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The Pathobiology of Cerebrovascular Lesions in CADASIL Small Vessel Disease

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ABSTRACT

Cerebral small vessel disease (cSVD) is a significant global health issue, accounting for approximately 25% of ischemic strokes and 20% of all dementia cases. CADASIL, the most common monogenic form of cSVD, is caused by stereotyped mutations in the NOTCH3 receptor that alter the number of cysteine residues in its extracellular domain (Notch3^{ECD}). The two hallmark features of CADASIL are the loss of arterial smooth muscle cells (SMCs) and the abnormal accumulation of Notch3^{ECD}, without associated accumulation of its transmembrane intracellular domain. Notably, cysteine-altering mutations in NOTCH3 are prevalent in the general population, and although they are not directly associated with classical CADASIL disease, they are still linked to an elevated risk of stroke and dementia. NOTCH3 is predominantly expressed in the mural cells of small blood vessels and plays an essential role in the development, maintenance, function and survival of arterial SMCs. Recent research has challenged the loss-of-function hypothesis, instead implicating Notch3^{ECD} aggregation, involving both mutant and wild-type NOTCH3, as the primary driver of vascular pathology in CADASIL. Consequently, therapeutic strategies targeting the reduction of Notch3^{ECD} levels in brain arteries, such as antisense therapies, are considered highly promising for clinical development.

Cerebral small vessel disease (cSVD) encompasses a heterogeneous group of diseases related to in situ pathology of brain vessels that impact their function or structure [1]. cSVD is commonly classified into two categories: cerebral amyloid angiopathy and nonamyloid cSVD. Cerebral amyloid angiopathy is characterized by the deposition of ß-amyloid in the walls of brain vessels and manifests primarily as lobar intracerebral haemorrhages [2]. Nonamyloid cSVD comprises a group of pathologies commonly related to ageing, hypertension or genetic factors. This form most commonly presents as lacunar strokes, which result from small deep infarcts occurring in the white matter or deep grey matter nuclei, and vascular cognitive impairment; other symptoms include depression, apathy, gait disturbances and urinary incontinence. Intracerebral haemorrhages may also occur in nonamyloid cSVD, however predominantly in the deep parts of the brain, unlike amyloid angiopathy [1]. On brain magnetic resonance imaging, cSVD manifests as a spectrum of lesions including acute deep infarcts, lacunes, white matter hyperintensities, cerebral microbleeds, enlarged perivascular spaces and cerebral atrophy [3].

cSVDs are chronic diseases that develop gradually over many years. The spectrum of cSVD manifestations ranges from lesions incidentally detected by neuroimaging, that is, without overt clinical manifestations and often referred to as silent or covert cSVD, to severe outcomes like dementia and disability. Silent

Summary

- Cerebral small vessel disease (cSVD) is a major health issue, causing about 25% of strokes and 20% of dementia cases worldwide.
- CADASIL, the most common hereditary form of cSVD, is caused by stereotyped mutations in the *NOTCH3* gene, which lead to protein changes and damage to small blood vessels.
- Damages involve the loss of smooth muscle cells and the buildup of NOTCH3 protein in blood vessels.
- These mutations are common in the population and increase the risk of stroke and dementia.
- Recent studies highlight NOTCH3 protein buildup in blood vessels as the primary cause of smooth muscle cell loss.

cSVD is particularly prevalent in ageing populations [4]. Overall, cSVD is one of the major health problems affecting all societies today, contributing to ~25% of ischemic strokes, the majority of spontaneous intracerebral haemorrhages, and about 20% of all dementia cases [1]. Despite this, there is no treatment of proven efficacy other than risk factor modification. One main reason is that the mechanisms of these diseases are still incompletely understood.

1 | CADASIL, the Most Frequent Hereditary cSVD, Caused by Cysteine Altering Variants in the NOTCH3 Receptor

A significant breakthrough in the field of cSVD has been the identification of monogenic forms of the condition, that is, cSVD caused by point mutations in a single gene. Familial cases were first reported in the 1970s, and the first causative gene has been identified in 1996. To date, 15 distinct diseases, caused by mutations in 13 different genes, have been described, and monogenic forms of cSVD are thought to account for about 2% of the total cSVD burden [5]. Mutations in *NOTCH3*, *HTRA1* or *COL4A1/COL4A2* genes account for the majority of the burden of adult-onset monogenic cSVDs, and among these, CADASIL, caused by dominant mutations in *NOTCH3*, emerges as the most prevalent hereditary form, accounting for up to 80% of genetically diagnosed cSVD [6].

CADASIL shares many clinical features with sporadic cSVD, but it has distinctive characteristics, particularly the occurrence of migraine with aura in up to 75% of mutation carriers in non-Asian populations, and an earlier age of onset of stroke and cognitive deficits, usually around the age of 50 [7]. Classical clinical features include recurrent ischemic strokes or transient ischemic attacks starting at 52 ± 10 years, mood disturbances, cognitive decline ranging from executive dysfunction to dementia, motor disability and gait disturbance with premature death occurring 15–20 years later, however with notable variability between and within affected families [7, 8]. Spontaneous intracerebral haemorrhage is rare, occurring in ~2% of Caucasian patients and 17% Asian patients [8, 9]. CADASIL shares also many neuroimaging features with sporadic cSVD, except for a predilection of white matter lesions in the temporal poles. White matter lesions often appear by age 30 in mutation carriers, whereas lacunar infarcts occur later in life [7].

