NOTCH3 cysteine correction : developing a rational therapeutic approach for CADASIL

Rutten, J.W.

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

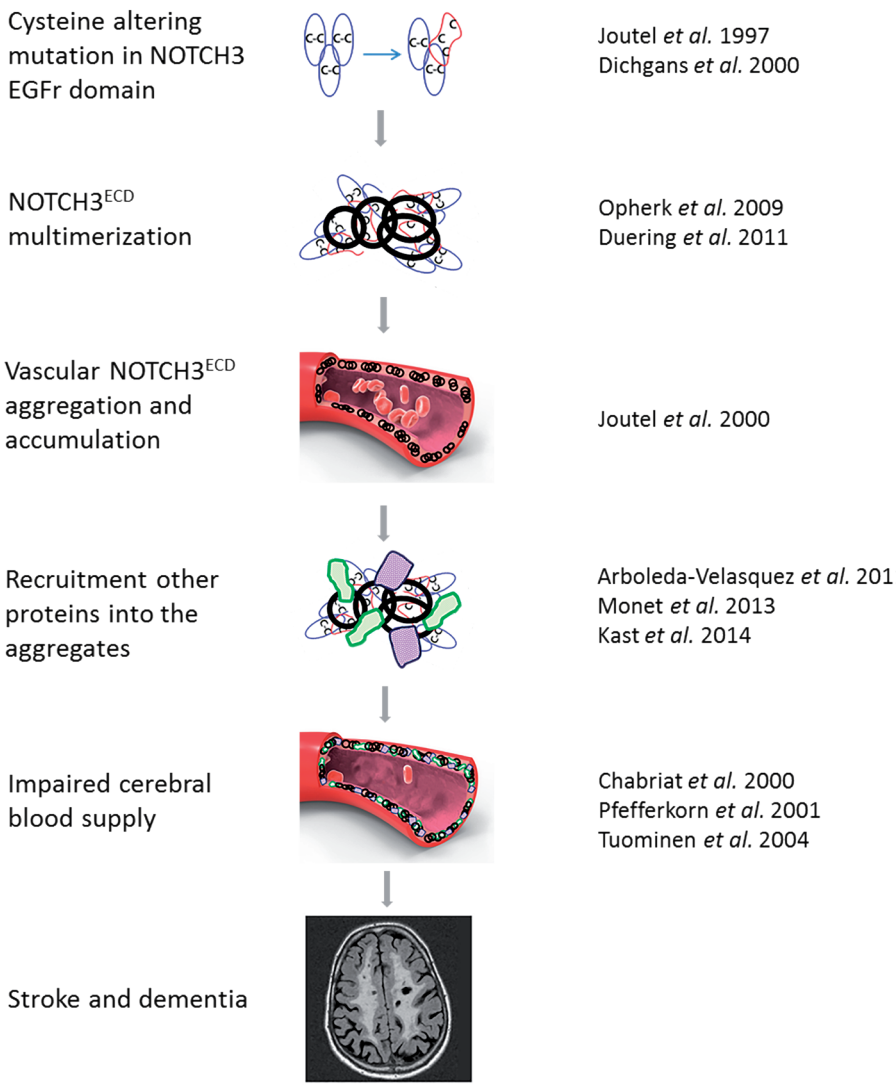

Figure 1. CADASIL pathophysiology. CADASIL-causing *NOTCH3* mutations alter the number of cysteines in one of the 34 EGFr domains of the NOTCH3^{ECD}. This causes multimerization and vascular aggregation of mutant NOTCH3^{ECD}, and recruitment of other proteins into the aggregates. This pathological 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.

