Preparing for CADASIL therapy

Gravesteijn, G.

Chapter 1

General introduction

CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) is the most prevalent hereditary small vessel disease.[1,2] CADASIL patients typically develop recurrent strokes from mid-adult age onwards, leading to progressive cognitive impairment and ultimately vascular dementia.[1] In 1996, archetypal cysteine altering missense mutations in *NOTCH3* (*NOTCH3*^{cys}) were discovered to cause CADASIL.[2] Since then, numerous CADASIL patients and families have been identified world-wide, leading to an estimated minimum prevalence of 2 - 5 CADASIL patients per 100,000 persons.[3–6] In The Netherlands, there are currently more than 275 diagnosed CADASIL families. To date, there is no therapy that can delay or prevent CADASIL.

The first chapter provides a background for the studies that are described in this thesis, which are all aimed at advancing pre-clinical CADASIL therapy development towards future clinical trials. CADASIL clinical signs and symptoms, neuroimaging features, molecular genetics and pathophysiology are described. In addition, the recent advances in therapy development and requirements for clinical trial readiness are discussed.

CADASIL clinical symptoms and neuroimaging features

CADASIL is characterized by recurrent (transient) ischemic events and cognitive decline. CADASIL patients typically suffer from their first ischemic stroke between 45-60 years of age, but age at first stroke can vary from the third decade and to the eight decade.[7–12] Ischemic events typically present as a classical lacunar syndrome with motor or sensory deficits.[13] Almost all patients with a classical CADASIL disease course ultimately develop gait disturbances, urine incontinence and vascular dementia.[7]

The first sign of cognitive decline is often impaired executive function, which can be present before the first stroke.[14–18] This is followed by slowed processing speed,[15,17] and later on by a decline in verbal fluency and visuospatial abilities.[15–17,19] Although recognition, semantic and episodic memory also deteriorate late in the disease course, they are well preserved compared to other domains.[15–17] Ultimately, the global cognitive impairment progresses towards vascular dementia with full care-dependency.

One-third to three-quarters of CADASIL patients develop migraine, often in the third decade, before the first ischemic event.[7,8,10,11,20] Migraine is accompanied by aura in ~80% of patients.[7,8,10,11,20] Women with CADASIL are more prone to develop migraine, and have a younger age at onset.[11,20] One-third of patients suffer from at least one atypical aura in their life, with motor deficits, confusion and decreased consciousness, which may be difficult to differentiate from transient ischemic attacks (TIAs).[10,20] In some cases, a migrainous encephalopathy may occur.[10,20]

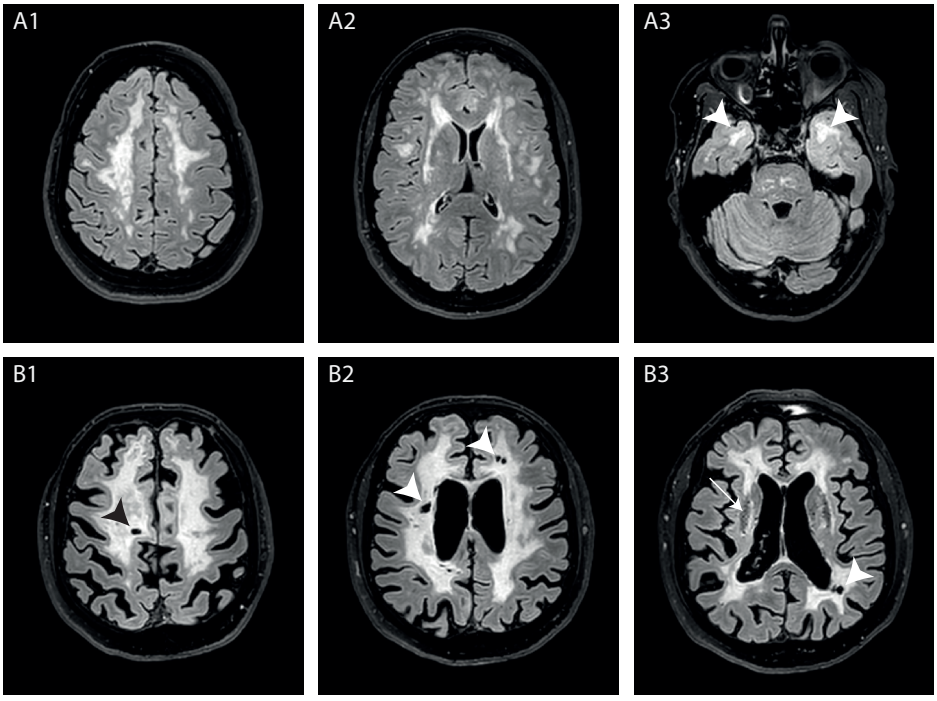

Figure 1.1: Characteristic neuroimaging features in CADASIL

(A) FLAIR images of a CADASIL patient without disability showing WMH in the semi-oval centre (A1), in the external capsules and periventricular areas (A2), and in the anterior temporal lobes (A3) (arrowheads). (B) FLAIR images of a CADASIL patient with disability showing multiple lacunes (arrowheads) and confluent WMH affecting almost all the supratentorial white matter. Multiple enlarged perivascular spaces are present in the basal ganglia (Virchow-Robin spaces, arrow), which are collectively also called état criblé or status cribrosum.