NOTCH3 encodes a single pass transmembrane receptor consisting of a large extracellular domain ($Notch3^{ECD}$) with 34 epidermal growth factor repeats (EGFr), noncovalently linked to a transmembrane intracellular domain ($Notch3^{TMIC}$) (Figure 1A) [10]. CADASIL-associated *NOTCH3* mutations are highly stereotyped and typically result in either the gain or loss of a cysteine residue, disrupting the conserved disulphide bond pattern within individual EGFr (Figure 1B,C). Mutations are predominantly located in EGFr 1–6 in Caucasian patients, although they can occur in any other EGFr as well [11]. Recent studies have identified specific high-risk EGFr, including EGFr 1–6, 8, 11 and 26, which are associated with a more severe disease phenotype compared to mutations in other EGFr [8, 12].

The disease process in CADASIL affects both leptomeningeal arteries––arteries running along the surface of the brain within the subarachnoid space of the meninges––as well as medium and small arteries/arterioles and veins throughout the brain. Arterial and arteriolar lesions are primarily characterized by prominent degeneration and loss of smooth muscle cells (SMCs), which is accompanied by hyaline thickening of the arterial media due to the accumulation of amorphous eosinophilic glassy (hyaline) material. Additionally, there is a thickening and fibrosis of the adventitia, resulting from the buildup of extracellular matrix proteins [13]. In the white matter, the lesions can be very severe, with nearly complete disappearance of SMCs and significant lumen narrowing. In the basal ganglia, lesions are severe, whereas in cortical arteries, they are typically mild [13–15]. A reduction in capillary density has been also observed in the affected white matter [16], as well as a collagenosis of periventricular veins [17]. Overall, these vascular lesions are similar to those observed in sporadic cSVD, but they tend to be more severe and more diffuse in CADASIL [15]. However, there are two pathological features that are specific to CADASIL: The presence of $Notch3^{ECD}$ aggregates and granular osmiophilic material (GOM) deposits [13]. Both $Notch3^{ECD}$ and GOM deposits accumulate around vascular mural cell of arteries, arterioles, capillaries and veins throughout the brain, as well as in small peripheral vessels across various organs. Importantly, there is seemingly no associated accumulation of $Notch3^{TMIC}$ in these deposits [10]. GOM deposits are located in the basement membrane of pericytes and SMCs and contain $Notch3^{ECD}$ and other extracellular matrix proteins, including but not limited to TIMP3 (tissue inhibitor of metalloproteinase 3), vitronectin and HTRA1 [18, 19].

Monogenic and sporadic cSVD are usually considered distinct entities, with monogenic cSVD being rare, whereas cardiovascular risk factors are believed to influence only sporadic forms. However, this assumption has been recently challenged, particularly in the case of CADASIL. First, typical cysteine-altering mutations in NOTCH3 have been found to be relatively common in the general population, with a frequency ranging from 1 in 1000 in European populations to as high as 1 in 100 in certain Asian populations [20, 21]. In contrast, the prevalence of CADASIL is much lower, around 2–6 per 100 000 [22, 23]. The mutations identified in the general population are predominantly located in EGFr 7–34 and are usually not associated with

