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REVIEW

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Signaling pathways and molecular mechanisms involved in the onset and progression of cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL); a focus on Notch3 signaling

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Abstract

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is an autosomal-dominantly inherited cerebral small-vessel disease (SVD). CADASIL has diverse clinical features such as migraine with aura, dementia, and recurrent strokes, and is caused by a pathogenic mutation in the *NOTCH3* gene which encodes a transmembrane receptor found in smooth muscle cells of small arteries and pericytes of brain capillaries. Pathogenic mutations alter the number of cysteine residues in the extracellular domain of *NOTCH3*, leading to the abnormal accumulation of granular osmiophilic material in the vessels of affected individuals. In addition, potential signaling pathways, such as transforming growth factor beta (TGF-β), may be involved in pathogenesis of the disease. This review aims to elucidate these mechanisms, particularly *NOTCH3*, in the context of CADASIL pathogenesis, providing insight into the role of *NOTCH3* signaling and discussing the significance of these pathways for potential future therapeutic interventions in CADASIL patients.

Key points

- CADASIL is a rare hereditary cerebral small-vessel disease caused by mutations in *NOTCH3* and its associated factors.
- Understanding the role of the *NOTCH3* signaling pathway may help in understanding that pathomechanisms of CADASIL and its manifestations.
- Recognizing the potential involvement of other signaling pathways, including TGF-β, that may contribute to the development or progression of CADASIL is important.
- Exploring the roles of these key signaling pathways associated with CADASIL provides a foundation for advancing management and treatment strategies.

Keywords CADASIL, NOTCH3, Signal transduction, Genetics, Cerebral small vessel disease

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Background

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a hereditary cerebral small-vessel disease, resulting in stroke, progressive cognitive impairment, migraine with aura (MA), and psychiatric disturbances [16, 18, 122]. Despite its rare occurrence in the general population, CADASIL remains the most common monogenic small-vessel disease. CADASIL is often considered a 'pure' model of cerebral small-vessel disease (cSVD) and vascular dementia [18, 22, 66] due to its occurrence without other concomitant age- and AD-related pathologies. CADASIL has commonly been reported as having prevalence of around 2–5/100 000 individuals, but recent advances in genetic testing, such as large-scale genome-wide association studies (GWAS) have suggested that the prevalence of *NOTCH3* mutations may be significantly higher than previously indicated due to underdiagnosis and underreporting [104, 132, 176], especially in certain cohorts such as Asian populations.

While CADASIL is primarily caused by a pathogenic variant in the *NOTCH3* gene, the underlying mechanism causing the disease's development and progression remains unclear [117]. The *NOTCH3* variant results in protein misfolding, leading to the accumulation of free $NOTCH3^{ECD}$ at the plasma membrane of vascular smooth muscle cells (VSMCs) and pericytes. A key pathological feature of CADASIL is the accumulation of granular osmiophilic material (GOM) on or near the degenerating vessel wall (Fig. 1) [95, 124]. GOM is comprised of oligomerized $NOTCH3^{ECD}$ and extracellular matrix proteins, in particular tissue inhibitor of metalloproteinase 3 (TIMP3) and clusterin, but also endostatin, vitronectin, serum amyloid P component (SAP), and latent transforming growth factor binding protein 1 (LTBP- 1). SAP co-localization with $NOTCH3^{ECD}$ in GOM hints at amyloid-like deposition, though its role remains unclear [108]. Although GOM has extensively been associated with CADASIL pathology, the pathogenic role of GOM

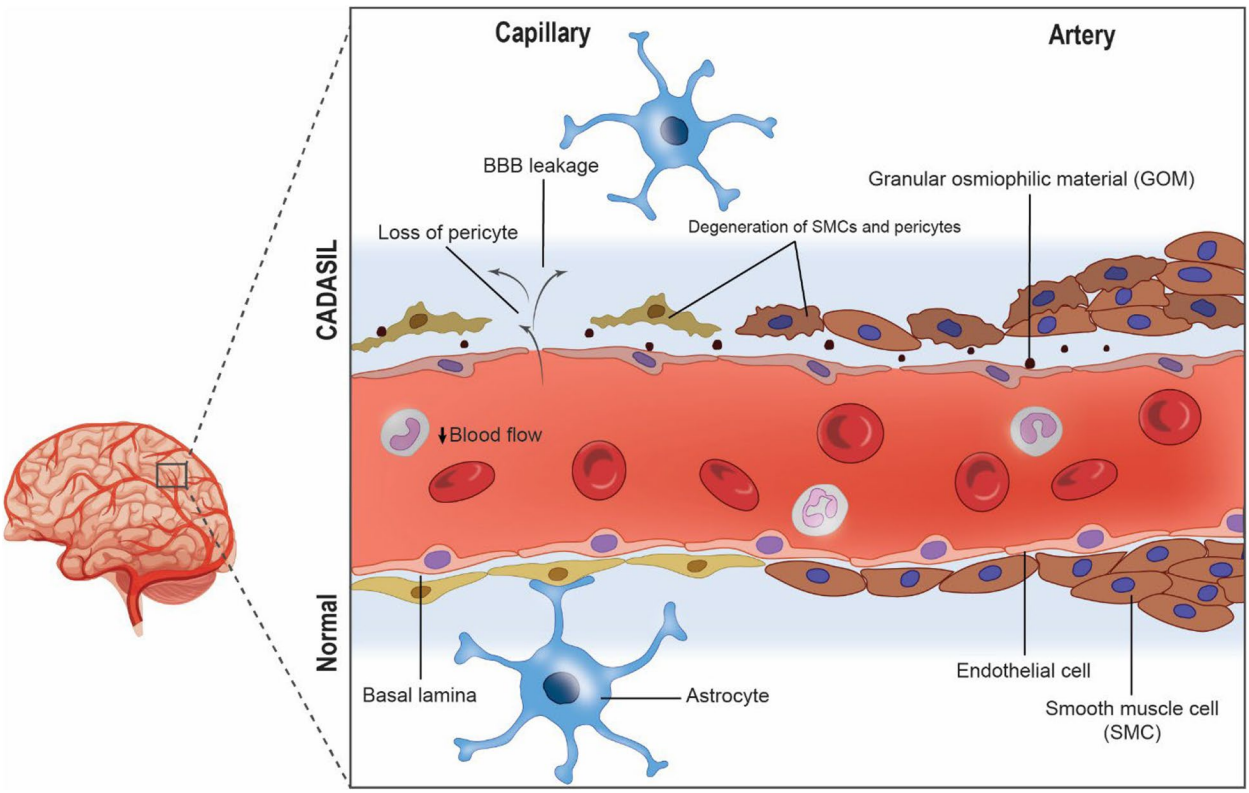

Fig. 1 Pathogenesis of CADASIL. The initial and progressive loss of anchorage of artery-type smooth-muscle cells (aSMCs) and pericytes to adjacent extracellular matrix cells plays a central role in the pathogenesis of CADASIL, leading to an early increase in the sub-endothelial space. The evolution of aSMC alterations includes dramatic separation of different wall cells and the presence of granular osmiophilic material (GOM). Brain capillary changes involve the detachment and fragmentation of pericytes, which leads to progressive loss of endothelium-pericyte contacts. Morphological observations suggest two stages in CADASIL: first, the impairment and loss of contacts between endothelium and pericyte, predicting neurovascular and gliovascular dysfunction; second, the degeneration of capillary pericytes impacting on contractile function, resulting in apparent blood–brain barrier (BBB) damage and hypo-permeability. These changes in aSMCs and pericytes contribute to vessel-wall alterations, decreased vascular compliance, and microbleeds. Dilatation of perivascular spaces is also characteristic, indicating widespread pericyte involvement