One-third of the patients suffer from mood disorders, most often a depressive episode.[7,10] Apathy occurs in 40% of the patients and is independent of depression. Other psychiatric disturbances are reported, including anxiety, psychotic disorders and adaptation disorders.[7,10]

The most prominent signs on MRI include white matter hyperintensities on T2-weighted and FLAIR images, and lacunes (Figure 1.1).[12,21–27] From the age of 20 onwards, focal subcortical WMHs can be present in the anterior temporal lobes and periventricular areas, and later also in the external capsules and semi-oval center.[12,27] Although anterior temporal lobe WMHs are frequently observed, they are not always present.[12,27–29] Over time, subcortical WMH lesion load increases and WMH may become confluent in almost all the white matter.[22,30] From a mean age of 40-50 years onwards, lacunes can be observed. Neuroradiological signs may also include brain atrophy, enlarged perivascular spaces,[24,31] and microbleeds on susceptibility-weighted imaging, the frequency of which seems to partially depend on the ethnicity of the population studied.[28,32,33] In the Asian population, for example, microbleeds are more frequent and there is a higher risk of intracerebral haemorrhage.[12,28,34,35]

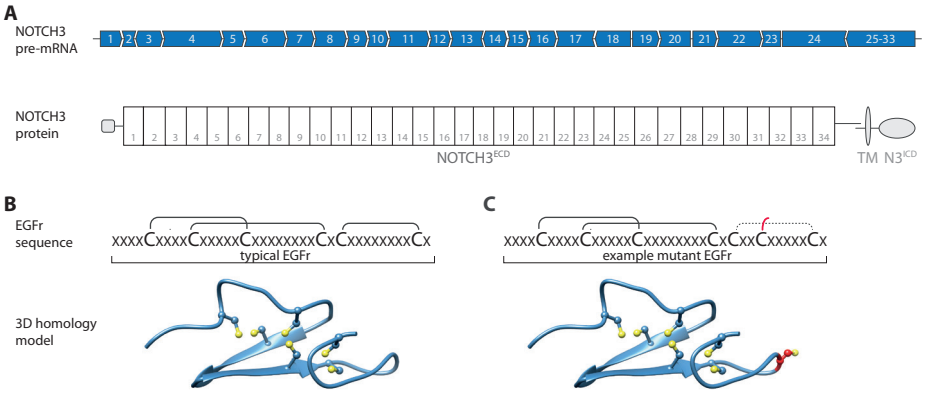

Figure 1.2: Schematic representation of *NOTCH3* exons, NOTCH3 protein and EGFr domains

(A) *NOTCH3* is one of the four *NOTCH* homologues in humans and encodes for a transmembrane receptor protein.[40,54] The *NOTCH3* gene consists of 33 exons. Exon 2-24 encode for 34 similar epidermal growth factor-like repeat (EGFr) domains, located within the ectodomain of the NOTCH3 protein ($NOTCH3^{ECD}$).[40,54] One exon can encode one or more, complete or partial, EGFr domains. (B) A schematic figure and 3D homology modelling of a wildtype EGFr domain showing the disulphide pairing of the six cysteine residues. Each wildtype EGFr domain contains six cysteine residues that form three disulphide bridges (C^1-C^3, C^2-C^4, and C^5-C^6), thereby maintaining the structural integrity of the protein.[47] The number of amino acids between C^4-C^5 and C^5-C^6 is highly conserved and is always 1 amino acid or 8 amino acids, respectively. The number of amino acids is variable between the other cysteine residues (typically 4-6 amino acids between C^1-C^2; 4-5 amino acids between C^2-C^3; and 6-9 amino acids between C^3-C^4). (C) A schematic figure and 3D homology modelling of a mutant EGFr domain, in which the number of cysteine residues is altered to an uneven number, leading to incorrect protein folding and increased multimerization.

