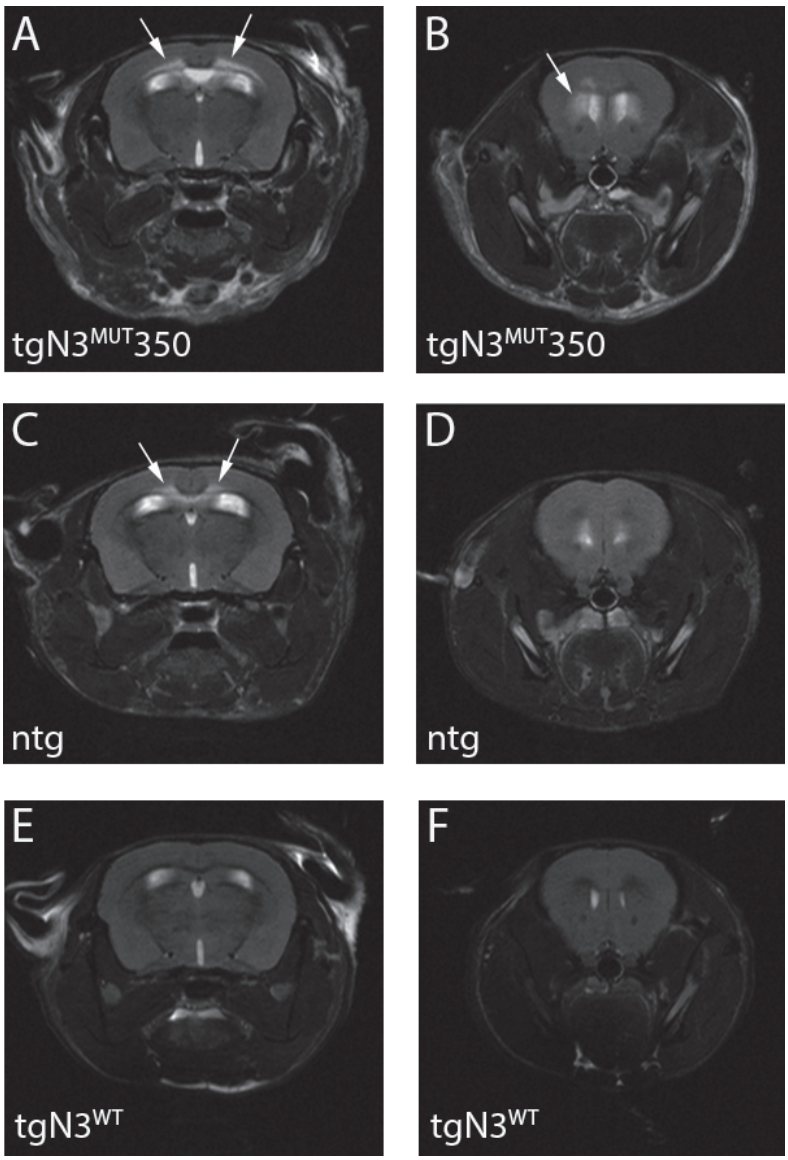
Supplementary figure 1. Validation of NOTCH3 protein quantification in transgenic human NOTCH3 mice. No differences in NOTCH3 score were seen upon image selection by two independent observers (score tgN3^{MUT}350: 1572 ±448 vs. 1394 ±318 p=0.54, unpaired *t*-test). Data represents the mean ±SD of 4 images.



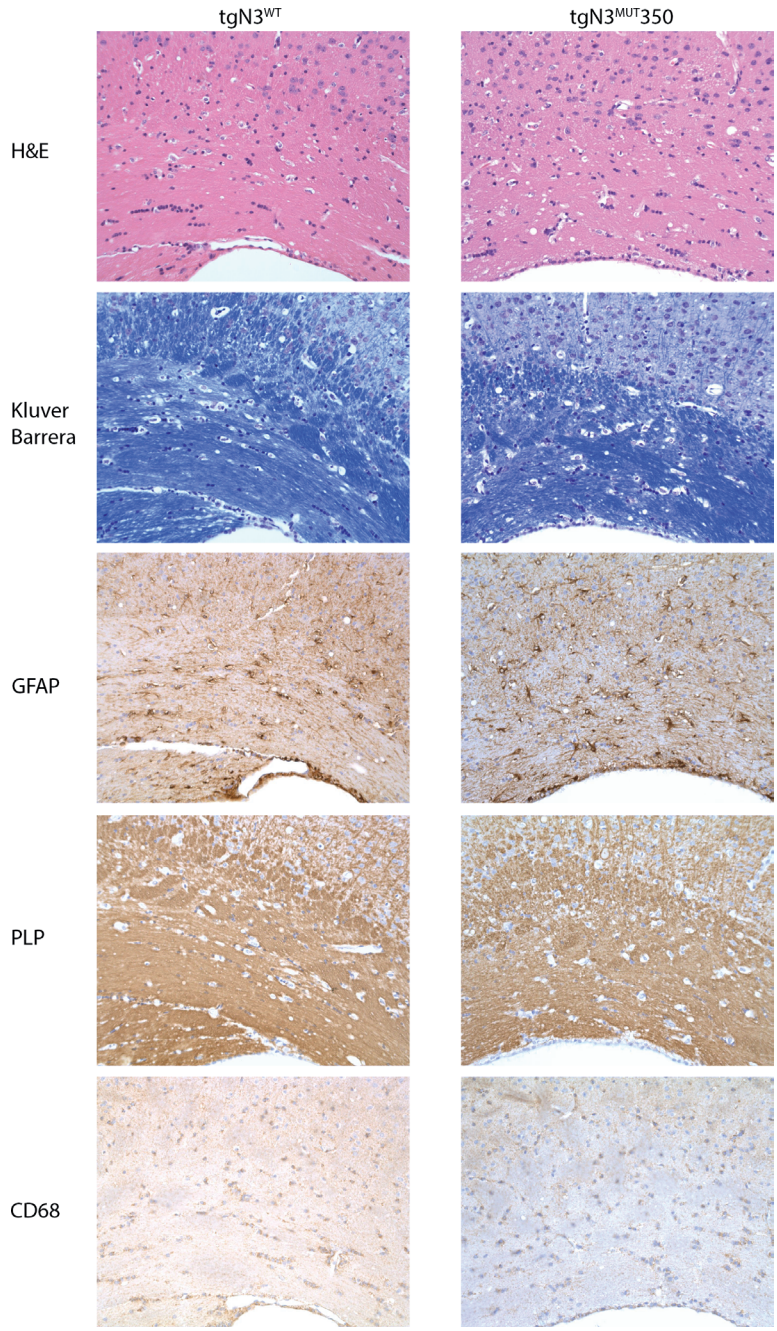
Supplementary figure 2. *NOTCH3* RNA expression in transgenic human *NOTCH3* mice. RT-PCR analysis using primers spanning the *NOTCH3* gene showed that the complete *NOTCH3* transcript is expressed in all transgenic mouse strains.



Supplementary figure 3. Endogenous *Notch3* RNA expression in transgenic human *NOTCH3* mice. qPCR analysis shows similar *Notch3* expression levels in brain tissue of transgenic and non-transgenic mice.



Supplementary figure 4. Brain MRI of transgenic human NOTCH3 p.Arg182Cys mice. (A-F) T2 weighted brain MRI images of mice aged 20 months. Six tgN3^{MUT} mice, five tgN3^{WT} mice and four non-transgenic littermates were analysed. Brain MRI abnormalities (indicated by the arrows) were detected in two of the six tgN3^{MUT}350 mice, and in one of the four non-transgenic mice. All mice with hyperintensities also had enlarged ventricles, which is a common finding in C57Bl/6J mice (The laboratory mouse, 2nd edition) (A-B) Representative pictures of one of the two tgN3^{MUT}350 mice with brain MRI abnormalities. T2 weighted brain MRI showed hyperintensities cranial to the corpus callosum, and in the frontal lobe around the ventricles. (C-D) Similar hyperintensities, although less severe, were seen in one of the non-transgenic littermates. (E-F) No MRI abnormalities were seen in tgN3^{WT} mice.



Supplementary figure 5. Brain parenchyma stainings of tgN3^{MUT}350 and tgN3^{WT} mice. No consistent differences were seen for any of the stainings analysed. Moreover, no histopathologic substrate for the observed MRI abnormalities was seen. All images are obtained at a 200x magnification.

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6

ARCHETYPAL NOTCH3 MUTATIONS FREQUENT IN PUBLIC EXOME: IMPLICATIONS FOR CADASIL

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ABSTRACT

Objective: To determine the frequency of distinctive EGFr cysteine altering *NOTCH3* mutations in the 60,706 exomes of the exome aggregation consortium (ExAC) database.

Methods: ExAC was queried for mutations distinctive for CADASIL, namely mutations leading to a cysteine amino acid change in one of the 34 EGFr domains of *NOTCH3*. The genotype-phenotype correlation predicted by the ExAC data was tested in an independent cohort of Dutch CADASIL patients using *NOTCH3* genotype and quantified MRI lesions. The Dutch CADASIL registry was probed for paucisymptomatic individuals older than 70 years.

Results: We identified 206 EGFr cysteine altering *NOTCH3* mutations in ExAC, with a total prevalence of 3.4/1000. More than half of the distinct mutations have been previously reported in CADASIL patients. Despite the clear overlap, the mutation distribution in ExAC differs from that in reported CADASIL patients, as mutations in ExAC are predominantly located outside of EGFr domains 1-6. In an independent Dutch CADASIL cohort, we found that patients with a mutation in EGFr domains 7-34 have a significantly lower MRI lesion load than patients with a mutation in EGFr domains 1-6.

Interpretation: The frequency of EGFr cysteine altering *NOTCH3* mutations is 100-fold higher than expected based on estimates of CADASIL prevalence. This challenges the current CADASIL disease paradigm, and suggests that certain mutations may more frequently cause a much milder phenotype, which may even go clinically unrecognized. Our data suggest that individuals with a mutation located in EGFr domains 1-6 are predisposed to the more severe 'classical' CADASIL phenotype, whereas individuals with a mutation outside of EGFr domains 1-6 can remain paucisymptomatic well into their eighth decade.