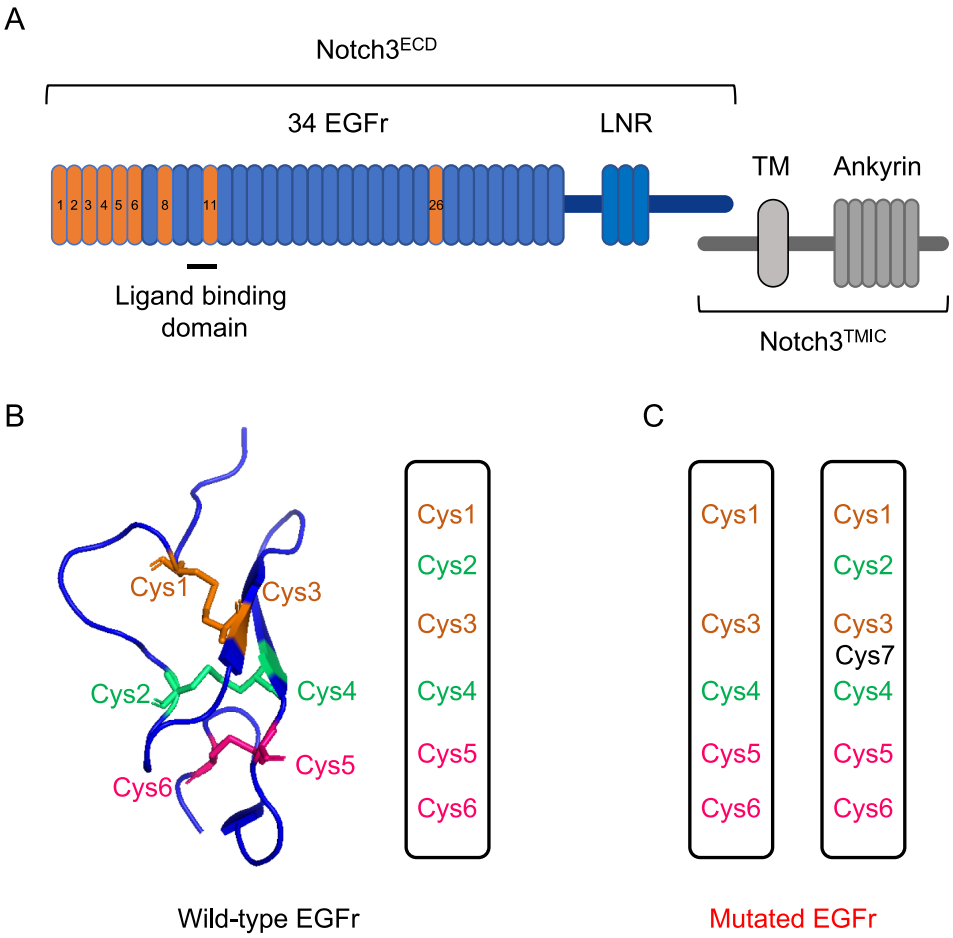

FIGURE 1 | Schematic representation of the NOTCH3 protein and CADASIL-associated mutations. (A) Schematic of the NOTCH3 protein highlighting its different domains. High-risk epidermal growth factor-like repeats (EGFr) are shown in orange. (B) Representation of a wild-type EGFr with its three disulphide bonds intact. (C) Schematic of mutated EGFr showing either the loss of a cysteine residue (left) or the gain of an additional cysteine residue (right).

a 'classical CADASIL'. This aligns with findings from CADASIL clinic cohorts, where cysteine mutations in EGFr 7–34 tend to be less common and are generally linked to a milder disease phenotype [8]. Despite this, typical cysteine-altering mutations in NOTCH3 are still associated with an increased risk of stroke and dementia in the general population [24]. Second, cardiovascular risk factors have been shown to act as modifiers of the disease phenotype in CADASIL families. For instance, hypertension has been found to double the risk of stroke and stroke to occur ~10 years earlier in active smokers [24, 25]. Interestingly, individuals with cysteine mutations in the general population are also more likely to develop stroke when cardiovascular risk factors are present [24]. Together, these data highlight the novel concept of blurring boundaries between monogenic and sporadic cSVD.

2 | NOTCH3, a Receptor Critically Involved in the Biology of Vascular SMCs

NOTCH3 is a member of the highly conserved Notch receptor family, which consists of four members (NOTCH1–4) in vertebrates. The core components of the Notch signalling pathway, in addition to the Notch receptors, include transmembrane ligands from the Delta/Jagged family and the DNA-binding protein RBPJκ [26]. Each Notch receptor is initially synthesized as a precursor protein, which is cleaved in the trans-Golgi, as it is transported towards the cell membrane. Notch receptor transmits signals by undergoing regulated proteolysis in response to binding to ligands expressed on the surface of adjacent cells. This ligand–receptor interaction imposes a mechanical force that unmasks a proteolytic site within the extracellular juxtamembranous region of TMIC. This cleavage leads to the dissociation of the 'TMIC-ECD' heterodimer, with the ECD being trans-endocytosed into the ligand-expressing cell. The TMIC is then further processed in the receptor-expressing cell, where it ultimately forms an active transcriptional complex with RBPJκ, which turns on the expression of target genes [26]. Moreover, there is compelling evidence of a 'noncanonical' mode of activation, which occurs in a ligand-independent manner as the receptor traffics through endocytic compartments [26]. Notch signalling is crucial for the development and homeostasis of almost every tissue and organ in Metazoa, playing a fundamental role in cellular differentiation, proliferation and apoptosis [26].

In late-stage embryos and adults, NOTCH3 is predominantly expressed in vascular mural cells, including arterial and venous SMCs as well as capillary pericytes, within both the brain and

peripheral organs. Remarkably, NOTCH3 is one of the few SMC markers specifically expressed in vascular SMCs, as opposed to visceral SMCs, and is primarily found in resistance arteries rather than large elastic arteries [10]. Within the vasculature, NOTCH3 activation occurs through its interaction with the ligand Delta4, expressed in arterial endothelial cells, and the ligand Jagged1, expressed in both endothelial cells and SMCs [26].