CADASIL genetics

CADASIL is caused by highly stereotypical mutations in the *NOTCH3* gene.[36,37] In adults, the transmembrane receptor NOTCH3 is mainly expressed in pericytes and vascular smooth muscle cells (VSMCs), which are collectively called mural cells.[38–40] *NOTCH3* expression is required for VSMC maturation, arterial identity, and blood vessel integrity.[39,41–44] The extracellular domain of NOTCH3 ($NOTCH3^{ECD}$) includes 34 epidermal growth factor-like repeat (EGFr) domains, with each EGFr domain having a fixed number of 6 cysteine residues (Figure 1.2). In CADASIL, mutations alter the canonical number of six cysteines in an EGFr domain to an uneven number of cysteines ($NOTCH3^{cys}$), usually five of seven.[1,36,45,46] This results in an unpaired cysteine residue, which leads to incorrect EGFr folding, abnormal protein folding and increased $NOTCH3^{ECD}$ multimerization.[47–50] Almost all $NOTCH3^{cys}$ mutations are missense mutations, the majority of which are located in exon 4. However, mutations can occur in exons that encode for any of the 34 EGFr domains (i.e. exon 2-24).[37,46] $NOTCH3^{cys}$ mutations located in EGFr domains 7-34 have recently been found to be associated with a milder CADASIL phenotype and increased survival compared to

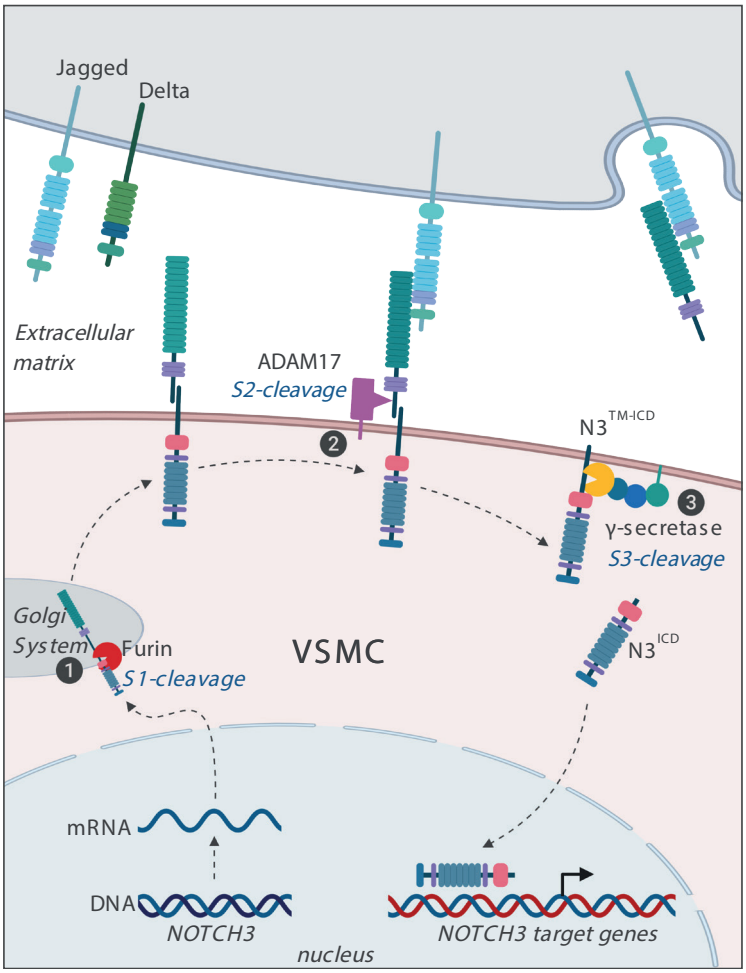

Figure 1.3: NOTCH3 processing and signalling

(1) The 280 kDa NOTCH3 precursor protein is cleaved in the Golgi system by Furin (S1 cleavage), resulting in a non-covalently bound heterodimeric protein, which is subsequently transported to the cell surface.[40,54] (2) Upon binding of a ligand (Jagged 1 or 2, Delta 1, 3 or 4) to EGFr domain 10-11, a mechanical traction force is applied to the $NOTCH3^{ECD}$, exposing the extracellular NRR near the cell membrane, that consists of LNR domains (light purple) and the heterodimerization domain. Next, the C-terminal part of the heterodimerization domain is cleaved by ADAM17 (S2-cleavage).[55] (3) Then, γ-secretase cleaves off the $NOTCH3^{ICD}$, which is comprised of a RAM domain (red) and several ANK (blue), two nuclear localisation signals, a transactivation domain (not shown) and a PEST domain (blue) involved in degradation regulation.[40,54,56] In the nucleus, the $NOTCH3^{ICD}$ interacts with the RBPJ protein and co-activator Mastermind-like (MAML) to activate downstream gene transcription.[40,54,56] ADAM17: a disintegrin and metalloproteinase domain-containing protein 17 (also known as TACE); ANK: ankyrin repeat; LNR: Lin12-Notch repeats; MAML: Mastermind-like; $NOTCH3^{ECD}$: NOTCH3 ectodomain; $NOTCH3^{ICD}$: NOTCH3 intracellular domain; NRR: negative regulatory region; PEST: proline, glutamate, serine and threonine (PEST)-rich; RAM: RBPJ-associated module; RBPJ: recombining binding protein suppressor of hairless.