REFERENCES

1. Joutel, A, Corpechot, C, Ducros, A, et al. Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. Nature. 1996; 383:707-710.
2. Tournier-Lasserve, E, Iba-Zizen, MT, Romero, N, et al. Autosomal dominant syndrome with strokelike episodes and leukoencephalopathy. Stroke. 1991; 22:1297-1302.
3. Baudrimont, M, Dubas, F, Joutel, A, et al. Autosomal dominant leukoencephalopathy and subcortical ischemic stroke. A clinicopathological study. Stroke. 1993; 24:122-125.
4. Chabriat, H, Vahedi, K, Iba-Zizen, MT, et al. Clinical spectrum of CADASIL: a study of 7 families. Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy. Lancet. 1995; 346:934-939.
5. Dichgans, M, Mayer, M, Uttner, I, et al. The phenotypic spectrum of CADASIL: clinical findings in 102 cases. Ann Neurol. 1998; 44:731-739.
6. Opherk, C, Peters, N, Herzog, J, et al. Long-term prognosis and causes of death in CADASIL: a retrospective study in 411 patients. Brain. 2004; 127:2533-2539.
7. Desmond, DW, Moroney, JT, Lynch, T, et al. The natural history of CADASIL: a pooled analysis of previously published cases. Stroke. 1999; 30:1230-1233.
8. Adib-Samii, P, Brice, G, Martin, RJ, et al. Clinical spectrum of CADASIL and the effect of cardiovascular risk factors on phenotype: study in 200 consecutively recruited individuals. Stroke. 2010; 41:630-634.
9. Amberla, K, Waljas, M, Tuominen, S, et al. Insidious cognitive decline in CADASIL. Stroke. 2004; 35:1598-1602.
10. Peters, N, Opherk, C, Danek, A, et al. The pattern of cognitive performance in CADASIL: a monogenic condition leading to subcortical ischemic vascular dementia. Am J Psychiatry. 2005; 162:2078-2085.
11. Guey, S, Mawet, J, Herve, D, et al. Prevalence and characteristics of migraine in CADASIL. Cephalalgia. 2015.
12. Lesnik Oberstein, SA, van den Boom, R, Middelkoop, HA, et al. Incipient CADASIL. Arch Neurol. 2003; 60:707-712.
13. Chabriat, H, Levy, C, Taillia, H, et al. Patterns of MRI lesions in CADASIL. Neurology. 1998; 51:452-457.
14. Dichgans, M, Holtmannspotter, M, Herzog, J, et al. Cerebral microbleeds in CADASIL: a gradient-echo magnetic resonance imaging and autopsy study. Stroke. 2002; 33:67-71.
15. Lesnik Oberstein, SA, van den Boom, R, van Buchem, MA, et al. Cerebral microbleeds in CADASIL. Neurology. 2001; 57:1066-1070.
16. Peters, N, Holtmannspotter, M, Opherk, C, et al. Brain volume changes in CADASIL: a serial MRI study in pure subcortical ischemic vascular disease. Neurology. 2006; 66:1517-1522.
17. van Den Boom R, Lesnik Oberstein, SA, van Duinen, SG, et al. Subcortical lacunar lesions: an MR imaging finding in patients with cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy. Radiology. 2002; 224:791-796.
18. Joutel, A, Andreux, F, Gaulis, S, et al. The ectodomain of the Notch3 receptor accumulates within the cerebrovasculature of CADASIL patients. J Clin Invest. 2000; 105:597-605.
19. Andersson, ER, Sandberg, R, and Lendahl, U. Notch signaling: simplicity in design, versatility in function. Development. 2011; 138:3593-3612.
20. Dichgans, M, Ludwig, H, Muller-Hocker, J, et al. Small in-frame deletions and missense mutations in CADASIL: 3D models predict misfolding of Notch3 EGF-like repeat domains. Eur J Hum Genet. 2000; 8:280-285.
21. Joutel, A, Vahedi, K, Corpechot, C, et al. Strong clustering and stereotyped nature of Notch3 mutations in CADASIL patients. Lancet. 1997; 350:1511-1515.
22. Rutten, JW, Haan, J, Terwindt, GM, et al. Interpretation of NOTCH3 mutations in the diagnosis of CADASIL. Expert Rev Mol Diagn. 2014; 14:593-603.