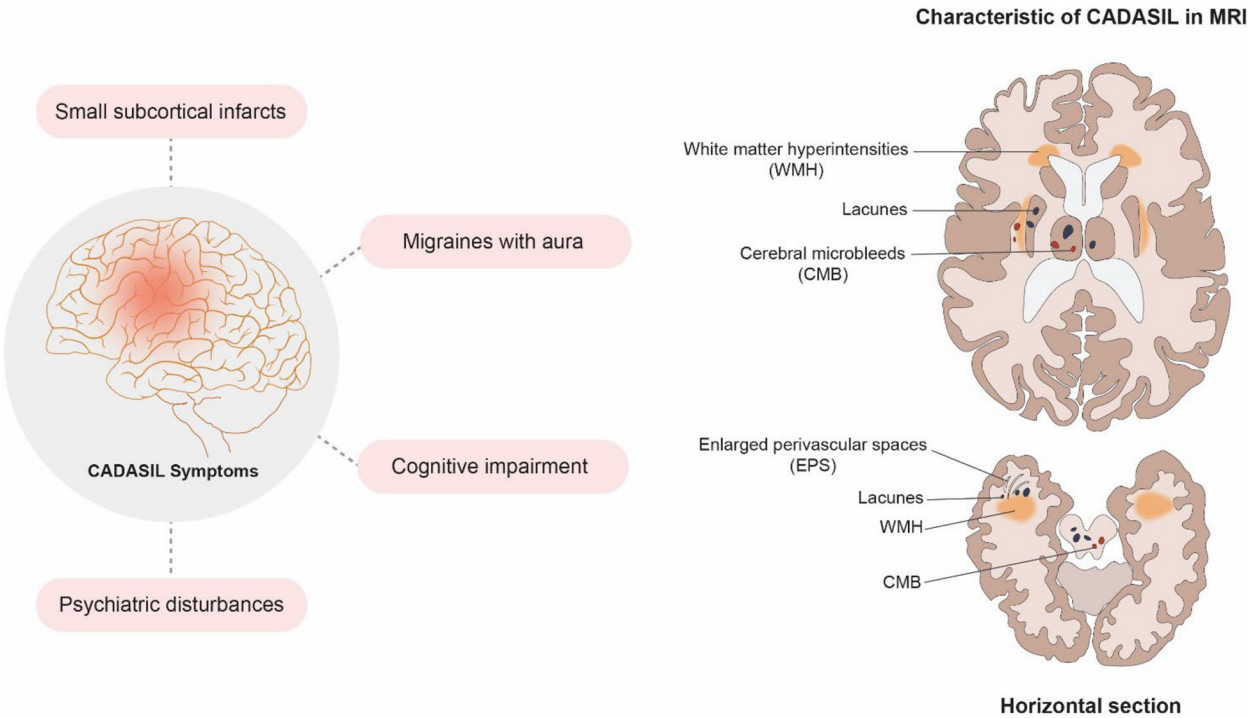

Fig. 2 Clinical presentations of CADASIL. White matter hyperintensity (WMH), lacunes, cerebral microbleeds (CMB), and enlarged perivascular spaces (EPS) are hallmark features in CADASIL. WMHs are frequently seen in the anterior temporal lobes, external capsules, and superior frontal gyrus. Moreover, lacunes are visible in the anterior temporal lobes, brainstem, lentiform nucleus, and thalamus. Microbleeds are also located in the brainstem, thalamus, and external capsules

deposits in CADASIL is still being explored. In addition to GOM toxicity, there are other proposed mechanisms which may be involved in CADASIL [44]. Firstly, abnormal *NOTCH3* signaling has been studied in several investigations [117], but a consistent conclusion has not been reached. Changes occur in receptor processing and presentation, ligand binding, and signal transduction [108, 183]. Secondly, CADASIL may be related to dysregulation of the signaling pathway of transforming growth factor-β (TGF- β) [72]. Thirdly, although not causative in nature, there are environmental factors (e.g., hypertension and smoking) which have been suggested to influence the CADASIL phenotype [105, 183].

The current review aims to explore the understanding of pathophysiological mechanisms in CADASIL, by investigating the *NOTCH3*-related signaling disruption and potential involvement of additional pathways.

Whilst CADASIL varies greatly between and within patients, the predominant signs and symptoms include MA, subcortical ischemic events, psychiatric disturbances, and cognitive decline (Fig. 2) [58, 168, 176]. When present, MA tends to be the first symptom, with an average age at onset of 30 years [16, 88, 93, 157]. MA is usually observed with visual or sensory symptoms and occurs less than once a month in majority of patients [20, 47, 191]. MA, prevalent in 40% of CADASIL patients, may stem from *NOTCH3*-driven cortical spreading depression [158]. Interestingly, before the age of menopause, MA appears to be a more frequent feature of CADASIL in women than men [48]. MA is a risk factor for ischemic stroke, possibly due to genetic predisposition, hypercoagulability, increased platelet aggregability, and hyperviscosity [20, 47, 191]. Ischemic stroke-induced motor dysfunction, apathy, and cognitive decline often arise between 40 and 70 years [29, 132, 169]. Up to 70% of biopsy-confirmed patients experience recurring ischemic events, presenting in diverse forms [33, 97, 122].

MRI scans of CADASIL patients typically show small lacunar infarcts and severe white matter hyperintensities (WMHs), the latter mostly in periventricular white matter, with involvement of the anterior temporal pole and external capsule [76]. Individuals with CADASIL also present with cerebral microbleeds (CMB) and enlarged perivascular spaces (EPS) [19], as well as global and cortical atrophy [17]. Cognitive deficits in executive function and processing speed are a central symptom of the disease which eventually progress into vascular dementia (VaD) [17, 140, 150, 151].

The clinical presentation of CADASIL exhibits high variability in terms of severity, the predominance of types of features, and progression [114, 183]. The evidence suggests that late-onset CADASIL with a mild phenotype is not uncommon [183]. The phenotypic spectrum of *NOTCH3* mutations has expanded to include mild cerebral small-vessel disease (SVD), an attenuated and delayed-onset CADASIL phenotype, as well as the classical CADASIL with middle-age-onset stroke and dementia [114, 142, 143].

While the position of *NOTCH3* mutations plays a substantial role in CADASIL severity, there remains evident phenotypic variation among patients carrying the same *NOTCH3* mutation, even within the same ethnic group [16, 183]. Pescini et al. reported a CADASIL patient with the p.Cys1131 Trp mutation who had their first minor stroke at 79 [126], while Lee et al. described a male with the p.Arg544 Cys mutation experiencing his first lacunar stroke at 86 and three asymptomatic carriers with subclinical leukoencephalopathy between 59–67 [84]. These studies indicate that late-onset CADASIL with a mild phenotype is increasingly recognized. The phenotypic spectrum now includes mild cerebral small-vessel disease, delayed-onset CADASIL, and classical CADASIL with middle-age onset stroke and dementia. Genotype–phenotype correlations show that p.Arg544 Cys mutation is associated with milder disease severity and later onset compared to other *NOTCH3* mutations. For instance, Taiwanese p.Arg544 Cys carriers had a 9.1-year delay to first symptom onset and fewer white-matter hyperintensities. Similarly, Japanese patients with the p.Arg75Pro mutation had a higher age at symptom onset and fewer anterior temporal pole involvements [87, 167].

Mutations in the exons 2–24 of *NOTCH3* with notable mutation hotspots in exons 2–6. In France, the UK, and Germany, 55%–72.9% of mutations are in exon 4 [121]. Dutch families also show half of the mutations in exon 4 and 15% in exon 11. In Japan, p.Arg133 Cys and p.Arg182 Cys in exon 4, and p.Arg75Pro in exon 3, are common, while Eastern China frequently sees the p.Arg607 Cys mutation in exon 11. Central Italy has a higher prevalence of p.Arg1006 Cys in exon 19. The p.Arg544 Cys mutation in exon 11 accounts for significant proportions of CADASIL cases in Jeju Island (90.3%), Taiwan (70.5%), and Southeastern China (15.5%), indicating a founder effect, where a single mutation in a common ancestor is prevalent in descendants [167]. This effect is also seen with the p.Arg133 Cys mutation in Finland and Kyushu, Japan. The p.Arg75Pro mutation appears unique to East Asia, suggesting regional founder effects [148]. These findings highlight the importance of population-specific genetic analysis for CADASIL [183].