NOTCH3^{cys} mutations in EGFr domains 1-6, which are largely encoded by exon 4.[51] Some patients have small *NOTCH3* in-frame deletions, insertions or splice site mutations, which also result in an uneven number of cysteines in the given mutant EGFr domain.[46] NOTCH3 signalling is considered to be intact for almost all *NOTCH3*^{cys} mutations.[50,52,53] Only when the mutation is located in the ligand binding domain of the NOTCH3 protein (i.e. EGFr domains 10 and 11), NOTCH3 signalling is found to be reduced (NOTCH3 signalling is described in Figure 1.3).[50,52,53]

CADASIL pathophysiology

NOTCH3^{cys} mutations lead to NOTCH3^{ECD} multimerization and aggregation in the blood vessel walls, in the vicinity of vascular smooth muscle cells and pericytes (Figure 1.4).[47,49,57–59] The NOTCH3^{ECD} aggregates are observed in the vessel walls of the small cerebral arteries, but also of the small arteries throughout the body.[60] Immunohistochemical NOTCH3^{ECD} staining of skin biopsies shows NOTCH3^{ECD} aggregates in skin arteries in almost all CADASIL patients, from at least early adulthood onwards.[60,61] Ultrastructurally, deposits of granular osmiophilic material (GOM) can be found near mural cells in the basement membrane of the small (brain) arteries and capillaries.[62–67] GOM deposits are considered to be pathognomonic for CADASIL.[64,66]

NOTCH3^{ECD} aggregates and GOM deposits sequester functionally important extracellular matrix proteins, such as TIMP3, vitronectin, HTRA1, endostatin and LTBP-1, thereby contributing to the disease pathology.[68–70] One potential mechanism contributing to disease pathogenesis involves the sequestration of HTRA1 in NOTCH3^{ECD} aggregates, resulting in an HTRA1 loss-of-function profile in the extracellular matrix with subsequent accumulation and dysfunction of HTRA1's substrates.[68,70,71] In addition, the abundance of TIMP3 in the cerebrovasculature is reported to have an effect via the ADAM17/HB-EGF/(ErbB1/ErbB4) pathway on vascular smooth muscle cell hyperpolarisation, pressure-induced myogenic tone and impaired cerebral blood flow autoregulation.[72,73]

Histologically, CADASIL is characterized by arterial wall thickening, especially of the small to medium-sized arteries (Figure 1.4). These vessels show thickened fibrotic walls with intense collagenous staining, marked vascular smooth muscle cell degeneration and a smooth muscle actin (SMA) positive intima, but not a substantially narrowed lumen.[62,74–78] These pathological changes are associated with impaired regulation of cerebral blood flow, altered VSMC morphology, white matter hyperintensities, blood-brain-barrier leakage and ultimately ischemic events and vascular cognitive impairment.[62,74–79]