INTRODUCTION

Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) is a dominant, monogenic small vessel disease caused by highly distinctive mutations in the *NOTCH3* gene.[1] *NOTCH3* mutations in CADASIL lead to (cerebro)vascular NOTCH3 protein aggregation, compromised cerebral blood flow, mid-adult onset of stroke and vascular cognitive impairment, and migraine with aura. Although most patients experience their first stroke at 45-50 years of age, there is a wide variability in age of onset and disease progression,[2] and a later-onset, milder disease course is increasingly recognised.[3]

CADASIL patients have progressive ischemic brain MRI abnormalities which correlate with disease severity.[4-6] In the pre-symptomatic stage of the disease, up to decades before onset of symptoms, symmetric periventricular white matter hyperintensities (WMH) are observed,[7,8] often also affecting the anterior temporal lobes. The MRI in symptomatic individuals often reveals multiple lacunar infarcts, microbleeds and brain atrophy.[9,10]

CADASIL has been reported worldwide, but only some countries in Europe and East Asia have compiled large series of CADASIL patients and their *NOTCH3* mutations.[11-17] Prevalence studies have been performed in relatively small populations, with a reported minimum prevalence of 2-5/100,000.[3,18,19] This number is held to be an underestimation.[3]

Over 98% of CADASIL patients have a distinctive missense mutation in one of *NOTCH3* exons 2-24, invariably leading to the gain or loss of a cysteine residue in one of the 34 epidermal growth factor- like repeat (EGFr) domains of the NOTCH3 protein. This changes the canonical number of six cysteines in one of these EGFr domains to an uneven number of five or seven cysteines.[12] The resultant unpaired cysteine is predicted to lead to abnormal disulphide bridge formation and NOTCH3 protein aggregation.[20] More than 200 distinct *NOTCH3* mutations have been identified in CADASIL patients, dispersed across EGFr domains 1-34, but with the highest prevalence in EGFr domains 1-6.[21] Non-penetrance has never been reported for this specific type of *NOTCH3* mutations. A definite, clinically relevant genotype-phenotype correlation has never been found.[13,14]

In this study, we determined the prevalence of EGFr cysteine-altering *NOTCH3* mutations in the large, publicly accessible exome sequencing dataset ExAC and performed comparative analyses with the over 200 *NOTCH3* mutations reported in both the Dutch and international CADASIL population. The genotype-phenotype association predicted by comparing ExAC and CADASIL mutation spectra was assessed in an independent cohort of Dutch CADASIL patients. We discuss the implications of our findings for the prevalence of CADASIL, the ever-broadening disease spectrum, and the discovery of a novel genotype-phenotype correlation.

MATERIALS AND METHODS

The Exome Aggregation Consortium dataset

NOTCH3 mutation frequencies were assessed using the browser of the Exome Aggregation Consortium (ExAC) (Lek *et al.*; http://dx.doi.org/10.1101/030338; http://exac.broadinstitute.org; accessed Oct/Nov 2015). This publicly accessible database contains exome data of 60,706 unrelated individuals; 33,644 males and 27,062 females. The exome data are compiled from 14 independent disease and control cohorts. No specific stroke or dementia cohorts are included in ExAC. Individuals in ExAC are assigned to one of six populations: European (n=36,677; Finnish n=3307, Non-Finnish n=33,370), Latino (n=5789), African/African American (n=5203), South Asian (n=8256), East Asian (n=4327) and Other (n=454). Data in ExAC are based on genome build GRCh37/hg19.

NOTCH3 variant ascertainment in ExAC

We queried ExAC for missense mutations in *NOTCH3* that passed all ExAC quality checks (Lek *et al.*; http://dx.doi.org/10.1101/030338). Next, these variants were filtered using stringent criteria for pathogenicity in CADASIL, namely missense mutations leading to the gain or the loss of a cysteine residue in one of the 34 EGFr domains of the NOTCH3 protein (amino acid residues 40-1373) (http://www.uniprot.org). Mean coverage of the *NOTCH3* canonical transcript in ExAC is 35.72x. Most of the EGFr encoding exons (exons 2-24) have a coverage of at least 10x, except for exon 15 and a part of exon 24, which have a coverage below 10x. Four independently accessible cohorts contributing to the ExAC database were also individually queried for pathogenic *NOTCH3* mutations: 1000 Genomes (http://www.1000genomes.org); NHLBI-GO Exome Sequencing Project (http://evs.gs.washington.edu); Sequencing Initiative Suomi (http://www.sisuproject.fi/) and Type 2 DiabetesGenetics 17k exome sequence analysis (http://www.type2diabetesgenetics.org).

Comparison of NOTCH3 mutations in ExAC to those reported in CADASIL patients

NOTCH3 mutations present in ExAC were compared to those reported in the Dutch CADASIL registry and the international CADASIL literature. To avoid bias through incomplete reporting of mutation frequencies or founder effects, the comparative analysis of the mutation distribution across the 34 EGFr domains was performed using distinct mutations rather than the prevalence of these mutations (i.e. each mutation was included only once in all analyses, independent of its total frequency in the CADASIL population). Next to a comparison with the mutation spectrum reported worldwide, we also separately compared the spectrum of ExAC mutations to those the Dutch registry alone because, in contrast to many

other countries, the *NOTCH3* mutation spectrum in the Dutch population is well characterized and complete, *NOTCH3* mutation screening in the Netherlands is comprehensive for exons 2-24, has been operational for more than 15 years and molecular diagnostics are readily accessible to all Dutch citizens. Moreover, all Dutch *NOTCH3* mutations are registered in a single national registry, which includes 383 individuals with 45 distinct mutations from 163 families. For mutation annotation, the reference sequence NM_000435.2 was used and sequence variants were described according to the HGVS nomenclature recommendations.

Genotype-phenotype analysis

The confirmation of an EGFr- location dependent genotype-phenotype correlation was performed in an independent CADASIL cohort, a detailed characterization of this cohort has been described elsewhere.[10] A standardized MRI protocol was performed at a field strength of 1.5T (Philips Medical System). Image acquisition and volumetric analysis of WMH, brain parenchyma, and lacunar infarcts were performed as previously described.[4] Briefly, WMHs were defined as white matter areas with increased signal intensity on T2-w MRI and FLAIR. WMH volume was calculated as a percentage of the brain parenchyma volume. Lacunar infarcts were defined as parenchymal defects not extending to the cortical grey matter, with a signal intensity corresponding to that of cerebrospinal fluid and having a diameter between 2 mm and 1.5 cm. Statistical analysis was performed using the SPSS statistical software package (version 23) (SPSS Inc., Chicago, USA). Comparison of quantitative brain MRI variables was performed using linear regression, co- varying for age, sex, smoking and hypertension.

NOTCH3 phenotypic spectrum in over 70 year olds

As ExAC genotypes cannot be traced back to the phenotypic data of individuals, we could not assess the correlation of genotype with (possible) CADASIL phenotype in ExAC. To determine whether *NOTCH3* mutations can be present in asymptomatic or paucisympomatic individuals and whether non-penetrance may occur, we queried our Dutch CADASIL registry for individuals of at least 70 years of age at the time of DNA-testing. We selected only those individuals with an available brain MRI or CT scan, determined the reason for *NOTCH3* mutation testing and reviewed their clinical records and brain scans.

RESULTS

High frequency of EGFr cysteine altering NOTCH3 mutations in ExAC

We found a total number of 642 *NOTCH3* missense variants in ExAC, of which 206 fit all the criteria for pathogenicity in CADASIL, namely mutations leading to

an uneven number of 5 or 7 cysteine amino acids in one of the 34 EGFr domains of the NOTCH3 protein (Table 1). The frequency of these mutations in ExAC is therefore 206 in 60,706 exomes, i.e. 3.4/1000. This is 100-fold higher than what would be expected based on the current CADASIL prevalence estimations of 2-5/100,000.[3,18,19] In the four independently accessible ExAC cohorts, we found the following frequencies of pathogenic *NOTCH3* mutations: Type 2 diabetes cohort (n=16,857): 3.9/1000; NHLBI-GO Exome Sequencing Project (n=6503): 1.2/1000; Sequencing Initiative Suomi (n=6118): 0.7/1000; 1000 Genomes (n=2504): 3.6/1000. This confirmation of high mutation frequencies in all four independently accessible cohorts shows that the high *NOTCH3* mutation frequency in ExAC is not attributable to a specific ExAC cohort.

Characteristics of NOTCH3 mutations in ExAC

The 206 EGFr cysteine altering missense mutations in ExAC are underlain by 25 distinct mutations (Table 1). Fourteen of the 25 distinct mutations are present in ExAC only once. The other mutations are recurrent, and are present between two and eight times, except for two mutations which have high frequencies of 32x and 120x. These two mutations are found across ethnicities, but are most frequent in the East Asian (p.Arg544Cys) and South Asian (p.Arg1231Cys) populations (Fig 1). The most frequent mutation (p.Arg1231Cys) is present in a homozygous state in three individuals, in a population reported to be high in consanguinity (Lek *et al.*; http://dx.doi.org/10.1101/030338).The mutations are located in 10 different *NOTCH3* EGFr encoding exons, encoding a total of 12 EGFr domains. Exons 11 and 22 harbor the most distinct mutations, namely six each, followed by exons 4 and 12 with three distinct mutations each. Overall, 17 of the 25 mutations lead to the gain of a cysteine residue in an EGFr domain and eight to the loss of a cysteine.