Experimental studies have demonstrated that, in the brain, NOTCH3 is critically required for every stage of intracranial vessel development in the brain. Specifically, in mice completely lacking NOTCH3 since the conception (*Notch3*$^{-/-}$), the Circle of Willis––a circulatory continuum of arterial anastomoses located at the base of the brain from which the major cerebral arteries originate––exhibits defective patterning, including variable dilations and constrictions of arterial segments, particularly in its anterior part, and a lack of functional communication between the left and right cerebral circulations [27]. Moreover, although SMCs appear normally recruited around the arterial lumen, their differentiation and postnatal maturation are strongly compromised in *Notch3*$^{-/-}$ mice. Mutant SMCs adopt a venous-like phenotype characterized by an abnormally thin shape, progressive loss of SMC contractile markers and increased extracellular matrix protein expression, and eventual cell death and loss [28–30]. Functionally, *Notch3*$^{-/-}$ arteries exhibit significantly reduced myogenic responses, and autoregulation of cerebral blood flow—a mechanism that maintains cerebral blood flow relatively stable in the face of moment-to-moment fluctuations in arterial blood pressure—is nearly abolished in response to increased arterial pressure [28]. Additionally, the glymphatic system––a fluid-clearance system involved in clearing metabolic waste from the brain interstitial space––is impaired as evidenced by reduced blood clearance of a fluorescent tracer injected into the cisterna magna in *Notch3*$^{-/-}$ mice compared to age-matched wildtype mice [30]. This defect likely stems from decreased contractility of mutant cerebral arteries, which is expected to decrease the CSF pumping into the brain [30]. Finally, recent studies in aged mice and humans suggest that age-related loss of arterial SMCs may result from age-related reduction in NOTCH3 expression, emphasizing its role in SMC survival [30]. Despite high expression of NOTCH3 in pericytes, *Notch3*$^{-/-}$ mice show unaffected capillary pericyte number and coverage, and the blood–brain barrier is preserved [29]. Together, these findings highlight that NOTCH3 is critically required for the development, homeostasis and function of arterial SMCs.

The results of these experimental studies have clinical relevance, as biallelic loss-of-function mutations in human NOTCH3 cause an extremely severe form of cSVD. However, this rare condition differs from CADASIL by its childhood onset, possible association with cutaneous manifestations and the absence of GOM deposits [31, 32].

3 | Modelling CADASIL in the Mouse

Various genetic manipulations have been attempted in mice to model CADASIL, including constitutive or conditional expression of mutant NOTCH3 under a SMC promoter, expression driven by the entire *Notch3* promoter or introduction of a typical cysteine mutation into the endogenous *Notch3* gene on different genetic backgrounds [33–36]. Among the most commonly used genetic backgrounds for developing mouse models are the FVB/N strain, known for its high efficiency in transgene expression, and the C57Bl/6 strain, which is widely employed in biomedical research due to its well-characterized genome and broad application in genetic studies. Most mouse models, except the Notch3$^{R142C/+}$ model (which may not have been optimally assessed), develop the pathological hallmarks of CADASIL: vascular deposition of GOM or Notch3^{ECD} aggregates, both of which worsen with age. Notably, Notch3^{ECD} accumulation is one of the earliest manifestations, appearing as soon as a few days after birth [37]. This observation aligns with findings in CADASIL patients, where Notch3^{ECD} accumulation, detectable in skin biopsies, precedes clinical and neuroimaging manifestations [38]. Strikingly, analysis of a transgenic mouse model expressing a mutant human NOTCH3 protein revealed that wildtype (murine) and mutant (human) Notch3^{ECD} both participate in the formation of aggregates, as shown using antibodies specific to murine and human forms of Notch3^{ECD} (Figure 2) [39]. This finding corroborates in vitro studies showing that wildtype and mutant peptides containing EGFr1–5 of NOTCH3 can multimerize to form aggregates [40]. It also suggests a thiol–disulphide exchange mechanism in Notch3^{ECD} aggregation. In this process, a free thiol from a mutant Notch3^{ECD} engages with a disulphide-bonded wildtype Notch3^{ECD} molecule, freeing a thiol group from the wildtype molecule; this freed thiol group can then react with another Notch3^{ECD} molecule, perpetuating the aggregation process [41].

Among the eight different CADASIL mouse models generated to date [33–36], convincing evidence of cerebral white matter lesions has only been observed in a FVB-Tg*Notch3*^{R169C}, a transgenic model developed on an FVB/N background, which overexpresses a rat NOTCH3 protein carrying the archetypal Arg169Cys mutation about fourfold over the endogenous murine NOTCH3 [37]. In these mutant mice, white matter lesions are detectable from 6 months of age, predominantly affecting the myelin sheath and the inner tongue, indicative of a primary myelin injury [42]. However, no lacunar infarcts or microbleeds have been observed in any of the CADASIL mouse models, and none have been systematically assessed for cognitive deficits.