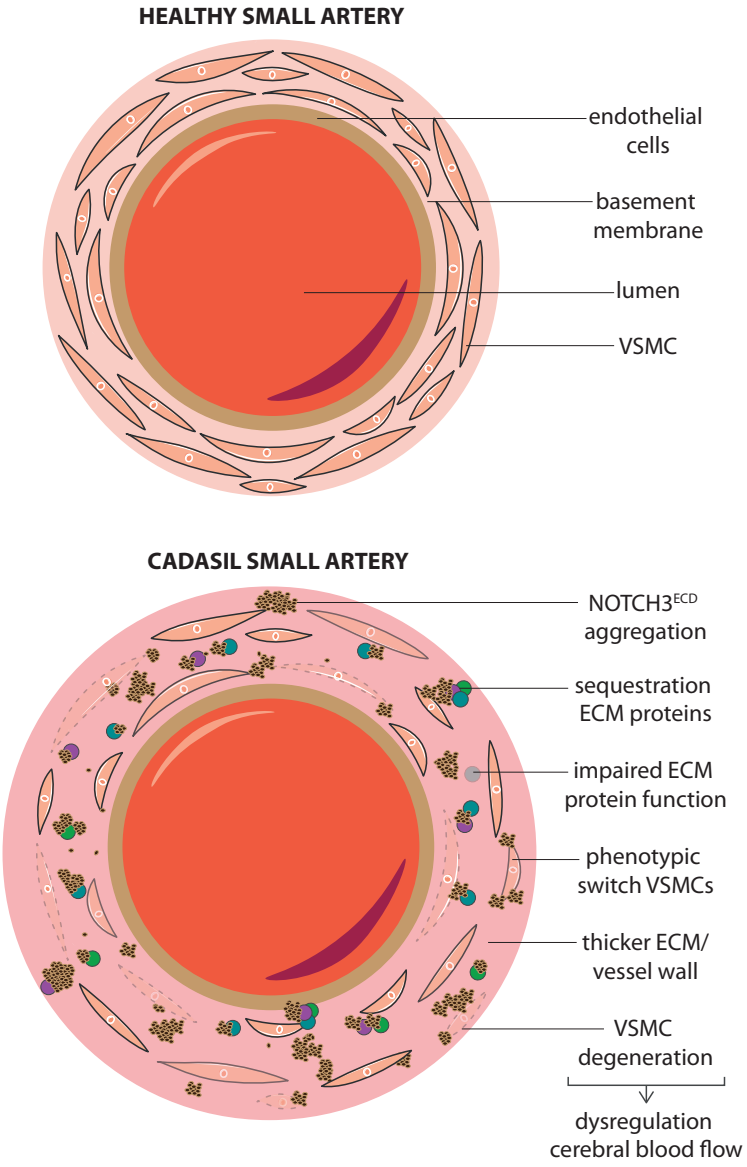

Figure 1.4: Hallmarks of CADASIL vessel wall pathology

NOTCH3 proteins with a cysteine altering mutation lead to extracellular aggregates of the NOTCH3^{ECD}, which sequester other extracellular matrix proteins, leading to extracellular matrix dysregulation, vessel wall thickening, blood-brain-barrier leakage, changes in VSMC phenotype and VSMC degeneration.[62,71,82,74–81]

Characterization of a CADASIL mouse model

Various CADASIL mouse models with a *NOTCH3^{cys}* mutation recapitulate the NOTCH3^{ECD} aggregates and GOM deposits found in CADASIL patients.[63,70,83–89] The humanized transgenic *NOTCH3^{Arg182Cys}* mouse model, that was developed in the Leiden University Medical Center, overexpresses the full length human *NOTCH3* gene with a typical CADASIL mutation from a genomic construct at expression levels of 100%, 150%, 200% and 350% relative to endogenous mouse *Notch3* expression. In brains of these mice, the amount of NOTCH3^{ECD} aggregates correlates with age and with the expression level of mutant *NOTCH3*, and NOTCH3^{ECD} aggregates can be observed as early as age 6 weeks in the mouse strain with 350% human *NOTCH3^{Arg182Cys}* expression. In this strain, GOM deposits were observed from the age of 6 months. Although the presence of GOM deposits in patients and animal models has been extensively reported, there have been no studies describing progression of GOM deposits from their incipience onwards.

Histological white matter abnormalities, cerebrovascular reactivity, myogenic tone, and blood brain barrier leakage have been studied in CADASIL mouse models, but had not yet been fully characterized in the Leiden mouse model.[63,70,83–89]

Chapter 2 describes the longitudinal characterization of GOM deposits in the Leiden humanized transgenic *NOTCH3^{Arg182Cys}* mouse model, and provides a five-tier classification system for GOM deposits in mice. Analysis of patient material showed that this classification system is translatable to GOM in patient tissue.[90] Chapter 2 also describes the characterization of the Leiden humanized transgenic *NOTCH3^{Arg182Cys}* mouse model in terms of histology, neuroimaging, cerebrovascular reactivity and cognition.[90]

Therapy development in CADASIL

NOTCH3^{ECD} aggregation is considered to have a pivotal role in CADASIL pathogenesis. Therefore, the focus of current therapeutic interventions is on counteracting NOTCH3^{ECD} aggregation (Figure 1.5).[91,92] Other therapeutic strategies aim at increasing NOTCH3 signalling and reducing endothelial and mural cell degeneration.[43,93]

The Leiden CADASIL research group developed a therapeutic approach aimed to prevent and halt the formation of mutant NOTCH3^{ECD} using an approach called 'NOTCH3 cysteine correction', aimed at correcting the number of cysteine residues in the mutant protein's EGFr domains. This is accomplished by removing the respective mutant EGFr domain from the NOTCH3 protein.[94] NOTCH3 cysteine correction can be achieved using a splice modulating therapy called exon skipping (Figure 1.5B$_1$). In exon skipping, short strands of modified RNA (antisense oligonucleotides, ASOs) bind to the *NOTCH3* pre-mRNA, thereby hiding an exon

from the splicing machinery, effectively excluding the mutant exon from the mature mRNA. This results in a NOTCH3 protein lacking the mutant EGFr domain. This modified protein is predicted to maintain canonical NOTCH protein structure, with correct disulphide bridge formation. *In vitro* proof-of-concept has been obtained for NOTCH3 cysteine correction by exon skipping, showing that the shorter NOTCH3 protein that is formed after *NOTCH3* exon skipping retains signalling function.[94] However, whether NOTCH3 cysteine correction also reduces $NOTCH3^{ECD}$ aggregation could not be assessed *in vitro*.