Comparison of NOTCH3 mutations in ExAC to those reported in CADASIL patients

We found pathogenic *NOTCH3* mutations in each of the populations included in ExAC (Fig 1). Mutation frequencies, however, differ between the populations and range from 0.4/1000 in African/African Americans to 11.7/1000 in South Asians. Some mutations are present in many different populations; the p.Arg578Cys mutation and the p.Arg1231Cys mutation, for example, are each found in four of the six ExAC populations. Mutations found in specific ethnic populations in ExAC have also been reported in CADASIL patients from the same ethnicity or geographical region. For example, the p.Arg544Cys mutation which is very frequent in East Asians in ExAC, has also been described frequently in CADASIL patients from Taiwan [22] and from Korea.[11] Likewise, the p.Arg1231Cys mutation, which is the most frequent mutation in Europeans in ExAC, has also been reported in three of the large European CADASIL cohorts.[12,13,15]

cDNA	Protein	Exon	EGFr	Frequency in ExAC	Mutation previously reported in CADASIL
c.350G>T	**p.Cys117Phe**	**4**	**2**	**1**	**yes** [31]
c.619C>T	**p.Arg207Cys**	**4**	**5**	**1**	**yes** [32]
c.635G>A	**p.Cys212Tyr**	**4**	**5**	**1**	**yes** [33]
c.931T>G	p.Cys311Gly	6	7	1	No
c.1045T>A	p.Cys349Ser	7	8	1	No
c.1630C>T	**p.Arg544Cys**	**11**	**14**	**32**	**yes** [32]
c.1672C>T	**p.Arg558Cys**	**11**	**14**	**5**	**yes** [12]
c.1732C>T	**p.Arg578Cys**	**11**	**14**	**5**	**yes** [12]
c.1759C>T	**p.Arg587Cys**	**11**	**15**	**2**	**yes** [34]
c.1819C>T	**p.Arg607Cys**	**11**	**15**	**1**	**yes** [35]
c.1823G>A	p.Cys608Tyr	11	15	1	No
c.1871G>C	p.Cys624Ser	12	16	1	No
c.1903C>T	p.Arg635Cys	12	16	1	No
c.1918C>T	**p.Arg640Cys**	**12**	**16**	**3**	**yes [LOVD]**
c.2149C>T	**p.Arg717Cys**	**14**	**18**	**3**	**yes [HGMD]**
c.2824G>T	p.Gly942Cys	18	24	1	No
c.2851C>T	p.Arg951Cys	18	24	1	No
c.3427C>T	**p.Arg1143Cys**	**21**	**29**	**2**	**yes [HGMD]**
c.3568C>T	p.Arg1190Cys	22	30	5	No
c.3601C>T	p.Arg1201Cys	22	30	7	No
c.3605G>C	p.Cys1202Ser	22	30	1	No
c.3628C>T	**p.Arg1210Cys**	**22**	**31**	**1**	**yes [Netherlands]**
c.3664T>G	**p.Cys1222Gly**	**22**	**31**	**8**	**yes**[3]
c.3691C>T†	**p.Arg1231Cys†**	**22**	**31**	**120**	**yes** [12]
c.3724C>T	p.Arg1242Cys	23	31	1	No
Total				*206*	

Table 1. Twenty-five distinct cysteine altering *NOTCH3* mutations in the ExAC database. Fourteen of the 25 mutations have been previously described in CADASIL patients. The others all fit the criteria for pathogenicity in CADASIL, namely leading to a cysteine amino acid change in one of the 34 EGFr domains of the NOTCH3 protein. Previously reported *NOTCH3* mutations in CADASIL patients are shown in bold. † Mutation reported three times in a homozygous state. LOVD= Leiden Open Variation Database (www.lovd.nl). HGMD= The Human Gene Mutation Database (http://www.hgmd.cf.ac.uk).

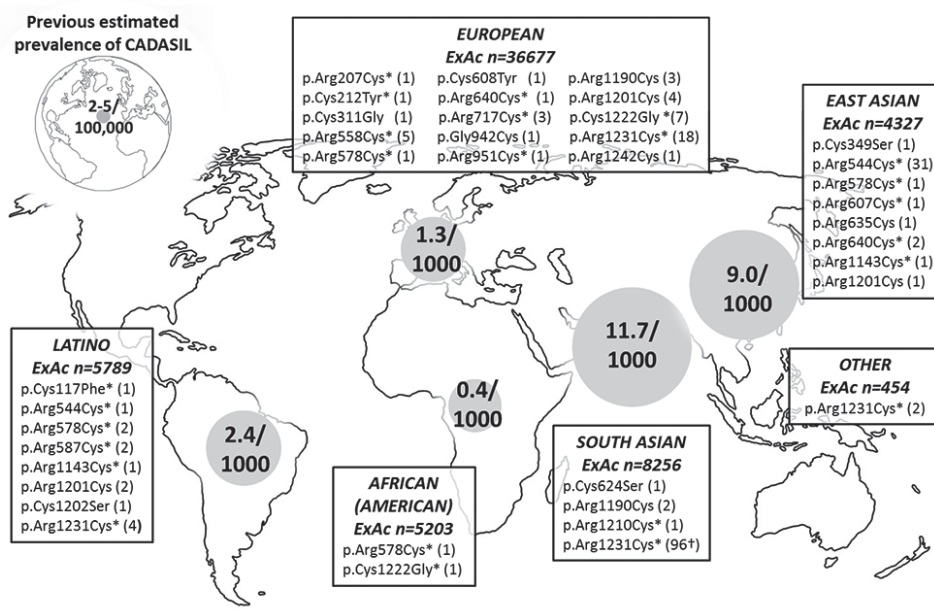

Figure 1. High frequency of cysteine altering *NOTCH3* mutations in all populations in the ExAC database. Frequencies range from 0.4/1000 individuals in the African/African American population to 11.7/1000 in the South Asian population, with an overall frequency of 3.4/1000.*: Mutation previously described in CADASIL patients; †: Mutation reported three times in a homozygous state; ( ): Allele counts

NOTCH3 mutations in ExAC are predominantly located outside of EGFr domains 1-6

Next, we compared the distribution of mutations in ExAC to those in the well characterized Dutch CADASIL registry and to those reported in CADASIL patients worldwide. In the Dutch CADASIL registry there are 45 distinct cysteine altering *NOTCH3* mutations, of which six have not been previously reported (Supplementary table 1). The distribution of Dutch CADASIL-causing *NOTCH3* mutations largely overlaps with those reported worldwide,[21] with the largest percentage of distinct mutations located in exon 4 (Fig 2). In ExAC, the percentage of distinct mutations in exon 4 is three times lower than in the Dutch CADASIL registry (12% versus 38%). Conversely, the percentage of distinct mutations in exon 22 is ten times higher in ExAC than in the Dutch CADASIL registry (24% versus 2%). There is a relatively high percentage of distinct mutations in exon 11 in both ExAC and the Dutch CADASIL registry (24% and 16%, respectively). Of note, although the distribution of mutations in ExAC differs from the distribution found in reported CADASIL patients, the majority of the exact same distinct EGFr

cysteine altering *NOTCH3* mutations in ExAC, namely 14 out of 25 (56%), have been previously reported in CADASIL patients (Table 1). Finally, we compared the mutation spectrum at the protein level using the distribution across the 34 NOTCH3 EGFr domains. This shows that mutations in ExAC are mostly clustered in EGFr domains 14-16 and 29-31, whereas reported CADASIL mutations cluster in EGFr domains 1-6.

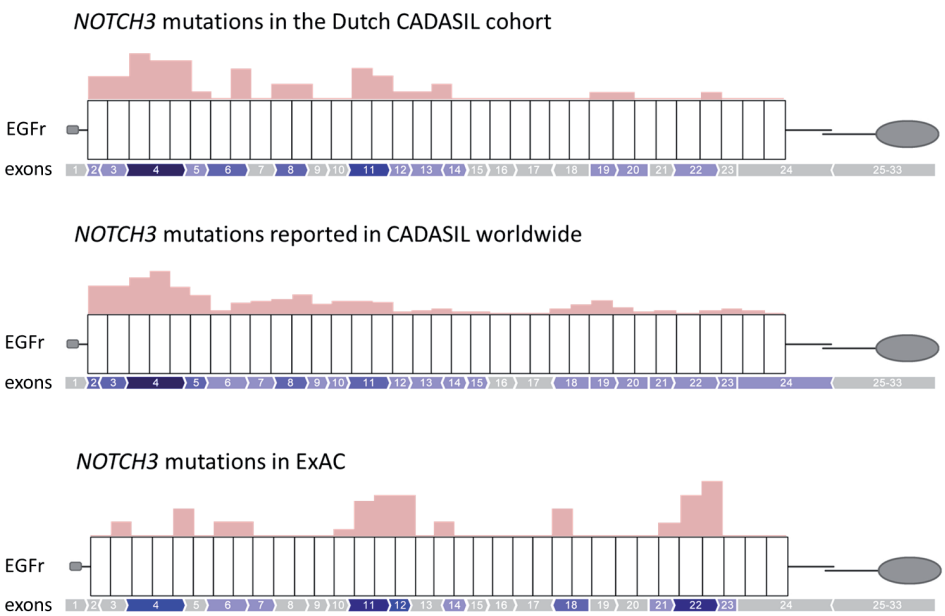

Figure 2. Distribution of distinct cysteine altering *NOTCH3* mutations in ExAC compared to those reported in CADASIL patients. Schematic representation of the NOTCH3 protein with 34 epidermal growth factor-like repeat domains (EGFr) and the NOTCH3 EGFr encoding exons (exon 2-24). Exons 25-33 (and part of exon 24) encode the transmembrane and intracellular domains of NOTCH3; these have never been found to harbour CADASIL-causing mutations. In the Dutch CADASIL registry and in CADASIL patients worldwide, most mutations are in exon 4, which largely accounts for the predominance of mutations in EGFr domains 1-6. In ExAC, this predominance is not seen, as mutations are most frequent in exons 11-12 and 22, encoding EGFr domains 14-16 and 29-31, respectively. Pink bars above the EGFr domains represent the distribution of mutations across EGFr domains, where the height of the pink bar reflects the percentage of mutations in the respective EGFr domain. Exon colours reflect the number of mutations in each exon: grey indicates exons without mutations; blue indicates exons with mutations. The darker the colour blue of the exon, the higher the number of distinct mutations.