Phenotypic variability in CADASIL arises from both genetic and environmental interplay. Mutations in epidermal growth factor-like repeats (EGFr) 1–6 correlate with earlier, severe phenotypes like stroke, while EGFr 7–34 variants often present milder, delayed-onset disease [183]. Environmental factors, such as hypertension, exacerbate vascular stiffness, accelerating GOM deposition and WMH burden, particularly in genetically predisposed individuals, while smoking amplifies oxidative stress, synergizing with *NOTCH3* mutations to worsen cognitive decline [154].

The role of *NOTCH3* in CADASIL

Each EGFr subunit of the NOTCH3^{ECD} contains six cysteine residues which partner to form disulfide bridges. When there is an uneven number of cysteines, as is common in CADASIL-causing *NOTCH3* mutations, the unpaired cysteine disrupts normal EGFr function and causes protein misfolding [67, 70]. It is thought that 98% of mutations in CADASIL are missense mutations leading to the gain or loss of a cysteine residue [144], though there are some reports of splice site mutations and small inframe deletions [23, 24, 37, 87]. The pathogenicity of non-cysteine altering mutations is as yet unknown. In CADASIL, the free NOTCH3^{ECD} accumulates at the plasma membrane of VSMCs and pericytes within or near GOM [65, 117, 182]. The aggregation of GOM is a key contributor to CADASIL pathology [62]. Over 300 pathogenic variants have been identified in patients with CADASIL, reported across the 34 EGFr domains. The strongest known modifying factor in disease presentation is position of the *NOTCH3* pathogenic variant [145]. The most severe CADASIL phenotypic presentation has been associated with variants in the first six EGFr domains, as compared to variants domains 7–34 [20, 142, 147]. However, CADASIL development is not limited to pathogenic variants in the first 6 EGFr domains, suggesting this cannot fully explain the disease-causing mechanism [49].

A study on neuroimaging and clinical features in individuals with cysteine-altering *NOTCH3* variants from the UK Biobank [145] showed that CADASIL represents the severe and rare end of the *NOTCH3*-associated disease spectrum. UK Biobank data from over 200 000 individuals revealed a higher-than-expected prevalence of *NOTCH3* variants (1 in 450 individuals) in the general population [21]. *NOTCH3* variants are frequently present in the general population, but individuals often exhibit a milder SVD phenotype or no indication of white matter disease [5, 21]. These large population-based studies suggest a broad spectrum of SVD severity influenced by position of the variant in the EGFr domains. Individuals with EGFr 7 to 34 variants still face an increased risk of cognitive deficits, possibly linked to

the burden of WMH [145]. While CADASIL variants affect the NOTCH3^{ECD}, leading to abnormal accumulation, the study acknowledges that variant position alone does not fully explain observed heterogeneity. Instead, there might be an influence of vascular risk factors alongside genetic modifiers [21].

CADASIL can be caused by mutations in the *NOTCH3* gene, located on chromosome 19 [65, 66]. The *NOTCH3* gene is one of four mammalian *NOTCH* homologs, essential for various developmental processes such as vasculogenesis, cell proliferation, fate determination, and apoptosis, which are expressed during VSMCs maturation and differentiation [31, 67, 70, 175, 183]. *NOTCH3* contains 33 exons which encode a transmembrane protein called NOTCH3. The NOTCH3 protein is comprised of an ectodomain (NOTCH3^{ECD}) and a C-terminal comprised of a transmembrane domain and an intracellular domain (NOTCH3^{ICD}) [144]. The NOTCH3^{ECD} contains 34 EGFrs, the ligand-binding site indicated at EGFr 10–11 [67, 125]. The most pathogenic mutations occur in exons 2–24, and some studies have revealed that mutations outside the EGFr coding region (exons 25–33) may also contribute to CADASIL [26, 60]. When the ligand binds to NOTCH3^{ECD}, this complex is dissociated into the interstitial space between cells. This process exposes the C-terminus for cleavage by A disintegrin and metalloprotease (ADAM) 10 and ADAM17 enzymes, and finally by γ-secretase which frees the NOTCH3^{ICD} from the transmembrane domain. The resulting NOTCH3^{ICD} enters the nucleus where it regulates gene transcription of target genes, assisting in VSMC homeostasis [37, 164].

Moreover, exons 2–6 are regarded as mutation hotspots, exhibiting ethnic variations. Among patients with CADASIL from France, the UK, and Germany, 55%–72.9% of the mutations were found in exon 4 of *NOTCH3*, while exons 3, 5, and 6 followed as the next most common locations in order [63, 175]. Studies mention that mutations that impact the ligand-binding domain of *NOTCH3* can lead to hypoactive and hyperactive *NOTCH3* signaling, which can contribute to the disruption of vascular integrity and function [12, 25]. This disrupted signaling pathway may result in the degeneration of VSMCs, defects of arterial structures, BBB leakage, and increased susceptibility to ischemic strokes [124, 150]. Reports of patients with *NOTCH3* mutations leading to *NOTCH3* loss of function demonstrate the role of this signaling pathway in cSVD, presenting symptoms such as leukoencephalopathy, stroke, and cognitive impairment [3, 38, 56, 122].

Immunohistological analyses of post-mortem brain samples from patients with CADASIL revealed significant differences in protein levels, particularly in ECM constituents, cytoskeleton, protein processing, vesicular traffic, and cell adhesion [183]. Proteomic analysis of human brain arteries in CADASIL patients with the p.Arg1031 Cys *NOTCH3* mutation identified 19 proteins with considerable level variations [108, 110]. ECM proteins such as collagen 1α2, collagen12α1, collagen14α1, collagen18α1, laminin α5, laminin γ1, lactadherin, clusterin, vinculin, leucine-rich repeat proteoglycan, and perlecan were increased, while neurofilament, neurofascin, internexin α, and solute carrier family 4 were decreased [5]. These proteins have been identified as GOM deposit components in postmortem brain vessels from French CADASIL patients. Proteomic analysis of brain arteries in transgenic mice and human samples enriched with NOTCH^{ECD} revealed the presence of TIMP3 and vitronectin in CADASIL and their absence in controls. TIMP3 activity significantly increased in postmortem CADASIL patients. Serum TIMP3 and related matrix metalloproteinase (MMP) levels in CADASIL patients remain unknown. Studies have identified novel or rare de novo mutations in the *NOTCH3* gene, which may lead to CADASIL in individuals without a prior family history. These mutations have been particularly noted in Japanese patients, providing further insights into the disease's underlying mechanisms [66, 91].

NOTCH3 mutations that lead to loss of function can result in clinical manifestations such as leukoencephalopathy, stroke, and cognitive impairment. These symptoms are often linked with specific mutations within the ligand-binding domain of NOTCH3, emphasizing the importance of this signaling pathway in the context of cSVD [5, 91, 108, 183].

Types of *NOTCH3* mutations

Typical cysteine-altering mutations

The altered bonding pattern within the EGFr, characterized by cysteine 1→ 3, 2→ 4, and 5→ 6 connections, leads to abnormal structural changes in *NOTCH3*. Studies on recombinant NOTCH3 proteins reveal consistent differences in mutant proteins, including increased oligomerization and heightened sensitivity to trans-reduction. These findings suggest a crucial role for cysteine and disulfide pairing in initiating structural abnormalities in NOTCH3. However, several aspects, such as the impact and range of cysteine mutations, the role of amino acid replacements, and the influence of unpairing or free cysteine, remain unclear [83]. Investigations of *NOTCH3* mutations affecting cysteine residues in the ligand-binding domain reveal varying clinical severity, with some individuals experiencing a milder phenotype, while others exhibit earlier onset strokes and widespread MRI abnormalities (Fig. 3) [37, 119, 140, 150].