The principle of NOTCH3 cysteine correction can also be applied at the DNA level using gene editing (Figure 1.5B_2). CRISPR/Cas9 has recently evolved rapidly into a useful tool for gene editing in *in vitro* model systems. In addition, CRISPR/Cas9 holds the promise of treating incurable genetic disease.[95] Chapter 3 describes the first in-human evidence that NOTCH3 cysteine correction is associated with reduced $NOTCH3^{ECD}$ aggregation, by analysis of a family with naturally occurring *NOTCH3* exon skipping.[96] Chapter 3 also describes *in vitro* proof-of-concept of NOTCH3 cysteine correction using CRISPR/Cas9-mediated genomic deletion of exons eligible for cysteine correction.[96]

Two alternative therapeutic approaches that are being developed by other laboratories are antibody-based. One approach aims to counteract $NOTCH3^{ECD}$ aggregation using passive immunization (Figure 1.5C).[92] Passive immunization with a monoclonal antibody targeting $NOTCH3^{ECD}$ in a CADASIL mouse model showed a protective effect on vasodilative cerebral blood flow response and pressure-induced myogenic tone, suggesting that immunization rescues the ADAM17/HB-EGF/(ErbB1/ErbB4) pathway. However, chronic passive immunization did not halt the formation of $NOTCH3^{ECD}$ aggregates, GOM deposits or myelin debris in mouse brain.[92]

Another antibody-based approach targets the negative regulatory region of the NOTCH3 protein, which thereby increases NOTCH3 signalling, but does not counteract $NOTCH3^{ECD}$ aggregation (Figure 1.5D).[43] This approach might be beneficial for the patients with a loss-of-function $NOTCH3^{cys}$ mutation in the ligand binding domain. Whether patients with a $NOTCH3^{cys}$ mutation outside the ligand binding domain would benefit from this approach remains uncertain, since it remains a matter of debate whether NOTCH3 signalling is reduced in CADASIL, and if it is, whether this contributes to the disease pathomechanism.[51,70,97]

Finally, another approach aims to diminish CADASIL-associated degeneration of endothelial cells and vascular smooth muscle cells by administration of two hematopoietic growth factors suggested to be neuroprotective, i.e. stem cell factor and granulocyte colony-stimulating factor.[93] Administration of these factors in a CADASIL mouse model resulted in

attenuated mural cell degeneration, increased vascular density, signs of reduced capillary endothelial cells damage, and retained cognition in a CADASIL mouse model.[93,98] However, it remains largely unclear whether the observed therapeutic effects are due to a specific effect of the treatment on CADASIL signs, or due to an aspecific effect not related to CADASIL.