NOTCH3 mutations outside of EGFr domains 1-6 are correlated with a lower MRI lesion load

The observed difference in mutation distribution between *NOTCH3* mutations in ExAC and reported CADASIL mutations led us to hypothesize that a genotype-phenotype correlation may play a role. Specifically, we hypothesized that mutations outside of EGFr domains 1-6, the classical CADASIL predominance region, predispose to a less severe phenotype. To test this hypothesis, we compared the WMH lesion load and number of lacunar infarcts between patients with a

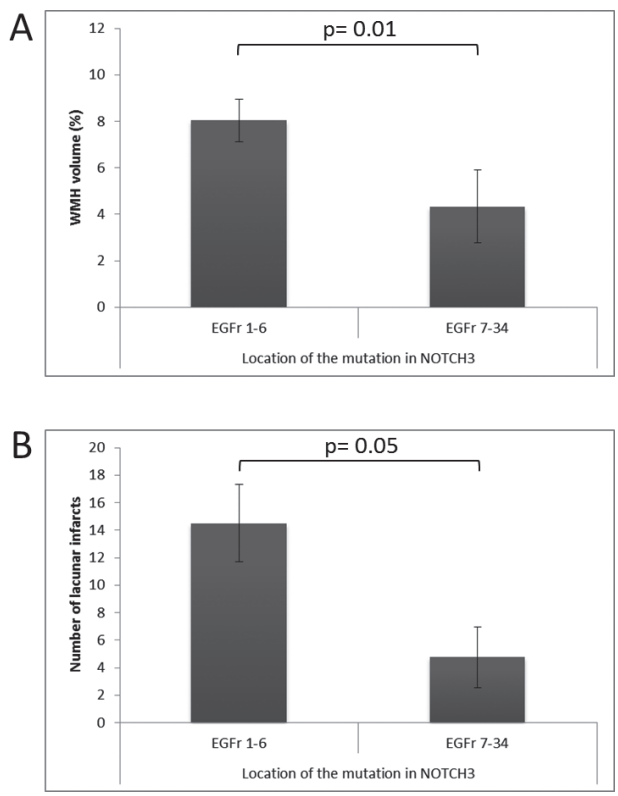

Figure 3. Correlation between EGFr location of the NOTCH3 mutation and MRI lesion load in CADASIL patients.

MRI quantification data from patients with a mutation in EGFr domains 1-6 (n=32) compared to patients with a mutation in EGFr domains 7-34 (n=8). (A) White matter hyperintensity volume (expressed as the % of total brain parenchymal volume), was significantly higher in patients with mutations in EGFr domains 1-6 than in patients with mutations in EGFr domains 7-34 (8.0% vs. 4.3%, p=0.01). (B) The number of lacunar infarcts showed a similar trend (14.5 vs. 4.8, p=0.05). Bars represent mean +/- SEM, corrected for age, sex, smoking and hypertension.

mutation in EGFr domains 1-6 to patients with a mutation in EGFr domains 7-34. We found that patients with mutations in EGFr domains 1-6 had a significantly higher WMH lesion load than patients with mutations in EGFr domains 7-34 (Fig 3A). A similar trend was seen for the number of lacunar infarcts, almost reaching statistical significance (Fig 3B). Although group size for this analysis is relatively small, these results suggest that mutations outside of NOTCH3 EGFr domains 1-6 are associated with a less severe brain MRI phenotype.

NOTCH3 mutations are penetrant on brain imaging in paucisymptomatic elderly individuals

To determine whether *NOTCH3* mutations can be associated with non-penetrance or an undiagnosed phenotype, possibly explaining the high ExAC mutation frequency, we selected individuals from the Dutch CADASIL registry who were 70 years or older at the time of DNA-testing and in whom a recent brain scan was available. We identified four such individuals. The reasons for DNA-testing were either predictive-testing because of the diagnosis of CADASIL in a family member, or because of a chance finding of WMHs on brain imaging, performed for unrelated signs or symptoms, such as hearing loss. The mutations in these patients were in exon 10 (p.Cys531Gly) and in exon 11 (p.Arg587Cys). None of them had a history of stroke. One patient reported mild complaints of memory loss, the other three had no cognitive complaints at all and no cognitive deficits were reported by their partners. All individuals lived at home and were fully independent for activities of daily living. One patient had a history of hypertension and hyperlipidemia, and one patient had a history of hypercholesterolemia. Their further clinical work-up and medical history was unremarkable. Extensive WMHs consistent with CADASIL were observed on brain imaging in all four patients. None of the patients had lacunes, which are typically seen up to 3 decades earlier in patients with classical CADASIL (Fig 4 A to C). The individuals who had an MRI scan, made at age 78 and 58 respectively, showed symmetric WMHs in both and microbleeds in one (Fig 4D to I). These individuals illustrate that *NOTCH3* mutations can be present in clinically asymptomatic or paucisymptomatic individuals over 70 years of age, who for this reason can easily remain undiagnosed with respect to CADASIL.

DISCUSSION

The frequency of EGFr cysteine altering *NOTCH3* mutations in the public exome database ExAC is 3.4/1000. This frequency is 100-fold higher than the current estimated prevalence of CADASIL. Although CADASIL is classically described as a mid-adult onset severe stroke and dementia syndrome, a later onset and milder disease course is increasingly recognised.[3] Our results suggest that this milder phenotype, which may

be indistinguishable from signs and symptoms of sporadic geriatric cerebral small vessel disease, is much more prevalent than recognised to date.

There are four possible explanations for the unexpected high frequency of *NOTCH3* mutations in ExAC, namely 1) the mutations in ExAC differ from those found in CADASIL and are not pathogenic; 2) all the individuals with a *NOTCH3* mutation in ExAC have an undiagnosed or unreported classical CADASIL; 3) some mutations have reduced penetrance in certain contexts, for example in certain

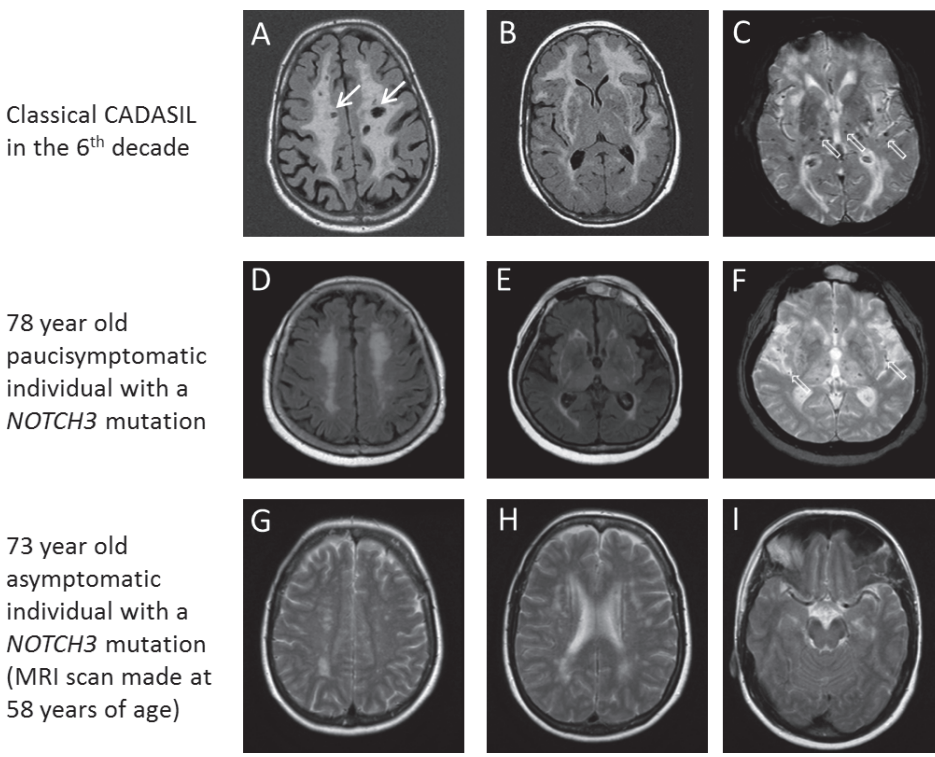

Figure 4. Brain MRI of elderly asymptomatic and paucisymptomatic individuals with a *NOTCH3* mutation compared to MRI in classical CADASIL. (A-C) Brain MRI images of a classical CADASIL phenotype in the 6th decade. (A-B) FLAIR images showing extensive confluent symmetric white matter hyperintensities and multiple lacunar infarcts (arrows indicate random samples). (C) T2*-weighted MRI showing multiple microbleeds (open arrows indicate random samples). (D-F) Brain MRI images of a female, diagnosed with CADASIL after predictive DNA testing at 78 years of age, with only very mild clinical symptoms. (D, E) FLAIR images showing symmetric white matter hyperintensities, but no lacunar infarcts. (F) T2- weighted MRI showing some small microbleeds (open arrows). (G-I) Dual echo images of a female who was still clinically asymptomatic at 73 years of age. MRI images were made at 58 years of age, showing very mild symmetric white matter hyperintensities, no lacunar infarcts and no microbleeds.

populations; 4) a late-onset, mild CADASIL phenotype which is less readily diagnosed, is much more prevalent than recognised to date.

The limitation of this study is that no phenotypic information is available for individuals in the ExAC database and we therefore cannot definitively confirm or reject any of these possible explanations. However, non- pathogenicity of any of the 25 EGFr cysteine altering *NOTCH3* mutations found in ExAC is highly unlikely, as more than half have been previously reported in CADASIL patients, all obey the criteria used by DNA-diagnostic laboratories to establish pathogenicity of a *NOTCH3* mutation and novel *NOTCH3* mutations in CADASIL are reported at a steady rate, with over 200 distinct EGFr cysteine altering missense mutations reported to date.[21]

As ExAC consists of various disease and control cohorts and does not contain any stroke or dementia cohorts, it is unlikely that the 206 individuals with a *NOTCH3* mutation in ExAC all have an undiagnosed classical mid-adult onset CADASIL phenotype. Non-penetrance in these individuals cannot be excluded but is also unlikely, as this has never been reported for this highly specific type of *NOTCH3* mutation and we did not find non-penetrance in asymptomatic or paucisymptomatic elderly individuals with a *NOTCH3* mutation in our Dutch registry. Therefore, in our opinion, the most likely explanation for the observed high mutation frequency in ExAC is a much higher prevalence than recognized to date of a very mild, late-onset *NOTCH3* mutation associated cerebral small vessel disease, which may remain undiagnosed into old age.[23]

Our finding that the EGFr distribution of mutations in ExAC differs from that found in diagnosed CADASIL patients, suggested a possible genotype-phenotype correlation. We hypothesized that mutations located in EGFr domains 1-6 predispose to 'classical' CADASIL and, conversely, mutations outside of EGFr domains 1-6 predispose to a much milder phenotype. This would also explain why most CADASIL patients diagnosed to date have a mutation in exon 4, as these have a more severe phenotype and are therefore much more readily diagnosed. As we could not confirm this EGFr- dependent genotype- phenotype correlation in the ExAC cohort, we confirmed our hypothesis in a well-characterized independent Dutch CADASIL cohort.[7] In this cohort, we found that patients with a mutation in EGFr domains 1-6 have a significantly higher MRI lesion load than those with mutations in EGFr domains 7-34. Interestingly, the paucisymptomatic elderly individuals in our Dutch cohort all had a mutation outside of EGFr domains 1-6, which is also the case in other mildly affected CADASIL patients reported in the literature.[23,24]