In Caucasian populations, MA is the predominant initial symptom of CADASIL, while in Asian cohorts, recurrent

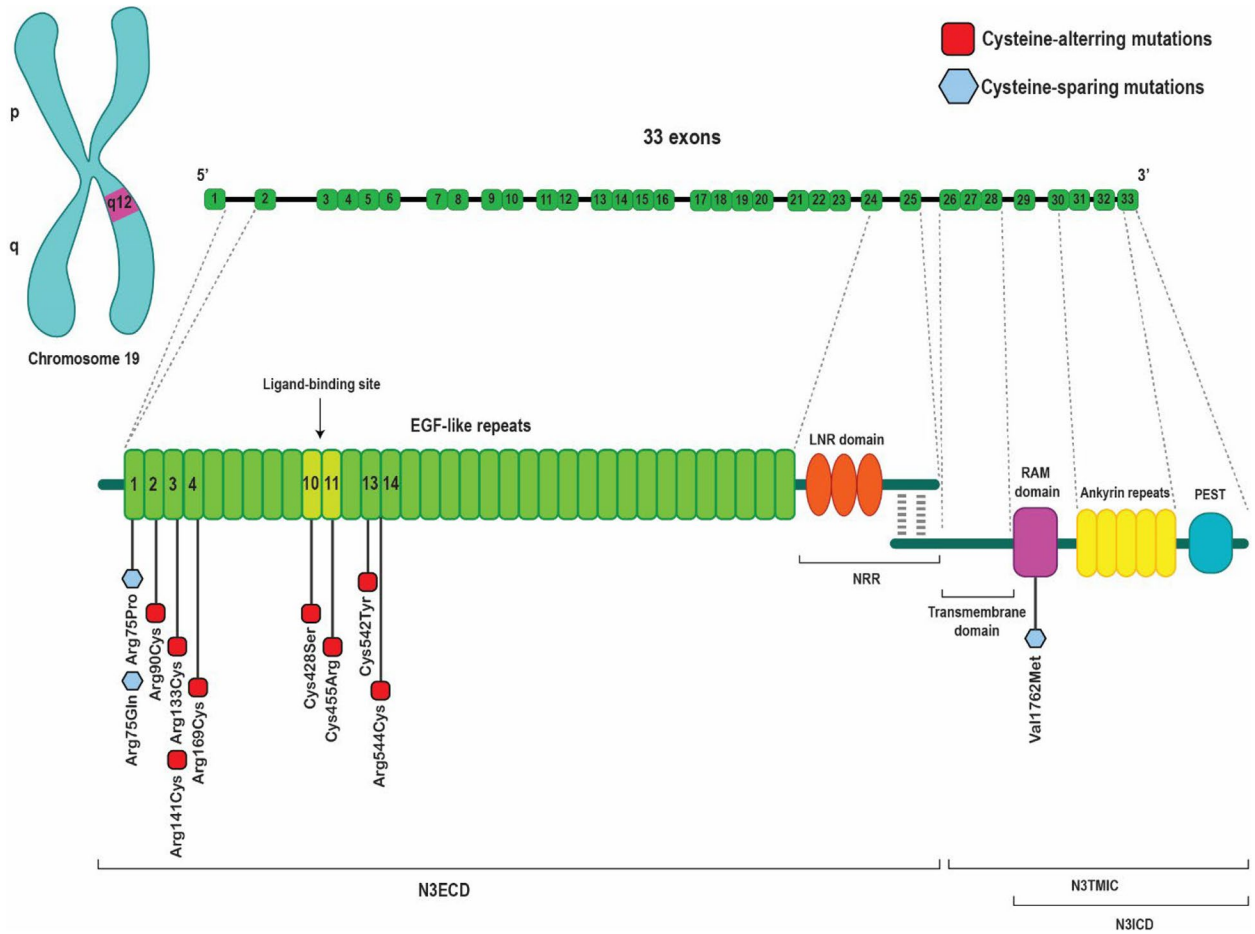

Fig. 3 Schematic view of Cysteine-dependent mutations, being responsible for the onset and progression of CADASIL. Each of the 33 exons that make up the *NOTCH3* gene encodes a part of a specific domain present in this receptor. Most mutations occur in the EGFr domain, encoded by exons 2–24. Cysteine-altering mutations are identified in red, and cysteine-sparing mutations are identified in blue. EGF, epidermal growth factor; LNR, LIN- 12/Notch repeats; NRR, negative regulatory region; RAM, RBP-Jkappa-associated molecule domain; PEST, domain rich in Pro, Glu, Ser and Thr

strokes, memory impairment, and cognitive decline often with minimal or no evidence of MA, are commonly observed. This highlights significant regional variations in the presentation of CADASIL, suggesting that genetic, environmental, and lifestyle factors may influence the clinical manifestations of the disease [75, 158].

Cysteine-sparing mutations

Cysteine-sparing mutations in *NOTCH3* have also been reported [77, 117]. CADASIL patients with cysteine-sparing mutations typically exhibit similar phenotypes to individuals with cysteine-affecting mutations, with reports of later onset and milder symptoms [58, 117]. However, a comprehensive understanding of the pathological role of cysteine-sparing mutations in *NOTCH3* in CADASIL is yet to be elucidated (Fig. 3) [112]. Cysteine-sparing mutations have mostly been observed in Asian cohorts [167, 174, 177], suggesting potential ethnic differences in genotype distribution [23, 24, 59, 111]. A cysteine-sparing mutation outside EGFr exons was identified in patient with GOM deposition in SVD [83]. There have been case reports of individuals with a clinical phenotype consistent with CADASIL, carrying non-cysteine mutations in *NOTCH3* [177]. While this may suggest a non-traditional mutation in CADASIL, it could also represent a CADASIL-like syndrome or may not be pathogenic; further investigation is ongoing [67, 70].

Non-genetic factors involved in CADASIL

Some cohort studies have revealed that within a family with the same genetic and mutation background, different phenotypes can occur, suggesting that CADASIL may also be influenced by non-genetic factors [92]. Certain non-genetic factors, such as smoking, sex, and arterial hypertension, have been linked to an increased risk of stroke and dementia [76, 115]. The role of

hypertension and smoking in arterial stiffness, as well as impaired vascular reactivity, has been associated with a severe phenotype of cognitive symptoms in CADASIL [136, 149, 154]. Furthermore, the effects of hypertension and smoking on VSMC result in a synthetic-like phenotype rather than a contractile one via nicotinic acetylcholine receptors and G protein-coupled receptors [79, 187]. It has been mentioned by few studies that nicotine stimulates the movement of VSMC from the tunica media to atheromatous plaques in the vascular intima [187]. This change enhances the progression of the disease [149, 154].

Hypertension and smoking significantly exacerbate the progression of CADASIL by increasing vascular stress on already compromised VSMCs due to NOTCH3 mutations. Hypertension contributes to elevated mechanical strain on arterial walls. This issue may accelerate the deposition of GOM within the vascular structure. Concurrently, smoking introduces oxidative stress and inflammation, further impairing VSMC function and promoting a more synthetic phenotype, which is characterized by increased proliferation and altered extracellular matrix production. These environmental factors likely interact with *NOTCH3* mutations by enhancing downstream effects such as dysregulation of the RhoA/Rho kinase pathway, ultimately leading to a more severe clinical phenotype in CADASIL patients [93].

S1 and S2 cleavage

Signal transduction in *NOTCH3* pathway occurs through receptor-ligand binding between adjacent cells [183]. Ligand binding leads to proteolytic cleavage of the transmembrane region, resulting in the production of a transcriptionally active ICD. The cleavage by γ-secretase releases NOTCH3^{ICD}, which translocates into the nucleus to activate downstream target genes [45, 52]. Several studies have shown changes in the S1 cleavage of mutant *NOTCH3*/*Notch3* (p.Arg133 Cys, p.Cys183 Arg, and mouse p.Arg142 Cys and p.Cys187 Arg) [69]. The decreased level of S1-cleaved mutant receptors (p.Arg142 Cys) compared to full-length receptors may be due to impaired receptor trafficking. This reduced ratio of S1-cleaved mutant receptors leads to intracellular aggregation and decreased cell-surface presentation, even though the ligand-induced signaling itself remains intact [52, 69, 96, 127].