The functional integrity of the cerebrovasculature has been examined in-depth primarily in the FVB-Tg*Notch3*^{R169C} model. Ex vivo studies reveal that both pial and penetrating cortical brain arteries exhibit diminished constriction and blunted membrane potential depolarization in response to pressure. This dysfunction is attributed to an increased number of voltage-gated potassium K_V1 channels in cerebral arterial SMCs [37, 43]. Additionally, mutant pial arteries demonstrate reduced distensibility and inward remodelling, despite the normotensive state of mutant mice [44]. In vivo, autoregulation of cerebral blood flow is impaired in mutant mice, with the lower and upper limits of autoregulation shifting to higher and lower arterial blood pressures, respectively [37, 45]. Neurovascular coupling—the mechanisms that mediate neuronal activity-dependent increases in blood perfusion—is defective due to impaired capillary-to-arteriole signalling. This defect is caused by reduced activity of the capillary endothelial inward-rectifier K^+ channel, Kir2.1 [46]. Notably, all functional defects are apparent in mutant mice starting at 6 months of age. Finally, a comprehensive analysis

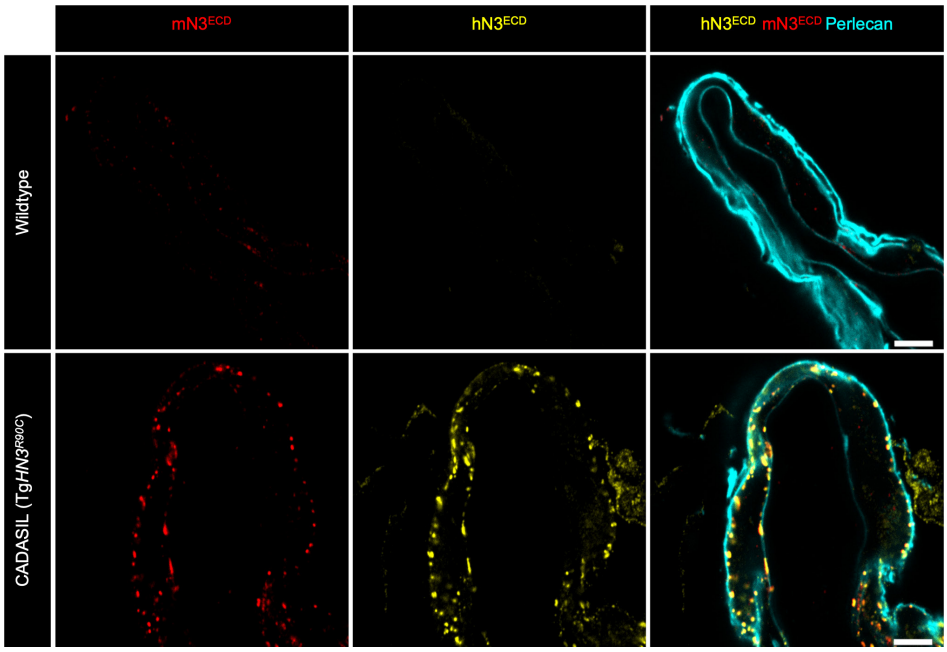

FIGURE 2 | Notch3^{ECD} aggregates contain both wild-type and mutant Notch3^{ECD}. Representative confocal images of brain arteries from a wild-type mouse (expressing murine NOTCH3) and a CADASIL mouse (Tg*HN3*^{R90C}, expressing human NOTCH3 with the R90C mutation alongside endogenous murine NOTCH3) stained with antibodies against mouse Notch3^{ECD} (mN3^{ECD}, red), human Notch3^{ECD} (hN3^{ECD}, yellow) and a basement membrane marker (Perlecan, cyan). The CADASIL artery shows Notch3^{ECD} aggregates containing both mutant human and wild-type murine Notch3^{ECD}.

of blood–brain barrier (BBB) integrity, including assessments of parenchymal staining of endogenous fibrinogen and leakage of intravenously injected tracers of various sizes, revealed no evidence of BBB leakiness in mutant mice [16].

As mentioned above, a key pathological feature of CADASIL (and cSVD in general) is the progressive degeneration and loss of SMCs in brain arteries. Until recently, pathological analyses of brain arteries in clinically relevant CADASIL mouse models with cysteine-altering mutations failed to detect any arterial SMC loss. This was notably the case for the well-characterized FVB-Tg*Notch3*^{R169C} model [37]. Recent work suggests that this failure stemmed from both technical limitations and genetic background differences. The Tg*Notch3*^{R169C} model, originally developed on an FVB/N background, was backcrossed onto the C57Bl/6 background (Bl6-Tg*Notch3*^{R169C}) based on prior studies indicating that this background may be more permissive for the expressivity of cSVD phenotypes [47]. Conventional histological analyses commonly employ a limited number of 7- to 12-μm-thick sections that insufficiently sample brain arteries. Instead, using 200-μm-thick brain sections immunostained with appropriate SMC markers allows to image long arterial segments (Figure 3A). Using this approach and optimized imaging modalities, our lab successfully detected SMC lesions in Bl6-Tg*Notch3*^{R169C} mice for the first time (Figure 3B). These lesions, observed as early as 6 months of age, were present in pial, cortical and subcortical arteries and worsened with age [39]. Importantly, the lesions were focal and segmental, explaining why they went undetected with conventional histological techniques [39]. Assessing arterial defects in the brain at the cellular level remains challenging, and the focal, segmental nature of the lesions makes quantification particularly labour intensive. To address this, our lab utilized the retina—a developmental extension of the brain with a vascular network resembling the cerebrovasculature. Remarkably, the superficial retinal vascular network in Bl6-Tg*Notch3*^{R169C} mice exhibited arterial SMC lesions strikingly similar to those in the brain [39]. Functional cerebrovascular changes in Bl6-Tg*Notch3*^{R169C} mice have yet to be reassessed to determine whether they are as severe or more pronounced compared to the FVB-Tg*Notch3*^{R169C} mice.