We can only speculate as to why mutations in certain EGFr domains may have more detrimental effects than others. Possibly, an unpaired cysteine in EGFr

domains 1-6, at the N-terminus of the NOTCH3 extracellular domain, may more readily interact with other proteins, leading to a stronger effect on CADASIL-associated protein multimerization.[25,26] Although this novel genotype-phenotype correlation may help predict a predisposition to a milder or a more severe CADASIL phenotype, this needs further delineating in larger cohorts before it can be used in the clinical setting. Also, clearly, factors other than *NOTCH3* genotype play an important role in CADASIL. For example, smoking and hypertension have been shown to be associated with CADASIL disease severity.[13]

A worldwide prevalence of pathogenic *NOTCH3* mutations of 3.4/1000 individuals, implies that these mutations may be a major contributor to cerebral small vessel disease in the general population. Indeed, a recent study in patients with adult-onset leukoencephalopathy showed that 21% of these individuals had a pathogenic *NOTCH3* mutation.[27] Similarly, high *NOTCH3* mutation frequencies (18%) were found in a Korean cohort of patients with subcortical vascular cognitive impairment.[28] Conversely, in a cohort of individuals with confluent white matter lesions or lacunes, no cysteine altering *NOTCH3* mutation was found.[29] Future studies in large, well-characterized cohorts are needed to determine the role of cysteine altering *NOTCH3* mutations in the development of white matter hyperintensities and vascular cognitive impairment in the general population.

Our study illustrates how large whole exome datasets can alter mutation-disease paradigms. This was recently also illustrated by a study reporting an unexpected high frequency of prion disease- associated *PRNP* mutations in ExAC.[30] Although our finding of high *NOTCH3* mutation frequencies shows some similarities to this study, there are also fundamental differences between *NOTCH3* mutations in CADASIL and *PRNP* mutations in prion disease. The most important difference is the fact that many different types of *PRNP* variants have been described, with variable levels of evidence for pathogenicity. This is in sharp contrast to the highly stereotyped nature of pathogenic *NOTCH3* mutations in CADASIL.

In conclusion, we show that the frequency of EGFr cysteine altering *NOTCH3* mutations in a large exome dataset is 3.4/1000 and we discovered the first genotype-phenotype correlation in CADASIL, which is related to the distribution of mutations across NOTCH3 EGFr domains. These findings fundamentally change the CADASIL disease paradigm and cause us to re-define and the phenotypic spectrum associated with EGFr cysteine altering *NOTCH3* mutations.

No.	Exon	Nucleotide change	Amino Acid change	Number of patients	Number of families
1	2	c.160C>T	p.Arg54Cys	5	2
2	2	c.179C>G	p.Ser60Cys	1	1
3	3	c.227G>A*	p.Cys76Tyr*	2	1
4	3	c.328C>T	p.Arg110Cys	11	5
5	4	c.350G>A	p.Cys117Tyr	2	1
6	4	c.353C>G	p.Ser118Cys	1	1
7	4	c.397C>T	p.Arg133Cys	12	4
8	4	c.402C>G	p.Cys134Trp	1	1
9	**4**	**c.421C>T**	**p.Arg141Cys**	**42**	**17**
10	4	c.431G>T	p.Cys144Phe	2	1
11	**4**	**c.457C>T**	**p.Arg153Cys**	**35**	**8**
12	4	c.464G>A	p.Cys155Tyr	1	1
13	4	c.486C>G	p.Cys162Trp	18	3
14	4	c.505C>T	p.Arg169Cys	3	2
15	**4**	**c.544C>T**	**p.Arg182Cys**	**32**	**10**
16	4	c.548G>A*	p.Cys183Tyr*	2	1
17	4	c.566A>G	p.Tyr189Cys	2	1
18	**4**	**c.619C>T**	**p.Arg207Cys**	**39**	**20**
19	4	c.634T>A	p.Cys212Ser	9	3
20	4	c.665G>A	p.Cys222Tyr	6	2
21	4	c.671G>A	p.Cys224Tyr	3	1
22	5	c.699T>G	p.Cys233Trp	2	2
23	5	c.773A>G	p.Tyr258Cys	1	1
24	6	c.953G>T*	p.Cys318Phe*	3	2
25	6	c.994C>T	p.Arg332Cys	4	1
26	6	c.1010A>G	p.Tyr337Cys	2	2
27	6	c.1019G>T*	p.Cys340Phe*	1	1
28	8	c.1241C>G	p.Ser414Cys	4	1
29	8	c.1261C>T	p.Arg421Cys	1	1
30	8	c.1337G>T	p.Cys446Phe	2	1
31	8	c.1345C>T	p.Arg449Cys	2	1
32	11	c.1630C>T	p.Arg544Cys	8	3
33	11	c.1645T>C	p.Cys549Arg	2	1
34	11	c.1672C>T	p.Arg558Cys	5	4
35	**11**	**c.1732C>T†**	**p.Arg578Cys†**	**62**	**27**
36	11	c.1759C>T	p.Arg587Cys	3	2
37	11	c.1790G>A*	p.Cys597Tyr*	2	1

No.	Exon	Nucleotide change	Amino Acid change	Number of patients	Number of families
38	11	c.1819C>T	p.Arg607Cys	5	5
39	12	c.1918C>T	p.Arg640Cys	2	1
40	13	c.1999G>T	p.Gly667Cys	5	4
41	13	c.2129A>G	p.Tyr710Cys	2	1
42	14	c.2182C>T	p.Arg728Cys	2	2
43	**19**	**c.3043T>C**	**p.Cys1015Arg**	**30**	**10**
44	20	c.3226C>T	p.Arg1076Cys	3	2
45	22	c.3628C>T*	p.Arg1210Cys*	1	1

Supplementary table 1. Pathogenic *NOTCH3* mutations in the Netherlands. Between 1998 and 2014, 45 distinct mutations have been detected in the Netherlands. Six mutations are especially prevalent (shown in bold), these include both (Dutch) founder mutations and recurrent mutations (Unpublished). *mutations which have not been previously reported, † mutation reported two times in a homozygous state.

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7

DISCUSSION AND FUTURE PERSPECTIVES

The aim of this thesis was to work towards pre-clinical proof-of-concept for NOTCH3 cysteine corrective exon skipping as a rational therapeutic approach for CADASIL. To address all aspects required for therapeutic development, the work performed for this thesis included not only *in vitro* testing of *NOTCH3* exon skipping in CADASIL patient derived vascular smooth muscle cells and studies into the function of the cysteine corrected proteins, but also the generation of a relevant humanized *in vivo* model, pre-clinical biomarker development, and studies defining prevalence, spectrum and characteristics of *NOTCH3* mutations worldwide.

There is a high unmet medical need in CADASIL. Currently, only palliative care and symptomatic treatment can be offered,[1] and there are no effective therapies that can delay the onset or progression of physical and cognitive dysfunction. The window of opportunity for treatment probably spans mainly the pre-symptomatic and early symptomatic stages of the disease, when no or only minimal brain white matter hyperintensities are present, and presumably no or only little irreversible damage has occurred in the vessel wall or to the brain tissue. The fact that asymptomatic family members of a CADASIL patient can have predictive molecular genetic testing, in young adults sometimes decades prior to the onset of clinical symptoms, in principle permits therapeutic intervention before irreversible damage has been done. The therapeutic approach described in this thesis is an antisense oligonucleotide- based strategy. However, many of the steps needed to bring this approach to clinical trial readiness also apply to other potential therapeutic approaches. Developing a more comprehensive framework for CADASIL therapeutic development is thus of wider benefit.

Cysteine correction of NOTCH3 is a therapeutic approach which directly targets the underlying defect in CADASIL: and uneven number of cysteines within EGFr. We have found that correcting the uneven number of cysteines using antisense-mediated exon skipping is feasible *in vitro* and does not abrogate normal protein processing and functionality. The crucial question that remains to be addressed is whether the modified NOTCH3 proteins formed after cysteine corrective exon skipping indeed have a reduced multimerization tendency and reduced aggregation properties. To address this question, we have generated stable cell lines with an inducible NOTCH3 skip protein expression. These cell lines can be used to measure NOTCH3 protein solubility and degradation as a read-out for protein aggregation.[2-4] An important limitation of such cell models, however, is protein overexpression. NOTCH3 overexpression is inherently associated with intracellular NOTCH3 protein aggregation, which may confound a reliable assessment of CADASIL- associated NOTCH3 protein aggregation. We are currently investigating an alternative *in vitro* model mimicking cysteine correction in (primary) cells with endogenous

NOTCH3 expression, using CRISPR-Cas9 genome editing technology.[5] Although potentially a more representative model, this approach is challenging as primary cells have a limited life-span and genome editing is not always efficient. Finally, we are collaborating with the LUMC mass-spectrometry group to apply advanced mass-spectrometry techniques in order to assess disulphide bridge formation in cysteine corrected NOTCH3 protein fragments.[6]

Proof of concept for the hypothesis that NOTCH3 cysteine correction reduces vascular toxic NOTCH3 aggregation will have to come from *in vivo* studies. Studies towards this *in vivo* proof of concept are now enabled by the human *NOTCH3* transgenic mouse model we generated (Chapter 5). The NOTCH3 score which we developed in this model can be used to determine the effect of a therapeutic intervention on NOTCH3 accumulation, as a marker for therapeutic efficacy. More detailed characterization of the *NOTCH3* transgenic mice is ongoing, including assessment of vascular smooth muscle cell degeneration and cerebrovascular reactivity. So far, the human *NOTCH3* transgenic mice have not shown any obvious signs of stroke or cognitive impairment. Studies formally assessing cognition and motor function are on-going, but we expect that, analogous to most other CADASIL mouse models, these mice will not develop measurable motor deficits or cognitive dysfunction. A different genetic background or additional aggravating factors, such as hypertension or nicotine exposure,[7, 8] may be needed to provoke a clinical phenotype in the mice. The role of hypotension in CADASIL is not well studied, but could theoretically provoke the occurrence of lacunar infarcts in watershed areas of the brain. However, the short life span and the anatomy of the mouse brain may be the most important limiting factors in recapitulating the full CADASIL phenotypic spectrum in mice. This is an important limitation, as stroke or cognition can therefore not be used as therapeutic read-outs. Given this limitation and considering the window of opportunity for pre-symptomatic intervention in CADASIL, it may be more important to focus on translational markers for disease progression in the pre- and early symptomatic stages of the disease. If these markers prove to correlate with disease severity and progression in humans, they can then be used as pre-clinical and clinical surrogate markers. The NOTCH3 score we developed in the mice is such a potential translatable biomarker, as it progresses as the mouse ages and this same NOTCH3 accumulation can also be detected in skin arterioles of CADASIL patients, decades before the onset of clinical symptoms.[9, 10] We are currently investigating whether the NOTCH3 score can be reliably measured in CADASIL skin biopsies and whether it correlates with disease severity and progression.