Homozygotes

Although CADASIL commonly arises from single mutations in the *NOTCH3* gene (heterozygous), but there have been a few reported cases which involve homozygous mutations [113, 166]. While, in some cases, homozygous mutations presented more severe clinical manifestations compared to heterozygous mutations, there is not complete agreement on this matter [53, 155]. For example, the homozygous *NOTCH3* R544 C mutation has been reported in a few cases from East Asia, which presented with similar clinical phenotypes to the more common heterozygous *NOTCH3* R544 C [82, 87].

NOTCH3 pathway, as the major signaling mechanism involved in CADASIL pathogenesis

Studies have mentioned that CADASIL-associated mutations enhance the multimerization of NOTCH3 contributing to the pathological process (Fig. 4) [25, 63]. NOTCH3 also interacts with the transcription factor RBP-Jk by activating the transcription of target genes, a process critical in arterial smooth muscle cells [1, 99, 125].

The *NOTCH3* signaling pathway is a highly conserved mechanism among species [10, 116]. It involves a sophisticated sequence of events, initiated by the interaction between NOTCH receptors and the Delta/Serrate/Lag- 2 (DSL) family of ligands. Receptor-ligand binding induces a series of intricate processes leading to transcriptional activation [135].

The process begins with triggering DSL ligands binding to NOTCH receptors ubiquitination and subsequent clathrin-mediated endocytosis [86, 172]. The activation of *NOTCH* signaling is a complex process with various regulatory steps including ligand-induced endocytosis, destabilization of the Negative Regulatory Region (NRR), and the interplay between activation and repression complexes in the nucleus [28, 42]. Regulated Intramembrane Proteolysis (RIP) is a critical step in the activation of NOTCH proteins, due to their typical inactivity in the absence of ligands. In response to ligand stimulation, RIP enables the release of NOTCH3^{ICD} to the nucleus. This process includes initial cutting by an ADAM protease in the NRR and subsequent cutting by γ-secretase in the intramembrane region. The NRR acts as an autoregulatory switch that maintains NOTCH quiescence, and its disruption can lead to autonomous receptor activation [184]. The NRR's combination is stabilized by calcium ions, and destabilization of this structure can activate Notch independent of ligands. Notch activation by DSL ligands is actively regulated within sender cells. MIB1, a major E3 ubiquitin ligase in mammals, is involved in ligand endocytosis and regulation of ligand activity. Ligand ubiquitination recruits the endocytic adapter protein Epsin, leading to clathrin-mediated endocytosis [43]. In addition, the NOTCH ligands can diffuse on the cell membrane, and impact signaling strength by influencing the number of receptor-ligand pairs at the cell contact area [57, 150].

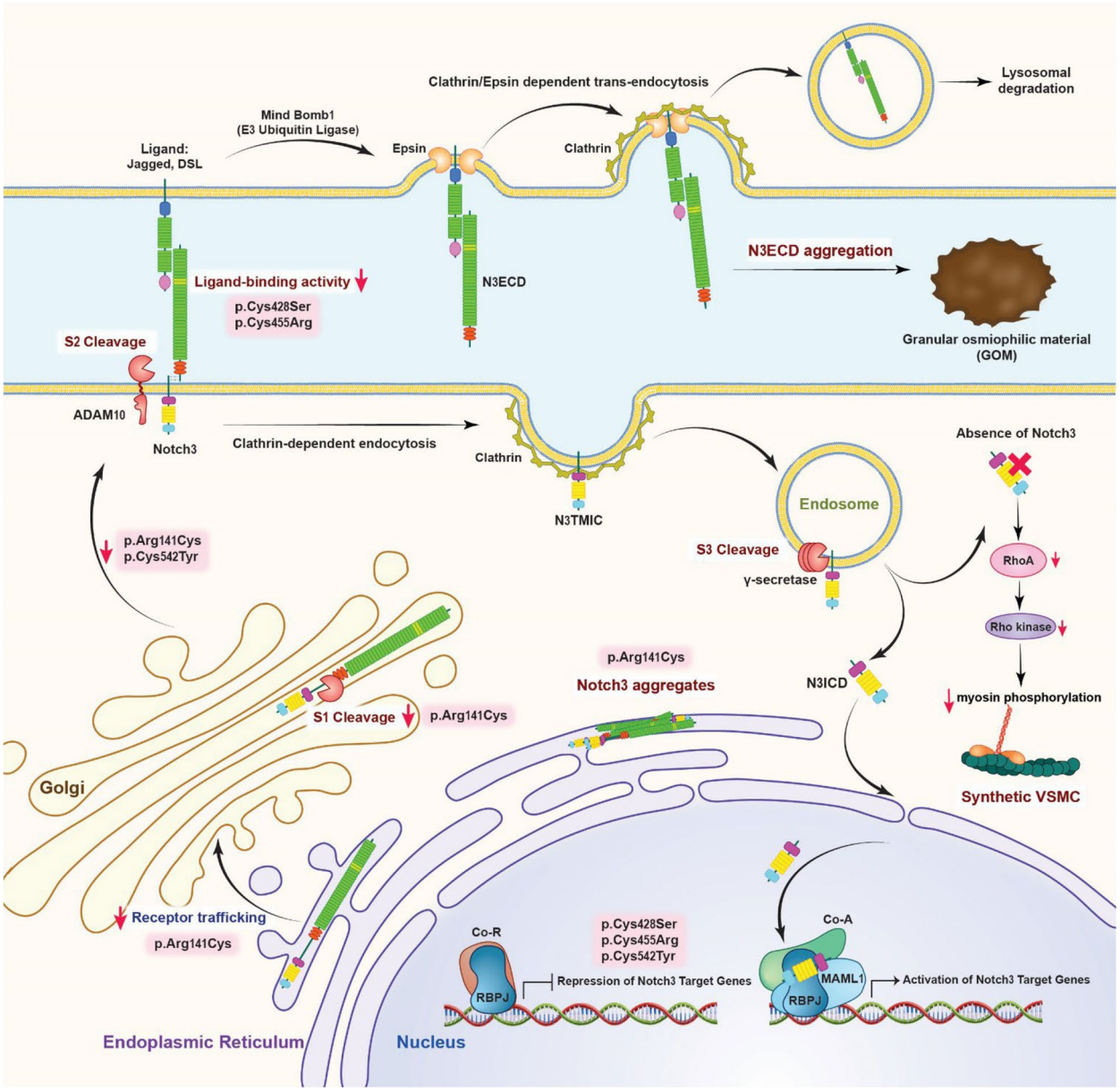

Fig. 4 A schematic illustration of *NOTCH3* signaling pathway involvement in the pathogenesis of CADASIL. NOTCH3 receptor is affected by a complex processing, including cleavage and activation steps. In the absence of *NOTCH3* mutations, NOTCH3 receptor binds to its ligand. Ligand binding results in the engulfment of N3 TMIC through the clathrin-dependent endocytosis. The N3ICD will then be cleaved by γ-secretase to be transferred into the nucleus to trigger the subsequent activation of NOTCH3 target genes. In the absence of NOCTH3, RhoA is downregulated, leading to the suppression of Rho kinase, which in turn decreases myosin phosphorylation. Contrarily, once the receptor, especially its extracellular domain is mutated (p.Cys428Ser and p.Cys455 Arg), the mentioned process is disrupted, affecting the ligand binding, and thus signal transduction mechanism. GOM deposition in the basement membrane involves the accumulation of NOTCH3.^{ECD} (N3ECD) multimers. The mutant NOTCH3 triggers the generation of these multimers, contributing to their intracellular (N3ICD) aggregation. When the removal process of these aggregates is impaired, ER stress, cell death, and abnormal vascular smooth muscle cell (VSMC) growth will be developed. GOM may also disrupt VSMC function, potentially affecting intramural periarterial drainage and contributing to white-matter degeneration. *NOTCH3* signaling interacts with TGF-β via LTBP- 1 in GOM and RhoA to regulate vascular tone, driving CADASIL pathology [9, 100]

When NOTCH3^{ICD} is released from the cell membrane, it combines with the transcription factor recombination signal binding protein for immunoglobulin Kappa J region (RBPJ) and a co-activator to form a NOTCH transcriptional complex (NTC). In the absence of NOTCH3^{ICD}, RBPJ acts as a transcriptional repressor,

forming complexes with co-repressor proteins. *NOTCH* signaling has an intricate interplay to balance activation and repression complexes [179, 180]. Studies suggest that the presence of $NOTCH^{ICD}$ enhances the recruitment of both activation and repression complexes to target genes. *NOTCH* activation also involves dimeric NTC complexes on sequence-paired sites, which further fine-tune the transcriptional response [133, 185, 189, 192].