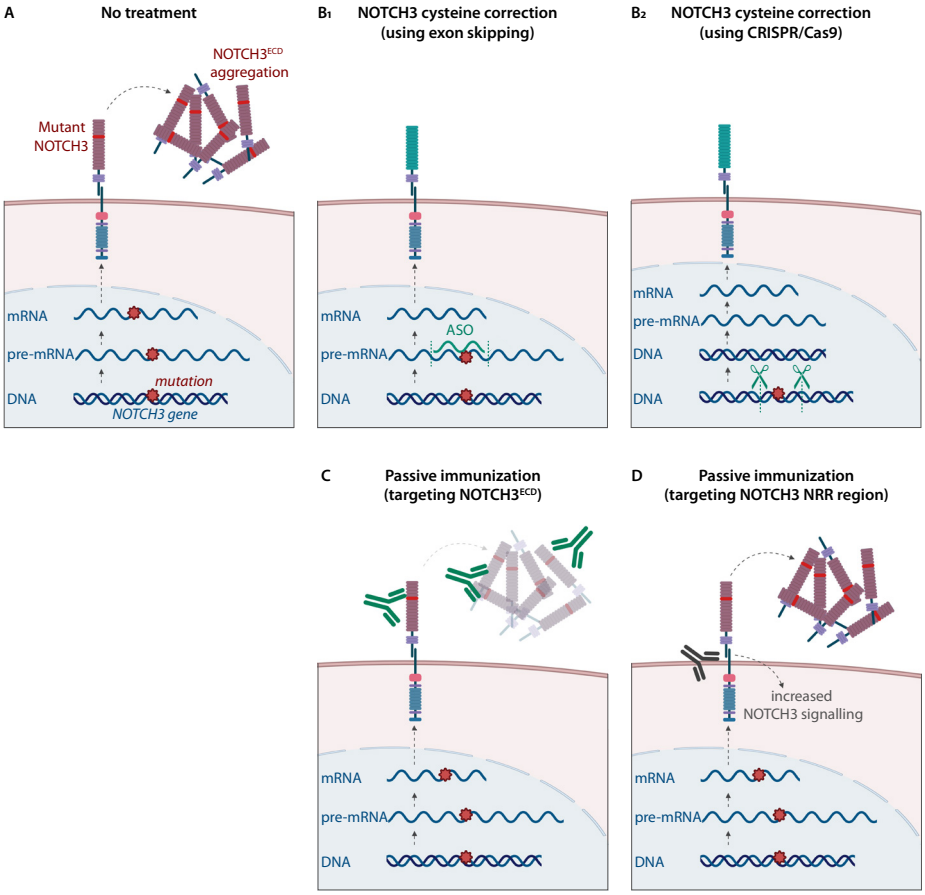

Figure 1.5: Therapeutic approaches which are currently being developed

(A) *NOTCH3* gene mutations are translated into mutant NOTCH3^{ECD} proteins, leading to NOTCH3^{ECD} aggregation. (B) NOTCH3 cysteine correction aims to prevent the formation of mutant NOTCH3^{ECD}. Mutant exons are excluded from the mature mRNA using short antisense oligonucleotide strands (ASOs) (B_1),[94] or are removed from the *NOTCH3* gene using CRISPR/Cas9 (B_2).[96] (C) Chronic passive immunization with antibodies targeting NOTCH3^{ECD} are aimed at counteracting the toxic effect of NOTCH3^{ECD} and NOTCH3^{ECD} aggregation.[92] (D) Antibodies targeting the negative regulatory region (NRR) of the extracellular NOTCH3 protein are aimed at restoring NOTCH3 signalling in the case of mutations affecting the ligand binding domain.[43]

CADASIL natural history

Before a therapeutic approach for CADASIL can be tested in clinical trials, information needs to be available on the natural history of CADASIL and the variability in disease progression. This information is required to select a relatively homogeneous patient (sub)group that is most likely to benefit from a potential therapy, and to identify disease measures that can be used as read-out for disease severity and therapeutic benefit.

Previously, follow-up studies of 2, 3 and 7 years were performed in CADASIL patients and controls, showing that lacunes and the level of brain atrophy – and not the extent of WMH – are associated with clinical deterioration, and that lacunes and brain atrophy could potentially be used as surrogate endpoints.[9,99–104] Moreover, signs of advanced CADASIL disease, such as gait disturbances, disability and dementia, are associated with signs of advanced disease on brain MRI and mortality.[19,101,102,105] These follow-up studies analysed disease progression on a group level, rather than studying interindividual differences. Taking this heterogeneity at the individual level into account is important, as there is a large variability in disease severity and progression between families and even between patients within families.

Chapter 4 describes an 18-year follow-up study in CADASIL patients, showing that disease course can remain remarkably stable over 18 years in some patients, and that disease progression is highly variable between patients: some patient had stroke and multiple lacunes before the age of 50 years, while others remained free of stroke and lacunes until well within their sixties.

Measuring CADASIL disease severity

For future clinical trials, a robust and sensitive read-out to assess disease severity and therapeutic benefit is required in order to get approval from regulators (such as the Food and Drug Administration [FDA, USA] and the European Medicines Agency [EMA]). These regulators will judge whether a therapy is safe to use, has a sufficient efficacy and leads to clinical benefit as experienced by the patients, based on data from clinical trials (on many aspects, including toxicity, pharmacokinetics, pharmacodynamics, dose finding, adverse effects, efficacy and effectiveness). This read-out is required to be able to measure a therapeutic effect in a short time frame, since clinical trials often do not span more than 2 years. In CADASIL, hard endpoints for clinical studies are for example incident stroke, disability, dementia, or survival. A major disadvantage of these hard endpoints is that, due to disease variability, they require large cohorts and a long follow-up in order to observe sufficient events (e.g. number of patients experiencing a stroke) to sufficiently power an interventional study.[106]

Other read-outs, such as biomarkers, could be used as surrogate endpoints, provided that these surrogate endpoints predict future clinical benefit. Biomarkers are defined as 'objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention'.[107] Several types of biomarkers exist, including diagnostic biomarkers, prognostic biomarkers, monitoring biomarkers and pharmacodynamic biomarkers (Figure 1.6). Especially monitoring and pharmacodynamic biomarkers are imperative to monitor target engagement and to measure efficacy of the therapeutic compound in future clinical trials.

Clinical measures

Measures for functional independence, such as the modified Rankin scale (mRS), and measures for cognitive function, such as the Cambridge Cognitive Examination (CAMCOG), have been reported to change over 2-, 3- or 7-year time span in CADASIL patients.[19,102,106] Furthermore, cognitive tests of specific domains, such as the Trail Making Tests for executive function, have been shown to be associated with an increase in MRI disease markers.[101,109] The disadvantage of clinical measures as monitoring markers, is that large sample sizes would be needed. In that respect, the use of neuroimaging measures would be more advantageous.[106,110]

Neuroimaging and cerebrohemodynamic biomarkers

Promising neuroimaging measures that could be used as monitoring biomarkers are lacune count or lacune volume and brain parenchymal fraction (BPF, brain volume relative to intracranial volume), as they reflect neuronal damage and are strongly associated with clinical deterioration and cognitive impairment in symptomatic CADASIL patients.[9,99–104]