4 | Controversy in CADASIL Pathogenesis: Loss of Function or Neomorphic Effect?

Whether vascular changes in CADASIL result from a defect in NOTCH3 signalling (loss of function) or from the deleterious effects of Notch3^{ECD} aggregation (neomorphic effect) has long been debated. This has driven research in two opposing directions: one favouring reduced NOTCH3 signalling as the root cause and the other emphasizing the pathological role of Notch3^{ECD} accumulation.

4.1 | Reasons for the Controversy

1. Lack of a suitable SMC cellular model

To date, no SMC model convincingly reproduces both Notch3^{ECD} aggregation and SMC defects. Importantly, a valid model must exhibit Notch3^{ECD} accumulation, the earliest abnormality in CADASIL, which occurs prior to SMC degeneration, and without concurrent Notch3^{TMIC} accumulation.

2. Lack of a mouse model exhibiting both pathologies

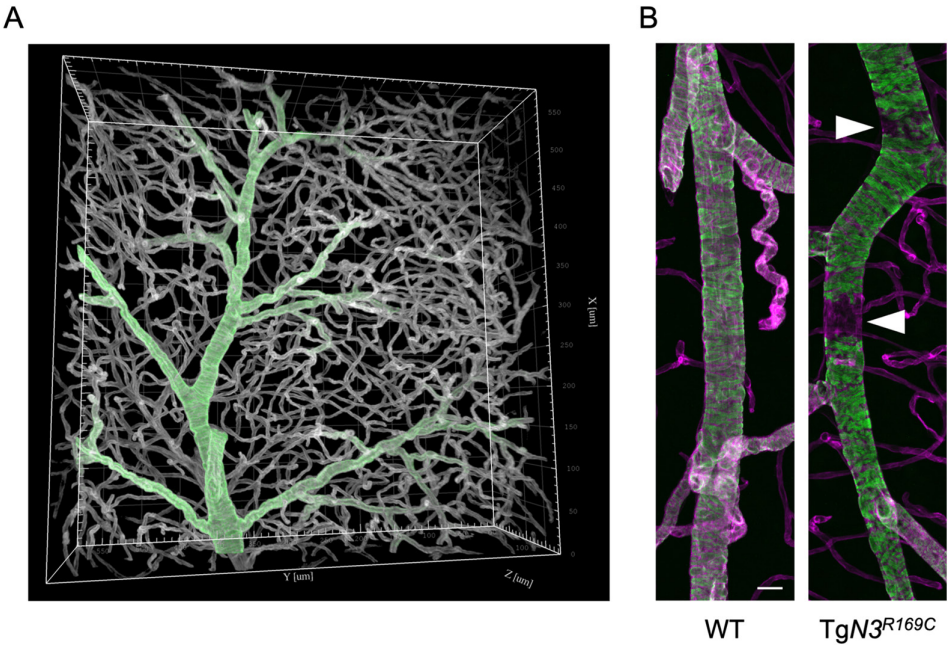

FIGURE 3 | Thick brain slices facilitate the analysis of long arterial segments and detection of focal arterial lesions in CADASIL brains. (A) Confocal image of a 200-μm-thick brain section from a wild-type mouse stained for α-SMA (green) and perlecan (white). A 3D rendering (Imaris software) shows a long α-SMA+ vascular segment within a single slice. (B) Confocal images of 200-μm-thick brain sections stained for α-SMA (green) and perlecan (purple). Representative subcortical brain arteries from 12-month-old wild-type (WT) and B6-Tg*Notch3*^{R169C} (Tg*N3*^{R169C}) mice are shown. The mutant artery exhibits SMC defects, indicated by discontinuities or gaps in α-SMA staining (arrowhead).

Until very recently, no mouse model developed both Notch3^{ECD} aggregation and SMC defects.

3. Overinterpretation of NOTCH3 loss-of-function phenotypes

The observations that *Notch3*$^{-/-}$ mice develop arterial SMC loss and that biallelic nonsense mutations in NOTCH3 cause a recessive form of cSVD in human fuelled the hypothesis of reduced NOTCH3 signalling. However, although these findings highlight the role of NOTCH3 in vascular health, they do not elucidate the mechanism by which CADASIL mutations cause pathology. Similarity does not imply causality.

4. Distinct effects of CADASIL mutations on NOTCH3 receptor activity, despite the highly stereotyped nature of mutations.