Studies have identified the regions of responsivities for receptor-ligand of NOTCH receptors and ligands result in differential activity [11, 106]. Various studies tried to quantify interactions with specific ligand-binding regions identified within the $NOTCH3^{ECD}$ of NOTCH receptors [32, 42]. Structural studies have illustrated the interactions between NOTCH receptors and ligands along with focusing on the role of O-linked fucose modifications in the receptor-ligand interface [94, 118, 156]. Cis-inhibition between receptors and ligands has been observed, with ligands expressed in the same cells as NOTCH receptors which illustrate some inhibitory effects. The involvement of fringe proteins in this process further diversifies the potential signaling states of cells [188]. Ligand Dll3 that exhibit only cis-inhibitory effects and others with same function are crucial for proper somitogenesis in mice. The structural basis for cis-inhibition lies in the binding of receptors and ligands in the same anti-parallel direction, preventing the exertion of a pulling force on the receptor by the ligand [64, 118, 125, 164].

Other research has mentioned that *NOTCH3* plays a critical role in vascular injury and VSMC survival [12]. Comparing wild-type *NOTCH3* and *NOTCH* 3R142 C revealed that the R142 C mutation led to reduced S1 cleavage and diminished cell surface expression of the NOTCH3 receptor [71]. Additionally, the mutation enhanced the formation of intracellular aggregates resembling aggresomes along with potential disruptions in receptor trafficking through the endoplasmic reticulum [9]. Despite these alterations, the study demonstrated that the R142 C mutation does not affect the signaling capacity of the NOTCH3 receptor in response to ligand induction [64, 71].

In order to investigate the signaling pathways in CADASIL, five mutations were examined and it was found that some mutations did result in impaired ligand-induced NOTCH3 activity, mediated by the RBPJ transcription factor [69]. Specifically, C428S exhibited impaired ligand-binding ability, while C542Y displayed reduced cell surface expression. The results indicate that the impaired activity of the mutations arises through different mechanisms. The C428S mutant lost its Jagged1-binding ability, whereas C542Y retained it but exhibited impaired presentation to the cell surface. In contrast, the R90 C, C212S, and R1006 C mutants retained the ability to bind Jagged1 and were associated with apparently normal levels of signaling activity [69].

It has been found that the *NOTCH3* signaling pathway regulates the expression of specific markers (incl. ephrin B2 (eph) and eph B4) expressed in both VSMCs and endothelial cells [130]. The results demonstrate that the genetic disruption of *NOTCH3* signaling in VSMCs leads to abnormal cerebrovascular development, characterized by defective arterial patterning, disrupted anastomoses, asymmetry in vessel caliber, and impaired collateral formation in the circle of Willis. Importantly, the reduced expression of eph B2 in VSMCs of mutant cerebral arteries highlights the essential role of *NOTCH3* signaling in guiding proper arterial maturation. These findings underscore the significance of *NOTCH3* signaling in vSMCs for the maintenance of normal cerebrovascular architecture and, consequently, for responding to ischemic challenges and ensuring cerebral perfusion.

The role of *NOTCH3* in arterial function was explored using a mouse model [9]. The study involved comparing the mechanical properties and vascular reactivity of arteries from wild-type and *Notch3*-null mice. *NOTCH3* deficiency was found to impair the response to pressure and flow in specific arteries, indicating its involvement in regulating myogenic tone and flow-mediated dilation. The absence of *NOTCH3* was associated with decreased RhoA activity, reduced myosin light chain phosphorylation, and altered integrin expression levels, emphasizing its critical role in modulating the RhoA/Rho kinase signaling pathway [9]. These insights indicate a potential molecular mechanism underlying NOTCH3-mediated vascular function.

The translocated $NOTCH^{ICD}$ forms complex with the DNA-binding transcription factor CSL inside the nucleus to regulate target genes. The multifaceted role of CSL (RBPJ in vertebrates) in *NOTCH3* signaling is context-dependent [179]. The RBPJ/L3MBTL3 interaction, identified through proteomic analyses, is critical in this process. L3MBTL3, a member of the MBT family, interacts with RBPJ to be co-localized on chromatin, recruiting the histone demethylase KDM1 A to *NOTCH* target genes for their transcriptional repression. The RBPJ/L3MBTL3 interaction is mediated by the N-terminal end of L3MBTL3, with specific residues in the RBPJ b-trefoil domain. The binding affinity between RBPJ and L3MBTL3 is moderate, and $NOTCH^{ICD}$ has a significantly higher affinity, possibly competing with L3MBTL3 for RBPJ binding. L3MBTL3 plays a critical role in the RBPJ-mediated repression of *NOTCH* target genes, acting via KDM1 A-mediated demethylation of H3 K4 me2. In vivo analyses in Drosophila highlighted

the conservation of the functional link between dL(3)mbt and NOTCH, validating the evolutionary conservation of this molecular mechanism across metazoans [179].

Investigating the role of NOTCH receptors in TLR-activated macrophages highlighted the differential impacts of DLL4 and JAGGED1 on *NOTCH* signaling, with DLL4 enhancing the process and JAGGED1 exhibiting inhibitory effects [94]. Additionally, the study has emphasized the essential role of ADAM10 in *NOTCH* signaling, especially post TLR activation, although a minor contribution of ADAM17 was observed. Notably, the research shed light on the distinct contributions of *NOTCH1*, *NOTCH2*, and *NOTCH3* in macrophage activation, with emphasis on the specific role of NOTCH3 in modulating NF-κB activation, potentially through p38 activation. Furthermore, the study pointed to the unique properties of *NOTCH3*, including its rapid activation and structural differences compared to *NOTCH1* and *NOTCH2*. The results also suggested a dynamic interplay between NOTCH1 and NOTCH3 during macrophage activation, with NOTCH3 playing a dominant role in the early stages and NOTCH1 taking control in later phases. Overall, the research contributes to the understanding of the complex interplay of NOTCH receptors in regulating macrophage activation and their potential implications in the context of inflammation [94].

Taken together, the central role of *NOTCH3* in the pathogenesis of CADASIL is known as a critical factor in the development of this disease. Mutations in the *NOTCH3* gene lead to the accumulation of the NOTCH3 protein, resulting in the selective degeneration of SMCs in blood vessels, which ultimately causes vascular damage and manifests as migraine headaches and early strokes [102]. Recent studies have shown that *NOTCH3* mutations can lead to structural changes in the protein and disrupt *NOTCH3* signalling, directly linking these alterations to the pathogenesis of CADASIL [13, 105]. Furthermore, new research indicates that unusual mutations in *NOTCH3* can result in varying clinical manifestations, requires more attention in the diagnosis and treatment of this condition [13].