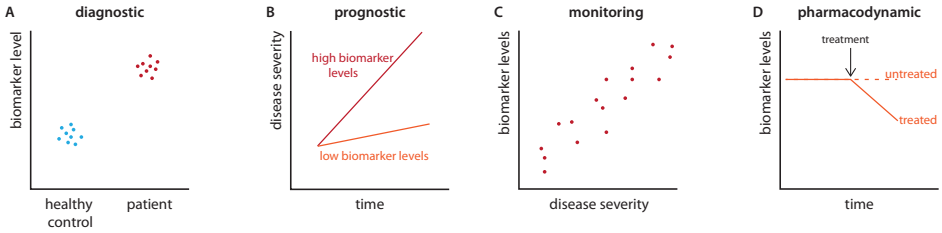

Figure 1.6: Types of biomarkers

(A) A diagnostic biomarker can detect or confirm the presence of a disease or a disease subtype. (B) Prognostic biomarkers are used to predict the likelihood of a clinical event, disease recurrence or progression in patients who have a certain disease. (C) A monitoring biomarker is determined serially to assess the status of a disease over time. (D) A pharmacodynamic biomarker is a type of monitoring biomarker that is able to show a biological response after exposure to a (therapeutic) product or agent.[108] Pharmacodynamic biomarkers do not necessarily have to be associated with anticipated future clinical benefit. The term therapeutic biomarkers is sometimes used to indicate a monitoring biomarker that shows a response after therapy that is anticipated to give clinical benefit.

WMH volume does not correlate with disease severity or disease progression, and therefore does not fulfill the criteria for surrogate marker.[101–104] Diffusion Tensor Imaging (DTI), which measures the microstructural integrity of the brain, has been shown to change over time in normal appearing white matter of CADASIL patients, correlating with cognitive function.[111–114] Most, but not all, studies have reported a reduction in resting cerebral blood flow (CBF) and cerebrovascular reactivity (CVR) in CADASIL patients [115–117] and CADASIL mouse models.[86,118,119]

The disadvantage of MRI-based markers is that MRI is relatively expensive, time consuming and often non-uniform across different sites.

Fluid biomarkers

A monitoring biomarker in blood could be a good alternative to MRI markers, as blood is easily accessible, blood withdrawal is minimally invasive, can be repeated at multiple time-points, is relatively cheap and can be uniformly analysed at one study site. A blood biomarker that reflects neuronal damage is Neurofilament Light-chain (NfL).[120] Serum NfL levels reflect active small vessel disease and could therefore also be promising as a biomarker for symptomatic CADASIL patients.[121,122] Other potential blood biomarkers for CADASIL that were identified in pre-clinical studies are levels of Notch3, Endostatin, Htra1 and Igf-bp1, as blood levels of these proteins were different between a CADASIL mouse model and wildtype mice.[43,123] These proteins, together with TGF-β-associated proteins, have also been found to be highly abundant in blood vessel walls of CADASIL patients.[68,71] Biomarkers in other fluids, such as the cerebrospinal fluid (CSF), have not been studied extensively.[124–126]

Chapter 4 describes a cross-sectional study of blood-based biomarkers, as well as a longitudinal analysis of cerebrovascular hemodynamics in CADASIL patients and controls. Chapter 5 describes that serum NfL levels in CADASIL patients correlate with disease severity, disease progression and survival. Chapter 4 describes a longitudinal analysis of serum NfL over 18 years in CADASIL patients.

Scope of this thesis

The aim of this PhD-project was to advance CADASIL therapy development, including natural history and biomarker identification. By analyzing a family with naturally occurring *NOTCH3* exon skipping, the first in-human evidence was obtained supporting the hypothesis that 'NOTCH3 cysteine correction' is associated with attenuated NOTCH3 protein aggregation, providing further rational for advancing this therapeutic approach.. An ultrastructural biomarker, a GOM deposit classification system, was developed in the transgenic human *NOTCH3*^{Arg182Cys} CADASIL mouse model that can be used as a monitoring or therapeutic marker in pre-clinical therapeutic studies. Further functional characterization of this mouse model was performed to explore other potential pre-clinical biomarkers. In CADASIL patients, multiple candidate blood biomarkers were tested, leading to the identification of Neurofilament Light-chain (NfL) as a biomarker for CADASIL disease severity. The longest follow-up study performed in CADASIL patients to date led to the observation that a subset of patients remain clinically remarkably stable over the course of almost two decades.

Overview of chapters in this thesis

- In chapter 2, the development of a GOM deposit classification system is described, as well as functional characterisation of the Leiden human *NOTCH3* transgenic mouse model.[90]

- In chapter 3, the first in-human evidence is provided supporting the hypothesis that *NOTCH3* exon skipping reduces mutant NOTCH3^{ECD} protein aggregation, and is associated with a milder CADASIL phenotype.[96]

- In chapter 4, a prospective 18-year follow-up study of CADASIL patients is described, showing that disease course can remain relatively stable over almost two decades, and blood biomarkers for CADASIL are evaluated.

- In chapter 5, Neurofilament Light (NfL) levels in blood of CADASIL patients are shown to be associated with disease severity and may therefore function as a biomarker for CADASIL.[127]

- In chapter 6, the findings of the research presented in this thesis are discussed, as well as the implications for future therapy development.

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