Although cysteine corrective exon skipping is an elegant strategy to bypass the pathogenic unpaired cysteine, there are some important limitations. Firstly,

it still remains to be determined whether this approach indeed reduces NOTCH3 aggregation *in vivo*. Also, the cysteine corrective exon skips we optimized require a combination of AONs for effective exon skipping and skipping efficiencies are not always high enough to ensure therapeutic efficacy. An alternative treatment strategy, which we are testing in parallel, is NOTCH3 downregulation. Our study on individuals with a *NOTCH3* stop mutation (Chapter 3) provides a solid rationale for this approach and has the added advantage that a single effective RNAse H inducing AON could be used for all CADASIL patients, irrespective of their mutation. The underlying concept is that a reduced expression of total NOTCH3 will also lower the amount of mutant NOTCH3, thereby theoretically decreasing NOTCH3 protein aggregation. However, a potential pitfall of this approach is that adult exposure to pharmacologically induced reduction of NOTCH3 is not comparable to a congenitally reduced NOTCH3 expression due to a stop mutation, with potential compensatory mechanisms. Moreover, a complete absence of NOTCH3, due to a homozygous stop mutation, does cause a severe cerebrovascular phenotype in humans,[11] and it is not well known how much NOTCH3 is required for normal vessel wall function in adults. Finally, it should be noted that the exact same stop mutation we identified in healthy individuals, was later found in a family with cerebral small vessel disease.[12] Therefore, although most studies indicate that there is some flexibility in the amount of NOTCH3 needed for normal functioning, the exact effects of loss of NOTCH3 function, both in the context of CADASIL and in the context of blood vessel development and maintenance, requires further study.

The major hurdle faced in obtaining *in vivo* proof of concept for NOTCH3 cysteine correction or NOTCH3 downregulation, is to attain sufficient levels of exon skipping or downregulation in the target cells, namely the vascular smooth muscle cells in the small-to medium sized arteries of the brain. Experiments are on-going in our lab to determine which route is most optimal for *NOTCH3* AON delivery into the cerebrovasculature, via the systemic circulation, or via the cerebrospinal fluid. Systemic administration of AONs, through intravenous, intraperitoneal of subcutaneous injection, is technically most straightforward. However, systemic administration causes the vast majority of AONs to be taken up by the liver and kidneys.[13] While the blood brain barrier precludes most AON chemistries from entering the brain, the cerebral vascular smooth muscle cells should in principle be accessible, but little is known about vessel wall delivery.[14] Administration to the cerebrospinal fluid, via intraventricular injection in mice or intrathecal injection in humans, is a more invasive administration route, but has the advantage that high concentrations of AONs in cerebrospinal fluid are easily achieved and half-life of AONs in cerebrospinal fluid is longer than in serum.[15] Next to delivery route, other ways to increase AON efficacy are improved AON chemistries, which are continually

being developed. For example, AONs with a 2'-O-methoxy-ethyl chemistry may be better tolerated in the cerebrospinal fluid than 2OMePS AONs, and a recent study demonstrated uptake of tricyclo AONs in the brain after systemic administration.[16]

Another challenge in CADASIL therapeutic development is the lack of feasible clinical read-outs. Clinical read-out development is hampered by a pronounced clinical variability, likely attributable to genetic and environmental modifiers. Despite studies in large groups of patients, feasible clinical read-outs or biomarkers have not yet been identified. Our finding of an unexpected high population frequency of *NOTCH3* mutations (Chapter 6) may have far-reaching implications for our understanding of CADASIL disease variability, as it shows that we have currently only identified a small subset of (severe) CADASIL patients. Probably most individuals with a *NOTCH3* mutation who have not yet been diagnosed, have a milder disease course than the classical mid-adult onset CADASIL. Such a milder disease course has been described[18, 19] and is increasingly encountered in our CADASIL outpatient clinic. A strong genotype-phenotype correlation would enable classification of *NOTCH3* mutations into predisposing to severe disease or predisposing to mild disease. In this way we might be able to specifically enrol only patients predisposed to severe disease into biomarker studies, which should be facilitated by the more narrow severe disease spectrum and reduced disease variability.

In the not so distant future, it is likely that every individual will have his or her exome or genome sequenced. For *NOTCH3* specifically, routine exome or genome sequencing will expose the large number of individuals with a cysteine altering *NOTCH3* mutation who now remain undiagnosed. Identification of these individuals will be important in our further understanding of the phenotypic spectrum associated with *NOTCH3* mutations and the identification of other genetic and environmental factors which mitigate disease progression, some of which may lead to strategic interventions.

KEY MESSAGES OF THIS THESIS

- Only cysteine altering *NOTCH3* mutations are indisputably causative of CADASIL, other variants should be considered polymorphisms until proven otherwise. Classification of non-cysteine altering *NOTCH3* mutations can only be attained in the context of brain MRI, clinical and family history and skin biopsy including NOTCH3 immunohistochemistry and electron microscopy analysis. (Chapter 2)
- *NOTCH3* loss of function mutations do not cause CADASIL and there is flexibility in the amount of NOTCH3 needed for normal health and development. (Chapter 3)
- Cysteine correction of NOTCH3 can be achieved via antisense- mediated exon skipping in CADASIL patient derived VSMCs. A cysteine corrected NOTCH3 protein lacks the corruptive mutated EGFr domain, whilst retaining functionality. (Chapter 4)
- Human genomic *NOTCH3* transgenic mice recapitulate the CADASIL vascular phenotype, and are a good model for studying therapies that target human NOTCH3 at the genomic, (pre-)mRNA or protein level. (Chapter 5)
- The NOTCH3 score is a quantitative measure for progressive NOTCH3 accumulation in mice, and can potentially be translated to humans. (Chapter 5)
- EGFr cysteine altering *NOTCH3* mutations have a worldwide frequency of 3.4/1000. The phenotypic spectrum associated with these *NOTCH3* mutations is probably much broader and milder than currently recognized. (Chapter 6)
- Mutations in EGFr domains 1-6 predispose to a more severe disease course, whilst mutations outside of these domains predispose to a milder disease course. This genotype-phenotype correlation may partially explain CADASIL disease variability. (Chapter 6)

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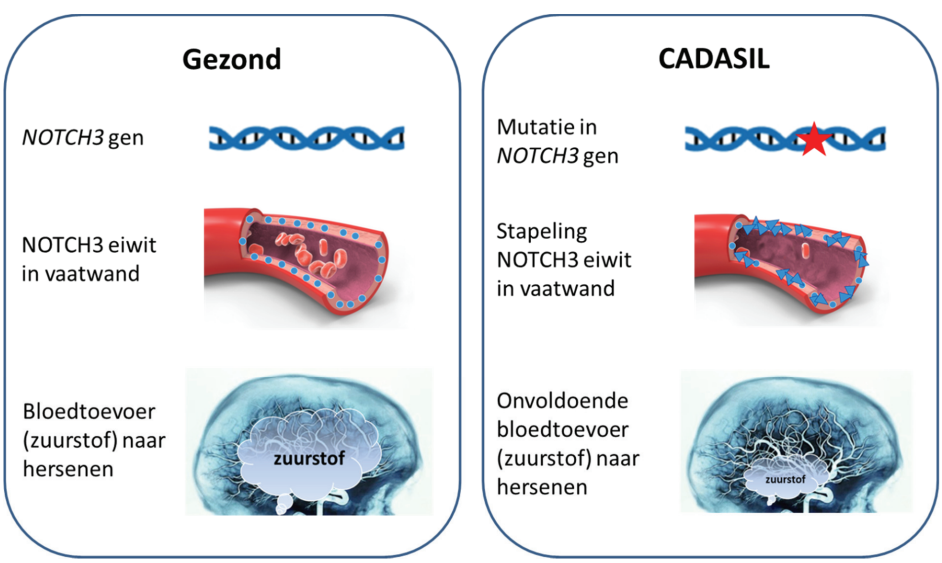
NEDERLANDSE SAMENVATTING

CADASIL is een erfelijke ziekte van de kleine hersenvaten, die leidt tot beroertes en cognitieve achteruitgang vanaf een gemiddelde leeftijd van 50 jaar. De ziekte erft autosomaal dominant over: dit betekent dat elk kind van een ouder met CADASIL 50% kans heeft om de ziekte te erven. Er is op dit moment geen medicijn dat het ziekteproces bij CADASIL kan vertragen. Het overkoepelende doel van de studies beschreven in dit proefschrift is het zetten van de eerste stappen richting een therapie voor CADASIL patiënten.

Hoofdstuk 1 bevat een algemene introductie over de ziekte CADASIL en beschrijft verschillende aspecten die van belang zijn bij het ontwikkelen van een therapie. De oorzaak van CADASIL is een verandering (mutatie) in een specifiek deel van de erfelijke code: het *NOTCH3* gen. Dit gen bevat de code voor het NOTCH3 eiwit, wat van belang is voor een goede aanleg en functie van bloedvaten. CADASIL patiënten hebben een mutatie in het *NOTCH3* gen, waardoor het NOTCH3 eiwit niet goed vouwt en gaat stapelen in de vaatwand. Dit zorgt voor een verstoorde bloedtoevoer naar de hersenen, met beroertes en dementie tot gevolg (Figuur 1).

In het LUMC wordt gewerkt aan een mogelijke therapie voor CADASIL, genaamd 'cysteine correctie'. Het doel van cysteine correctie is de verkeerde vouwing van het NOTCH3 eiwit te voorkomen, om zo de schadelijke eiwitstapeling in de hersenvaten tegen te gaan en het ontstaan van beroertes en dementie uit te stellen.