Transforming growth factor-B (TGF-B) signaling pathway may also contribute to CADASIL progression

In CADASIL, the TGF-β pathway has been reported to be dysregulated, as indicated by the recruitment of latent transforming growth factor binding protein (LTBP- 1) into $NOTCH^{ECD}$ deposits and the increased expression of latency associated peptide (LAP) in affected vessels (Fig. 5) [72, 153]. This sequestration of LAP shows that the TGF-β pathway may be involved in CADASIL pathogenesis, by altering TGF-β bioavailability. Notably, the bioavailability of TGF-β is regulated by various molecules such as fibronectin, fibrillin- 1, and LTBP-1 [16, 67, 70]. In CADASIL, TGF-β pathway dysregulation affects VSMCs and vessel thickening, impaired functionality, and increased fibrotic thickening of vessels. LTBP-1 sequestration and altered TGF-β bioavailability suggest its role in CADASIL pathogenesis [28, 122, 134]. Similar dysregulation of the TGF-β pathway has been observed in other vascular diseases, such as Marfan syndrome and the recessively inherited, CARASIL [72, 164]. Further investigations into the TGF-β pathway in CADASIL and its potential role in disease pathogenesis are necessary. Insights from molecular evaluations investigating the pathology of CADASIL have unveiled several crucial mechanisms contributing to the clinical manifestations of the disease and its therapeutic implications. The role of GOM, *NOTCH3* transendocytosis, and downstream signaling related to TGF-β have been identified as essential components for comprehending disease progression [72, 104, 156]. Studies suggest that the dysregulation of LTBP-1 and other proteins involved in TGF-β biology, such as Emilin and Nidogen, may play a role in CADASIL development (Fig. 5).

In-vitro and in-vivo evaluations

In-vitro analyses investigating CADASIL-related signaling pathways

Various in-vitro structures have been used for studying CADASIL, including VSMCs [51, 165], skin fibroblasts [131], myoblasts [2], oligodendrocytes [161], and human embryonic kidney (HEK293) cells [31]. Table 1 has summarized key in-vitro studies, as well as in-vivo investigations, on CADASIL-related signal transduction.

Studies that have used induced pluripotent stem cells (iPSCs) derived from CADASIL patients have demonstrated structural and functional aberrations, including dysregulated activation of *NOTCH* signaling and NF-κB pathway, resulting in inflammatory responses and vascular dysfunction [36, 89, 159]. These studies highlighted anomalies in mediators and microfilament structures, such as linking to *NOTCH3*-mediated expression, in addition to defects in innate immunity and cellular adhesion assessed in endothelial cells during inflammatory conditions [89]. Moreover, iPSC-derived vascular mural cells exhibited alterations in platelet-derived growth factor receptor beta (PDGFRβ) and VEGF levels potentially due to *NOTCH3*-related gain-of-function mechanisms, in turn affecting capillary stabilization [138]. In a study of CADASIL patient-derived iPSC-derived VSMCs (iPSC-MCs), the iPSC-MCs illustrated dysfunction compared to those from healthy individuals [73]. Specifically, CADASIL iPSC-MCs were unable to stabilize angiogenic capillary structures and support the survival

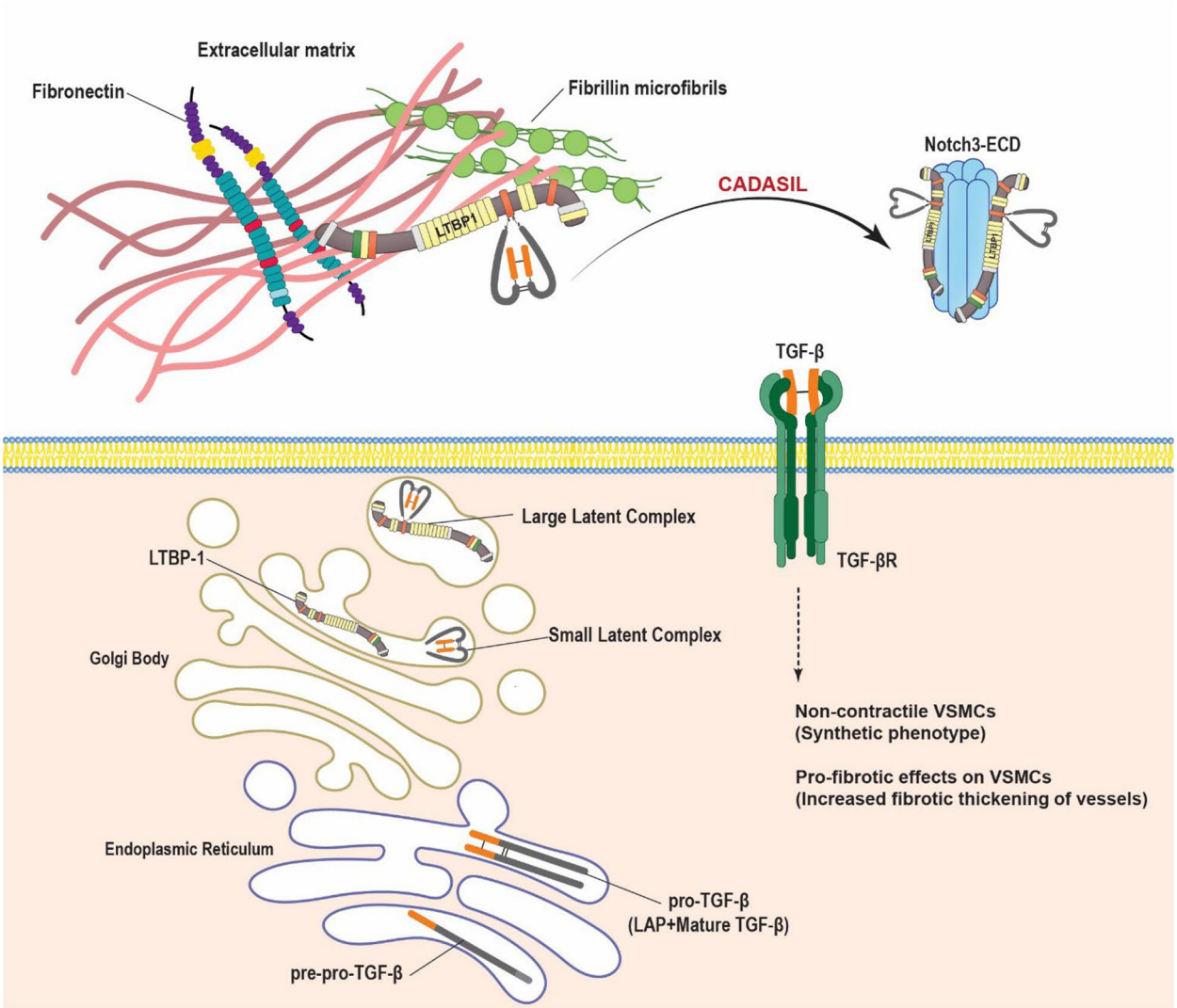

Fig. 5 TGF-β signal transduction, the other signaling mechanism contributed to CADASIL development. TGF-β pathway is reported to be dysregulated, as evidenced by the recruitment of latent transforming growth factor binding-protein (LTBP-1) into NOTCH extracellular domain (Notch-ECD) deposits and increased expression of latency associated peptide (LAP) in affected vessels. The dysregulation of the TGF-β pathway in CADASIL affects vascular smooth muscle cells (VSMCs), leading to vessel thickening, impaired functionality, and increased fibrotic thickening of vessels. Various molecules, including fibronectin, fibrillin- 1, and LTBP- 1, regulate the bioavailability of TGF-β. In CADASIL, the dysregulation of the TGF-β pathway and LTBP- 1 sequestration contribute to pathogenesis

of iPSC-derived endothelial cells. Further investigations revealed that CADASIL iPSC-MCs had decreased PDGFRβ and reduced secretion of VEGF, both crucial for maintaining the stability of the capillary network. To rescue these phenotypes, the researchers supplemented VEGF and conducted siRNA knockdown of *NOTCH3*, which significantly improved the stability of the capillary structures formed by the CADASIL iPSC endothelial cells and iPSC-MCs. This suggests that the *NOTCH3* mutation plays a role in the observed defects in the CADASIL iPSC-MCs, indicating its involvement in the capillary network stabilization. An in-vitro study of the role of glucose transporters (GLUTs) in VSMCs in CADASIL was also investigated [123]. Using cerebral VSMCs derived from CADASIL patients and control subjects, along with post-mortem brain tissues, researchers underscored the role of glucose metabolism in CADASIL and its possible implications for disease progression. The study examined the expression levels of GLUT2 and GLUT4, finding that both GLUT2 and GLUT4 expressions were reduced in