The impact of CADASIL mutations has been investigated through both in vitro and in vivo studies. In vitro analyses used cells that did not recapitulate Notch3^{ECD} accumulation as typically seen in patients. In vivo studies have employed mouse models that exhibit Notch3^{ECD} accumulation but no associated arterial pathology. Most mutations, including those located in EGFr1–6, appear to preserve or slightly enhance NOTCH3 receptor activity, even amidst Notch3^{ECD} accumulation. Conversely, mutations located in or around EGFr10–11––the ligand binding domain of NOTCH3––accounting for ~5%–10% of patients, result in a loss of receptor signal transduction activity, regardless of Notch3^{ECD} accumulation [34, 48–51]. Here, it is important to stress that CADASIL mutations occur mainly at the heterozygous state. Therefore, a patient carrying a mutation in or around EGFr10–11 will have 50%–50% of inactive and active NOTCH3 receptors. Notably, mutations in EGFr10–11 have not been documented in homozygous states among CADASIL patients.

4.2 | Key Findings Supporting a Neomorphic Effect

1. A series of functional studies combined with pharmacological and genetic interaction experiments identified an excess of tissue inhibitor of metalloproteinases-3 (TIMP3)—which coaccumulates with Notch3^{ECD} in GOM deposits—as a causal factor in cerebrovascular functional deficits observed in CADASIL mouse models. Specifically, defective myogenic tone and impaired neurovascular coupling in Tg*Notch3*^{R169C} mice stem from an excess of TIMP3 protein that suppresses the activity of the ADAM17/HB-EGF/(ErbB1/ErbB4) pathway and results in an increased density of K_V1 channels in arterial SMCs and a decreased activity of Kir2.1 in capillary endothelial cells, respectively. Consequently, reducing TIMP3 expression has been shown to restore myogenic tone and neurovascular coupling in mutant mice [46, 52].
2. Overcoming the challenge of detecting arterial pathology in CADASIL mouse models, a recent study explored the relationship between Notch3^{ECD} accumulation, NOTCH3 activity and arterial SMC loss [39]. The study focused on the Arg169Cys mutation, situated in EGFr4—a mutational hotspot associated with a severe and penetrant phenotype. The first experiment involved profiling the transcriptome of brain arteries dissected from Bl6-Tg*Notch3*^{R169C} mice, at an age when arterial

pathology was present, and from *Notch3*$^{-/-}$ mice in order to assess the expression of *Notch3*-regulated genes on a genome-wide scale. Findings revealed no overlap between genes downregulated in *Notch3*$^{-/-}$ mice and those upregulated in Bl6-Tg*Notch3*^{R169C} mice, and vice versa, suggesting that reduced NOTCH3 activity is not the underlying mechanism of CADASIL arterial pathology. The second experiment examined the correlation between the extent of Notch3^{ECD} accumulation and the severity of arterial pathology by comparing two different CADASIL mouse models expressing varying levels of the same mutant NOTCH3 receptor, on the same genetic background. Results indicated that both Notch3^{ECD} accumulation and arterial pathology were more pronounced in transgenic mice overexpressing four copies of the mutant receptor on a wild-type *Notch3* background than in knock-in mice expressing the mutant receptor at the homozygous state. The final experiment aimed to establish a causal link between Notch3^{ECD} aggregation and arterial pathology. By leveraging the fact that wild-type Notch3^{ECD} coaccumulates with mutant Notch3^{ECD}, genetic reduction of murine *Notch3* in the Bl6-Tg*Notch3*^{R169C} model was used to decrease Notch3^{ECD} accumulation, without affecting mutant NOTCH3 expression. This approach involved creating Bl6-Tg*Notch3*^{R169C} mice haploinsufficient for murine *Notch3* (Bl6-Tg*Notch3*^{R169C};*Notch3*$^{+/-}$). The results demonstrated that a reduction in Notch3^{ECD} accumulation in Bl6-Tg*Notch3*^{R169C};*Notch3*$^{+/-}$ mice, compared to Bl6-Tg*Notch3*^{R169C};*Notch3*$^{+/+}$ mice, was associated with a mitigation of arterial pathology [39].

4.3 | Epilogue

Collectively, these findings provide strong evidence against a loss-of-function mechanism and point to Notch3^{ECD} aggregation as the key driver of CADASIL vascular pathology. The question remains whether EGFr10–11 mutations cause pathology through a similar mechanism. The conclusion that Notch3^{ECD} accumulation is central to CADASIL aligns with clinical studies linking severe mutations in high-risk EGFr domains to greater Notch3^{ECD} accumulation but not to modification in NOTCH3 activity [12, 53]. Several questions remain to be addressed, including the following: Why is there a selective accumulation of Notch3^{ECD}, that is, without associated accumulation of Notch3^{TMIC}? Is there a single or several Notch3^{ECD} species that accumulate, and do all these species have deleterious effects? The toxic effect of Notch3^{ECD} on SMCs seems to be indirect because there is a substantial delay between Notch3^{ECD} accumulation and arterial SMC degeneration. Why does explain this delay and why SMCs degenerate?

5 | Implication for Therapeutic Developments and Perspectives

CADASIL remains without effective, proven treatments. Management is primarily focused on mitigating risk factors, including hypertension control and smoking cessation. Interestingly, a recent study conducted from a cohort of CADASIL patients prospectively recruited over a 23-year period showed improvements in disease outcomes, particularly with increasing age at onset for the first stroke and dementia [54]. It is possible that such an improvement in disease severity is related to a reduction in risk factor exposure and better treatment of risk factors nowadays [54].