Figuur 1. De oorzaak van CADASIL is een mutatie in het *NOTCH3 gen*. Deze mutatie zorgt voor verkeerde vouwing en stapeling van het NOTCH3 eiwit in de vaatwand. Dit leidt tot een verstoorde bloedtoevoer naar de hersenen, waardoor de hersenen te weinig zuurstof krijgen.

Om te kunnen onderzoeken of cysteine correctie in de toekomst als therapie voor CADASIL zou kunnen worden gebruikt, moeten velen stappen worden gezet in het wetenschappelijk onderzoek. Zo moet er voldoende kennis zijn over de mutaties die de ziekte veroorzaken en hoe deze mutaties tot de ziekte leiden. Daarnaast moet cysteine correctie uitgebreid getest worden in cel- en muismodellen, en zijn uitleesmaten nodig om de effectiviteit van het medicijn te kunnen meten. Tot slot moet de variabiliteit in het natuurlijk beloop van de ziekte bekend zijn, alsmede welke factoren van invloed zijn op het ziektebeloop. De hoofdstukken van dit proefschrift gaan over deze verschillende aspecten die van belang zijn voor het begrijpen van de ziekte en de ontwikkeling van een therapie.

Hoofdstuk 2 beschrijft welke onderzoeken belangrijk zijn voor het stellen van de diagnose CADASIL. Hierbij ligt de nadruk op de genetische diagnose: welk type mutaties in *NOTCH3* veroorzaakt CADASIL, en welke veranderingen in *NOTCH3* leiden niet tot ziekte. Er zijn inmiddels meer dan 200 *NOTCH3* mutaties bekend die CADASIL kunnen veroorzaken. Al deze mutaties hebben één belangrijke overeenkomst: ze zorgen voor een cysteine aminozuurverandering in het NOTCH3 eiwit. Elk eiwit in het lichaam is opgebouwd uit aminozuren. Cysteine is één van deze aminozuren, en is belangrijk voor een normale vouwing van het eiwit. Wanneer het aantal cysteines in het NOTCH3 eiwit verandert, veroorzaakt dit een verkeerde vouwing en schadelijke stapeling van het eiwit in de vaatwand.

Het vinden van een cysteine- veranderende mutatie in het *NOTCH3* gen bij een patiënt bevestigt de diagnose CADASIL. Er zijn in de literatuur ook een aantal patiënten beschreven met een mutatie in het *NOTCH3* gen die niet tot een cysteine-aminozuurverandering leidt. Deze niet-cysteine veranderende mutaties worden echter ook vaak gevonden in gezonde personen. Er is discussie over de vraag of deze niet- cysteine veranderende mutaties ook tot CADASIL kunnen leiden.

Wanneer een niet-cysteine veranderende mutatie wordt gevonden in een patiënt, is er aanvullend onderzoek nodig om te bepalen of er wel of niet sprake is van CADASIL. De medische voorgeschiedenis van de patiënt moet goed in kaart gebracht worden, alsmede de familiegeschiedenis. Een her-beoordeling van de MRI scan door een in CADASIL gespecialiseerd radioloog en het afnemen van een huidbiopt geven vaak belangrijke informatie. In het huidbiopt wordt gekeken of er sprake is van NOTCH3 eiwitstapeling. Wanneer de uitkomsten van al deze onderzoeken passen bij CADASIL, dan moet met aanvullende technieken gekeken worden of er kleine stukken van het *NOTCH3* gen ontbreken (deletie) of juist dubbel aanwezig zijn (duplicatie). In zeldzame gevallen kunnen dergelijke deleties en duplicaties namelijk ook CADASIL veroorzaken. De conclusie van hoofdstuk 2 is dat er tot op heden er geen overtuigend bewijs is dat niet-cysteine veranderende mutaties CADASIL

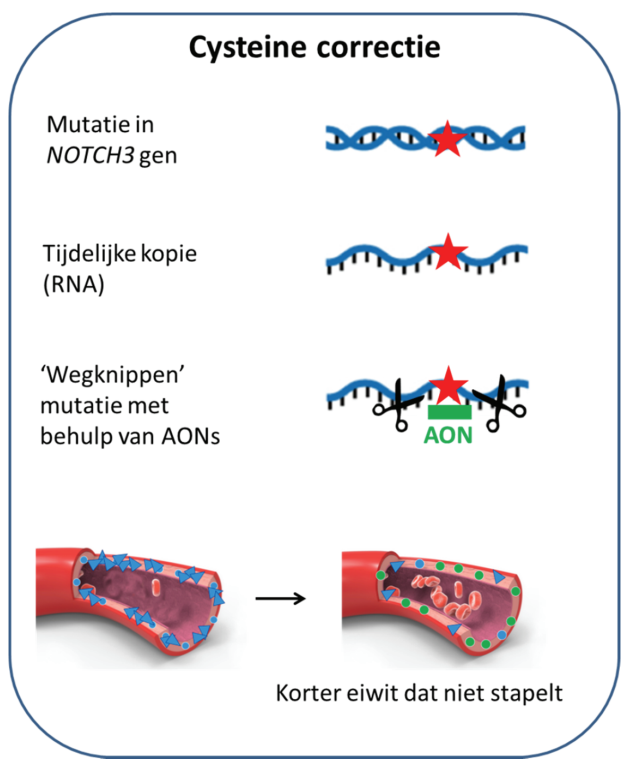
kunnen veroorzaken. Wanneer een dergelijke mutatie wordt gevonden, is er altijd aanvullend onderzoek nodig voordat de diagnose kan worden gesteld.

Hoofdstuk 3 beschrijft de identificatie van een aantal personen met een speciaal type *NOTCH3* mutatie, namelijk een zogenoemde hypomorfe mutatie. Deze mutaties leiden niet tot een cysteine aminozuurverandering, maar zorgen ervoor dat er minder NOTCH3 eiwit wordt aangemaakt. Hoofdstuk 3 toont aan dat dergelijke hypomorfe mutaties geen CADASIL veroorzaken, en ook niet tot duidelijke andere symptomen leiden. Deze bevinding is van belang voor het correct interpreteren van *NOTCH3* mutaties, en geeft daarnaast belangrijke inzichten in het ontstaan van CADASIL en mogelijkheden voor therapieën. De personen met een hypomorfe *NOTCH3* mutatie zijn gezond, hetgeen suggereert dat een lagere hoeveelheid NOTCH3 eiwit al voldoende is voor normaal functioneren. Een mogelijk aangrijpingspunt voor therapie, naast cysteine correctie, zou daarom NOTCH3 downregulatie kunnen zijn. Hierbij wordt de totale productie van het NOTCH3 eiwit verminderd, waardoor in theorie de schadelijke NOTCH3 eiwitstapeling ook minder zal zijn.

In hoofdstuk 4 wordt de rationale achter de genetische interventie cysteine correctie beschreven en wordt getest of cysteine correctie werkt in cellen van CADASIL patiënten. Het idee voor cysteine correctie is voortgekomen uit het gegeven dat *NOTCH3* mutaties bij CADASIL patienten altijd leiden tot een verandering in het aantal cysteines in het NOTCH3 eiwit. Met cysteine correctie wordt geprobeerd dit aantal cysteines te herstellen, om zo de schadelijke NOTCH3 eiwitstapeling tegen te gaan. Cysteine correctie kan worden bereikt met behulp van antisense oligonucleotiden (AONs). AONs binden aan het RNA, dit is een tijdelijke kopie van het DNA die nodig is om eiwitten te kunnen produceren. Door aan het RNA te binden kunnen de AONs ervoor zorgen dat de mutatie uit het RNA geknipt wordt (Figuur 2). Hierdoor wordt een korter NOTCH3 eiwit aangemaakt, met het correcte aantal cysteines. Software programma's die de vouwing van het NOTCH3 eiwit kunnen voorspellen laten zien dat dit kortere eiwit een normale vouwing zou moeten hebben. In theorie zou dit herstelde NOTCH3 eiwit dus niet gaan stapelen in de vaatwand. De experimenten beschreven in hoofdstuk 4 tonen aan dat NOTCH3 cysteine correctie technisch mogelijk is in cellen van CADASIL patiënten. Daarnaast wordt aangetoond dat het herstelde NOTCH3 eiwit dat gevormd wordt zijn normale functie in de cellen behoudt.

Om cysteine correctie verder te kunnen ontwikkelen is een relevant muismodel nodig. Het genereren en karakteriseren van een dergelijk muismodel is beschreven in hoofdstuk 5. Dit CADASIL muismodel is gemaakt door het menselijke *NOTCH3* gen in een bevruchte eicel van een muis te injecteren. Hierdoor wordt het menselijke gen in de erfelijke code van de muis ingebouwd, en maakt de muis niet alleen

het muizen-Notch3 eiwit aan, maar ook het menselijke NOTCH3 eiwit. Omdat het menselijke *NOTCH3* gen permanent is ingebouwd in de erfelijke code van de muis, wordt het ook doorgegeven aan de volgende generatie: hierbij spreken we van een muizenlijn. Met deze techniek zijn in totaal 5 verschillende muizenlijnen gemaakt: 1 lijn die een normaal menselijk *NOTCH3* gen bevat en 4 lijnen die het menselijke *NOTCH3* gen met een mutatie bevatten. De muizen die het *NOTCH3* gen met een mutatie hebben krijgen dezelfde NOTCH3 eiwitstapeling als ook bij CADASIL patiënten wordt gezien. De NOTCH3 stapeling is hoger naarmate er meer mutant NOTCH3 eiwit wordt aangemaakt, en neemt duidelijk toe met de leeftijd. Om de NOTCH3 stapeling te kunnen kwantificeren is de 'NOTCH3 score' ontwikkeld: een maat voor de hoeveelheid NOTCH3 stapeling in de hersenvaten. Omdat het nieuwe CADASIL muismodel het menselijke *NOTCH3* gen bevat en al op jonge leeftijd NOTCH3 eiwitstapeling laat zien, is het een goed model voor het testen van mogelijke therapieën voor CADASIL, zoals bijvoorbeeld cysteine correctie. Hierbij kan NOTCH3 score kan worden gebruikt als een uitleesmaat om het effect van de therapie op NOTCH3 eiwitstapeling te meten.