23. Opherk, C, Duering, M, Peters, N, et al. CADASIL mutations enhance spontaneous multimerization of NOTCH3. Hum Mol Genet. 2009; 18:2761-2767.
24. Duering, M, Karpinska, A, Rosner, S, et al. Co-aggregate formation of CADASIL-mutant NOTCH3: a single-particle analysis. Hum Mol Genet. 2011.
25. Kalimo, H, Ruchoux, MM, Viitanen, M, et al. CADASIL: a common form of hereditary arteriopathy causing brain infarcts and dementia. Brain Pathol. 2002; 12:371-384.
26. Ruchoux, MM, Chabriat, H, Bousser, MG, et al. Presence of ultrastructural arterial lesions in muscle and skin vessels of patients with CADASIL. Stroke. 1994; 25:2291-2292.
27. Kast, J, Hanecker, P, Beaufort, N, et al. Sequestration of latent TGF-beta binding protein 1 into CADASIL-related Notch3-ECD deposits. Acta Neuropathol Commun. 2014; 2:96.
28. Monet-Lepretre, M, Haddad, I, Baron-Menguy, C, et al. Abnormal recruitment of extracellular matrix proteins by excess Notch3ECD: a new pathomechanism in CADASIL. Brain. 2013; 136:1830-1845.
29. Arboleda-Velasquez, JF, Manent, J, Lee, JH, et al. PNAS Plus: Hypomorphic Notch 3 alleles link Notch signaling to ischemic cerebral small-vessel disease. Proc Natl Acad Sci U S A. 2011; 108:E128-E135.
30. Pfefferkorn, T, von Stuckrad-Barre, S, Herzog, J, et al. Reduced cerebrovascular CO(2) reactivity in CADASIL: A transcranial Doppler sonography study. Stroke. 2001; 32:17-21.
31. Chabriat, H, Pappata, S, Ostergaard, L, et al. Cerebral hemodynamics in CADASIL before and after acetazolamide challenge assessed with MRI bolus tracking. Stroke. 2000; 31:1904-1912.
32. Tuominen, S, Miao, Q, Kurki, T, et al. Positron emission tomography examination of cerebral blood flow and glucose metabolism in young CADASIL patients. Stroke. 2004; 35:1063-1067.
33. Domenga, V, Fardoux, P, Lacombe, P, et al. Notch3 is required for arterial identity and maturation of vascular smooth muscle cells. Genes Dev. 2004; 18:2730-2735.
34. Haritunians, T, Chow, T, De Lange, RP, et al. Functional analysis of a recurrent missense mutation in Notch3 in CADASIL. J Neurol Neurosurg Psychiatry. 2005; 76:1242-1248.
35. Haritunians, T, Boulter, J, Hicks, C, et al. CADASIL Notch3 mutant proteins localize to the cell surface and bind ligand. Circ Res. 2002; 90:506-508.
36. Joutel, A, Monet, M, Domenga, V, et al. Pathogenic mutations associated with cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy differently affect Jagged1 binding and Notch3 activity via the RBP/JK signaling Pathway. Am J Hum Genet. 2004; 74:338-347.
37. Karlstrom, H, Beatus, P, Dannaeus, K, et al. A CADASIL-mutated Notch 3 receptor exhibits impaired intracellular trafficking and maturation but normal ligand-induced signaling. Proc Natl Acad Sci U S A. 2002; 99:17119-17124.
38. Low, WC, Santa, Y, Takahashi, K, et al. CADASIL-causing mutations do not alter Notch3 receptor processing and activation. Neuroreport. 2006; 17:945-949.
39. Peters, N, Opherk, C, Zacherle, S, et al. CADASIL-associated Notch3 mutations have differential effects both on ligand binding and ligand-induced Notch3 receptor signaling through RBP-Jk. Exp Cell Res. 2004; 299:454-464.
40. Voit, T, Topaloglu, H, Straub, V, et al. Safety and efficacy of drisapersen for the treatment of Duchenne muscular dystrophy (DEMAND II): an exploratory, randomised, placebo-controlled phase 2 study. Lancet Neurol. 2014; 13:987-996.
41. Ruchoux, MM, Domenga, V, Brulin, P, et al. Transgenic mice expressing mutant Notch3 develop vascular alterations characteristic of cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy. Am J Pathol. 2003; 162:329-342.
42. Monet-Lepretre, M, Bardot, B, Lemaire, B, et al. Distinct phenotypic and functional features of CADASIL mutations in the Notch3 ligand binding domain. Brain. 2009; 132:1601-1612.
43. Lundkvist, J, Zhu, S, Hansson, EM, et al. Mice carrying a R142C Notch 3 knock-in mutation do not develop a CADASIL-like phenotype. Genesis. 2005; 41:13-22.

44. Joutel, A, Monet-Lepretre, M, Gosele, C, et al. Cerebrovascular dysfunction and microcirculation rarefaction precede white matter lesions in a mouse genetic model of cerebral ischemic small vessel disease. J Clin Invest. 2010; 120:433-445.
45. Wallays, G, Nuyens, D, Silasi-Mansat, R, et al. Notch3 Arg170Cys knock-in mice display pathologic and clinical features of the neurovascular disorder cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy. Arterioscler Thromb Vasc Biol. 2011; 31:2881-2888.
46. Rutten, JW, Klever, RR, Hegeman, IM, et al. The NOTCH3 score: a pre-clinical CADASIL biomarker in a novel human genomic NOTCH3 transgenic mouse model with early progressive vascular NOTCH3 accumulation. Acta Neuropathol Commun. 2015; 3:89.
47. Peters, N, Herzog, J, Opherk, C, et al. A two-year clinical follow-up study in 80 CADASIL subjects: progression patterns and implications for clinical trials. Stroke. 2004; 35:1603-1608.
48. Opherk, C, Gonik, M, Duering, M, et al. Genome-wide genotyping demonstrates a polygenic risk score associated with white matter hyperintensity volume in CADASIL. Stroke. 2014; 45:968-972.
49. Mykkanen, K, Junna, M, Amberla, K, et al. Different clinical phenotypes in monozygotic CADASIL twins with a novel NOTCH3 mutation. Stroke. 2009; 40:2215-2218.
50. Chabriat, H, Herve, D, Duering, M, et al. Predictors of Clinical Worsening in Cerebral Autosomal Dominant Arteriopathy With Subcortical Infarcts and Leukoencephalopathy: Prospective Cohort Study. Stroke. 2016; 47:4-11.
51. Razvi, SS, Davidson, R, Bone, I, et al. The prevalence of cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy (CADASIL) in the west of Scotland. J Neurol Neurosurg Psychiatry. 2005; 76:739-741.
52. Moreton, FC, Razvi, SS, Davidson, R, et al. Changing clinical patterns and increasing prevalence in CADASIL. Acta Neurol Scand. 2014; 130:197-203.
53. Narayan, SK, Gorman, G, Kalaria, RN, et al. The minimum prevalence of CADASIL in northeast England. Neurology. 2012; 78:1025-1027.