CADASIL patients have been included as a subgroup in a recent clinical trial of cSVD. The TREAT-SVDs trial is a randomized, open-label, blinded-endpoint crossover study that investigated the effects of different antihypertensive drug classes (calcium channel blockers, angiotensin receptor blockers and beta blockers) on cerebrovascular reactivity (CVR) in patients with cSVD [55]. The results revealed that 4 weeks of treatment with amlodipine, losartan or atenolol had comparable effects on CVR in patients with sporadic cSVD. In contrast, a significant improvement in CVR was observed in CADASIL patients, with both amlodipine and losartan showing better effects than atenolol. This significant effect in the CADASIL group may be attributed to the relatively homogeneous nature of this cohort compared to the more heterogeneous sporadic cSVD group. The findings underscore the value of including CADASIL patients in clinical trials on cSVD, as their inclusion provides a more controlled group that could yield clearer insights into treatment efficacy. Furthermore, studying CADASIL—a younger-onset form of cSVD—offers the advantage of minimizing confounding factors like coexistent neurodegenerative changes, which are more prevalent in the older population with sporadic cSVD. However, although CADASIL and sporadic cSVD may share some clinical features, they may also be driven by distinct underlying mechanisms. That said, emerging evidence increasingly suggests shared pathophysiological features [56]. Several Phase II clinical trials are currently investigating potential treatments for CADASIL. These trials include Cerebrolysin (ClinicalTrials.gov ID: NCT05755997), a porcine brain-derived peptide preparation with properties similar to endogenous neurotrophic factors. Another trial is evaluating Tocotrienols (NCT04658823), a derivative of vitamin E known for its antioxidant properties. Additionally, Adrenomedullin (NCT06072118), a vasoactive peptide with angiogenic, vasodilatory, anti-inflammatory and antioxidative effects, is being tested. Although these agents have interesting mechanisms of action, the rationale for their specific application in CADASIL remains limited.

Accumulating evidence indicates that Notch3^{ECD} accumulation is a major driving force in CADASIL pathology. Consequently, strategies aimed at reducing Notch3^{ECD} levels in brain arteries are considered highly promising for clinical development. Immunotherapies, which are highly effective at neutralizing or clearing extracellular protein aggregates, have become a central focus of research in neurodegenerative diseases. Passive immunization involves chronic administration of monoclonal antibodies, whereas active immunization utilizes specific antigens to stimulate an adaptive immune response. Numerous antibodies have entered clinical trials [57], and some have recently been approved by the US Food and Drug Administration for Alzheimer's disease [58]. In CADASIL, immunotherapy targeting Notch3^{ECD} has been explored in mouse models. Passive immunization against Notch3^{ECD} improved cerebrovascular function in FVB-Tg*Notch3*^{R169C} mice. However, it failed to clear Notch3^{ECD} deposits in brain vessels despite achieving almost complete

antibody engagement with the deposits [59]. Active immunotherapy showed significant reduction in Notch3^{ECD} deposits in brain capillaries but did not impact deposits in brain arteries of Tg*Notch3*^{R182C} mice, a model expressing the Arg182Cys mutation [60]. These findings suggest that although immunotherapy shows potential in addressing some pathological features of CADASIL, its overall efficacy in clearing Notch3^{ECD} deposits remains limited. Additional studies are needed to determine whether immunotherapy is a viable and effective treatment strategy for CADASIL.

Antisense oligonucleotides (ASOs) are short synthetic nucleic acid analogues designed to modulate the expression of targeted genes through various mechanisms. Numerous ASO-based therapies are under clinical development for neurological diseases, with some are already approved for clinical use, such as treatments for spinal muscular atrophy and Duchenne muscular dystrophy [61]. One mechanism of action for ASOs is exon skipping, which enables selective skipping of specific exons during mRNA processing. In 2016, Lesnik Oberstein and colleagues have proposed to use antisense oligonucleotide-mediated exon skipping of specific exons of NOTCH3 to restore an even number of cysteine residues in mutant NOTCH3, a concept termed 'cysteine correction'. Although proof-of-concept has been demonstrated in cultured cells, ability of this approach to reduce NOTCH3 accumulation in vivo has not yet been established [62]. Another mechanism of ASOs involves recruiting RNase H, which induces transcript cleavage and degradation [63]. This approach is particularly relevant for CADASIL, as it could reduce Notch3^{ECD} expression by targeting both mutant and wild-type NOTCH3 transcripts, circumventing the need for allele-specific targeting. Because both mutant and wild-type NOTCH3 abnormally accumulate in CADASIL, this strategy holds significant therapeutic potential. Further preclinical studies are urgently needed to evaluate the efficacy of these ASO-based approaches in reducing Notch3^{ECD} levels and improving CADASIL outcomes.

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Conflicts of Interest

Financial reimbursement for a scientific advisory board was provided by Biogen.

Data Availability Statement

Data sharing is not applicable to this article, as no datasets were generated or analysed during the current study.

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