Figuur 2. Cysteine correctie met behulp van AONs.

De tot op heden geschatte prevalentie van CADASIL is 2-5 per 100.000 personen. Echter, de ziekte wordt vaak niet herkend door artsen, hetgeen leidt tot onder-diagnostiek. CADASIL komt dus waarschijnlijk veel vaker voor. **Hoofdstuk 6** laat zien dat de frequentie van CADASIL- veroorzakende mutaties 100-maal hoger ligt dan de tot nu toe geschatte prevalentie van de ziekte. Deze bevinding toont aan dat er waarschijnlijk veel meer CADASIL patiënten zijn dan gedacht, maar ook dat veel van deze patiënten waarschijnlijk een relatief mild beeld hebben. De meeste CADASIL patiënten die tot nu toe zijn gediagnostiseerd krijgen een eerste beroerte rond de leeftijd van 50 jaar. De leeftijd waarop de eerste symptomen zich manifesteren varieert echter sterk. Hoofdstuk 6 laat zien dat er CADASIL patiënten zijn die tot de leeftijd van 70 jaar nauwelijks klachten hebben. Daarnaast toont hoofdstuk 6 aan dat de plek van de mutatie in het *NOTCH3* gen waarschijnlijk één van de factoren is die een rol speelt bij de variatie in de ernst van de ziekte.

Hoofdstuk 7 bevat een samenvatting van de verschillende studies beschreven in dit proefschrift, en bespreekt welk vervolgonderzoek nodig is om cysteine correctie verder te kunnen ontwikkelen als mogelijke toekomstige therapie voor CADASIL patiënten. Een belangrijke volgende stap die moet worden gezet is het aantonen dat cysteine correctie inderdaad NOTCH3 stapeling vermindert. Dit kan deels worden onderzocht in speciale celmodellen die momenteel worden gemaakt, maar zal uiteindelijk moeten worden bepaald door experimenten in muizen. Hiervoor is het van belang dat er voldoende AON terecht komt op de juiste plek: de gladde spiercellen in de hersenvaten. Vervolgonderzoek zal moeten aantonen wat de beste toedieningsmethode is om voldoende AON in de hersenvaten te krijgen. Een andere belangrijke stap is het verder ontwikkelen van uitleesmaten. De NOTCH3 score zoals beschreven in hoofdstuk 5 van dit proefschrift is een uitleesmaat voor NOTCH3 stapeling in de muizen, maar kan potentieel ook worden gebruikt als uitleesmaat in mensen. Daarnaast zijn er mogelijk uitleesmaten in bloed.

In de toekomst zal het steeds gangbaarder worden om bij mensen de gehele erfelijke code te analyseren. Hiermee zal duidelijk worden hoeveel personen in de populatie een *NOTCH3* mutatie hebben die nu, deels vanwege een mild beeld, niet gediagnostiseerd worden. Het identificeren van meer personen met een *NOTCH3* mutatie is van belang om beter te begrijpen hoe variabel de ziekte CADASIL precies is, en welke factoren hierop van invloed zijn. Zo kunnen beschermende factoren worden geïdentificeerd, die als aangrijpingspunt kunnen dienen voor leefstijladviezen en nieuwe mogelijke therapieën voor CADASIL.

CURRICULUM VITAE

Julie Rutten is geboren op 1 augustus 1984 te Breda. Na 1 jaar Werktuigbouwkunde te hebben gestudeerd aan de TU Delft, begon zij in 2004 met de studie Geneeskunde aan het Leids Universitair Medisch Centrum (LUMC). Vanaf 2008 heeft zij dit gecombineerd met de studie Biomedische Wetenschappen, eveneens aan het LUMC. Een deel van haar opleiding volgde zij aan het Karolinska Institutet in Zweden en aan de Universidade de Coimbra in Portugal. In 2011 en 2012 behaalde Julie het artsexamen en het masterdiploma Biomedische Wetenschappen (cum laude), waarna zij startte met haar promotieonderzoek op de afdelingen Humane Genetica en Klinische Genetica aan het LUMC, onder supervisie van dr. Saskia Lesnik Oberstein, prof. Annemieke Aartsma- Rus en prof. Gert-Jan van Ommen. Binnen dit promotietraject heeft Julie gewerkt aan therapieontwikkeling voor de erfelijke ziekte CADASIL, waarbij zij zowel klinische als preklinische studies heeft verricht. Tijdens haar onderzoek is Julie een aantal maanden werkzaam geweest aan het Institute for Stroke and Dementia Research in München. De resultaten van haar promotieonderzoek zijn beschreven in dit proefschrift en gepresenteerd op meerdere (inter)nationale congressen. Voor deze presentaties ontving Julie in 2014 de Annual award van de Nederlandse Vereniging voor Humane Genetica en in 2015 de Young Investigator Award van The European Society of Human Genetics. Tijdens haar promotieonderzoek is Julie actief betrokken geweest bij het opzetten van de patiëntenvereniging Stichting Platform CADASIL. Tevens heeft zij via verschillende media uitleg gegeven over de ziekte en het onderzoek naar een mogelijke therapie. In 2016 is Julie gestart met de opleiding tot Klinisch Geneticus aan het LUMC, hetgeen zij zal combineren het onderzoek naar therapieontwikkeling voor CADASIL.

LIST OF PUBLICATIONS

Papers

1. A.H. Hainsworth, S.M. Allan, J. Boltze, C. Cunningham, C. Farris, E. Head, M. Ihara, J.D. Isaacs, R.N. Kalaria, S.A.J. Lesnik-Oberstein, M.B. Moss, B. Nitzsche, G.A. Rosenberg, J.W. Rutten, M. Salkovic-Petrisic, A.M. Troen. *Translational Models for Vascular Cognitive Impairment and Dementia (VCID). A Position Statement Including Larger Species.* Submitted.

2. J.W.Rutten, J.G. Dauwerse, G. Gravesteijn, M. van Belzen, J. van der Grond, J. Polke, M. Bernal-Quiros, S.A.J. Lesnik Oberstein. *Archetypal NOTCH3 mutations frequent in public exome: implications for CADASIL*. Annals of Clinical and Translational Neurology. 2016; in press.

3. J.W.Rutten, J.G. Dauwerse, D.J.M. Peters, D.J.M. Peters, A. Goldfarb, H. Venselaar, C. Haffner, G.J.B. van Ommen, A.M. Aartsma-Rus, S.A.J. Lesnik Oberstein. *Therapeutic NOTCH3 cysteine correction in CADASIL using exon skipping: in vitro proof of concept.* Brain 2016; Apr 139:1123-35.

4. J.W.Rutten, R.R. Klever, I.M. Hegeman, D.S. Poole, J.G. Dauwerse, L.A.M. Broos, C. Breukel, A.M. Aartsma-Rus, J.S. Verbeek, L. van den Weerd, S.G. van Duinen, A.M.J.M. van den Maagdenberg, S.A.J. Lesnik Oberstein. *The NOTCH3 score: a pre-clinical CADASIL biomarker in an novel human genomic NOTCH3 transgenic mouse model with early progressive vascular NOTCH3 accumulation.* Acta Neuropathologica Communications. 2015; 293(1):89.

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S.A.J. Lesnik Oberstein. *Hypomorphic NOTCH3 alleles do not cause CADASIL in humans.* Human Mutation. 2013; 34(11):1486-9.

9. M.C.H. De Visser, S. Roshani, J.W. Rutten, A. van Hylckama Vlieg, H.L. Vos, F.R. Rosendaal, P.H. Reitsma. *Haplotypes of VKORC1, NQO1 and GGCX, their effect on levels of vitamin K-dependent coagulation factors, and the risk of venous thrombosis.* Thrombosis and Haemostasis. 2011; 106(3):563-5.

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Oral presentations

1. J.W. Rutten and S.A.J. Lesnik Oberstein. *De klinisch geneticus van de toekomst vind je in de behandelkamer.* VKGN/VKGL dag, June 2016, Utrecht, The Netherlands. (Invited speaker)

2. J.W. Rutten, S.J. van Duinen, R.R. Klever, I.M. Hegeman, S.J. Verbeek, A.M.J.M. van den Maagdenberg, S.A.J. Lesnik Oberstein. *Quantitative analysis of vascular notch3 accumulation in a novel human notch3 transgenic mouse model; a pre-clinical biomarker for CADASIL.* The 9th international congress on vascular dementia, October 2015, Ljubjana, Slovenia.

3. J.W. Rutten, J.G. Dauwerse, D. J.M. Peters, M. Overzier, A.Goldfarb, R.R. Klever, H. Venselaar, C. Haffner, J.S. Verbeek, G.B. van Ommen, Arn M.J.M. van de Maagdenberg, Annemieke Aartsma-Rus and Saskia Lesnik Oberstein. *Cysteine Correction of NOTCH3: exon skipping as a potential therapeutic strategy for CADASIL.* 11th Annual meeting of the Oligonucleotide Therapeutics Society, October 2015, Leiden, The Netherlands

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Poster presentations

1. J.W. Rutten, E.M.J. Boon, M.J. Pont, J.G. Dauwerse, P. Lakeman, M.K. Liem, M.J. Vollebregt, A. de Kort, M.H. Breuning, S.G. van Duinen, G.M. Terwindt, S.A.J. Lesnik Oberstein. *Interpretation of atypical NOTCH3 mutations: lessons from patients with novel NOTCH3 alterations but no CADASIL*. Joint Clinical Genetics Meeting, March 2014, Leiden, The Netherlands.

2. J.W. Rutten, J.G. Dauwerse, H. Venselaar, M.H. Breuning, S.G. van Duinen, D.J.M. Peters, G.B. van Ommen, A.M. Aartsma-Rus, S.A.J. Lesnik Oberstein.

Cysteine Quantity Correction in CADASIL. Modification of the NOTCH3 protein using exon skipping. IRDiRC conference, April 2013, Dublin, Ireland.

3. J.W. Rutten, E.M.J. Boon, M.J. Pont, J.G. Dauwerse, P. Lakeman, M.K. Liem, M.J. Vollebregt, A. de Kort, M.H. Breuning, S.G. van Duinen, G.M. Terwindt, S.A.J. Lesnik Oberstein. *Interpretation of atypical NOTCH3 mutations: lessons from patients with novel NOTCH3 alterations but no CADASIL.* ESHG, June 2012, Nürnberg, Germany.

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