Table 1 In-vitro and in-vivo studies of the involvement of signaling pathways in CADASIL

Type of study	Model/System used	Key findings	Reference(s)
***In-vitro* analyses**			
iPSCs	VSMCs, Fibroblasts, Myoblasts, HEK293, iPSCs	- Dysregulated activation of NOTCH signaling and NF-κB pathway - Structural and functional aberrations in iPSCs - NOTCH3^{ECD} aggregates observed - Altered PDGFRβ and VEGF levels in vascular mural cells - Accumulation of NOTCH3^{ECD} and GOM in CADASIL pathology - Anomalies in mediators and micro-filament structures	[15, 36, 138]
Bri2 BRICHOS domain	Stable cell lines, Protein modeling	- Bri2 BRICHOS interacts with Aβ aggregates - Counteracts Aβ42-induced neurotoxicity and fibrillization - Bri2 BRICHOS inhibits non-fibrillar aggregations - Potential as a molecular chaperone for NOTCH3 - In vitro testing with stable cell lines showed reduced aggregation kinetics with Bri2 BRICHOS	[120]
Glucose Transporters	Cerebral VSMCs	- Reduced GLUT2 and GLUT4 expressions in CADASIL VSMCs - ↓ glucose uptake in CADASIL patients	[123]
***In-vivo* animal analyses**			
Drosophila Lethal-Abruptex	Drosophila	- Illustrates alterations in *NOTCH3* signaling and vascular structure	[7]
Zebrafish *NOTCH3* Mutants	Zebrafish	- Demonstrates alterations in *NOTCH3* signaling and vascular structure	[190]
NOTCH3 Knock-Out Mice	Mice *NOTCH3*$^{-/-}$	- Exhibits CADASIL-related pathological features, such as VSMC degeneration, impaired cerebral vasoreactivity, changed myogenic response, and white matter lesions	[6, 9, 27, 35, 54, 55, 78, 90, 141]
Transgenic mouse models	Mice R90 C, R142 C, R169 C, C428S, C455R, R1031 C	- Express specific mutations in *NOTCH3* gene - Replicate CADASIL pathological hallmarks - VSMC degeneration, - Impaired cerebral vasoreactivity, - The lack of myogenic response - Increased white matter lesions - GOM accumulation and modified *NOTCH3* activity	[8, 30, 44, 46, 68, 109, 119, 128, 139]
Knock-In Conventional	Mice R170 C, C142R	In C170 C: the absence of the CADASIL phenotype was seen In C142R: elevated expression of *NOTCH3* target genes - Decreased cerebral artery diameter and impaired dilator capacity - Increases in *NOTCH3* signaling activity,	[8, 98, 170]
Knock-In Conditional	Mice R1031 C C455R	C455R results in early ischemic events R1031 C leads to age-dependent hypomorphic phenotype,	[5]

Table 1 (continued)

Type of study	Model/System used	Key findings	Reference(s)
GOM Pathology Study	CADASIL mouse model	- Longitudinal study on GOM pathology in CADASIL mouse model - Temporal increase in GOM size and density - No other typical CADASIL-related pathologies observed	
Impact of CADASIL mutations	Transgenic mice	- Investigated effects of *NOTCH3* mutations (R90 C and R169 C) on ischemic stroke outcomes - No influence on residual tissue perfusion - ↑ sensitivity to ischemia and susceptibility to spreading depolarizations	[119]

CADASIL VSMCs and brain microvessels, potentially contributing to the observed decrease in glucose uptake among CADASIL patients.

The correlation between CADASIL mutations, $NOTCH3^{ECD}$ accumulation, and disease severity has been investigated [15, 31, 102]. Increased levels of TIMP3, which co-aggregates with $NOTCH3^{ECD}$ has also been suggested to disrupt cerebrovascular reactivity, indicating a shared mechanism in CADASIL [43, 181].

The findings showed significant alterations in cellular phenotypes, such as reduced gene association with Wnt and TGFβ signaling, and the formation of $NOTCH3^{ECD}$ aggregates resembling the characteristic GOM observed in CADASIL patients. Additionally, the CADASIL blood vessel organoids exhibited modified gene expression patterns associated with angiogenesis and vasculogenesis, underscoring the influence of the p.Arg75Gln mutation on vascular network formation. These insights highlighted the potential of iPSC-based 2-D and 3-D models in replicating disease-associated features and elucidating the underlying molecular mechanisms [15, 28, 43].

The diverse functionalities of Bri2 BRICHOS domain structures in Alzheimer's disease and CADASIL were highlighted in a recent study [120]. The research revealed that Bri2 BRICHOS monomers and dimers interacted with amyloid-β (Aβ) aggregates, counteracting Aβ42-induced neurotoxicity and fibrillization. Furthermore, Bri2 BRICHOS oligomers were found to inhibit non-fibrillar aggregations. The observed molecular interactions of BRICHOS with aggregates suggest its potential as a molecular chaperone for the NOTCH3 protein, potentially capable of delaying CADASIL progression. The researchers generated stable cell lines expressing *NOTCH3* EGF1–5 wild type (WT) and R133 C. The tertiary structures of *NOTCH3* EGF1–5 was modeled, and circular dichroism spectra were recorded. Co-incubation with Bri2 BRICHOS and turbidity assays indicated reduced aggregation kinetics in the presence of Bri2 BRICHOS. Negative-stain preparation and transmission electron microscopy imaging was also conducted to visualize the samples.

In parallel, comprehensive analyses of the NOTCH3 EGF1–5 proteins which focused on both the WT and R133 C mutant, by including protein generation and characterization, structural predictions, assessments of secondary structure, and aggregation studies with and without the presence of the Bri2 BRICHOS molecular chaperone, also revealed that under reducing conditions, both WT and R133 C proteins primarily existed in monomeric states, indicating the absence of disulfide bridge-dependent oligomer formation [36, 128]. Nonetheless, under non-reducing conditions, both proteins exhibited slower migration patterns, suggesting the formation of intramolecular disulfide bonds. Structural modeling unveiled a linear structure for both proteins, with the R133 C mutation having no significant impact on the overall tertiary structure of EGF1–5 [128, 140, 173]. The circular dichroism spectra demonstrated that two recombinant proteins adopted similar random coil-like structures, with the R133 C mutant appearing slightly more unstructured at higher temperatures [150, 186]. The study also demonstrated that the R133 C mutant displayed a higher propensity for aggregation compared to the wild-type counterpart [171].

The presence of the Bri2 BRICHOS chaperone stabilized the mutant NOTCH3 protein, resulting in the formation of soluble monomeric proteins. The effects of BRICHOS were dose-dependent, with a higher molar ratio leading to increased stabilization of the mutant proteins [186]. Additionally, the turbidity assay indicated that the presence of BRICHOS reduced the aggregation kinetics of the R133 C mutant by 50%. Transmission electron microscopy imaging further supported the findings, demonstrating that BRICHOS could stabilize the mutant

Table 2 Experimental models of CADASIL

Knock-out	
NOTCH3$^{-/-}$	1. Arboleda-Velasquez JF, et al. (2008) [6] 2. Romay, et al. (2023) [137] 3. Eikermann-Haerter K, et al. (2011) [35] 4. Kofler N, et al. (2015) [35] 5. Domenga et al. (2004) [27] 6. Belin de Chantemèle EJ, et al. (2008) [35] 7. Henshall et al. (2015) [55] 8. Helle, 2022 (***renal vasculature***) [54] 9. Krebs et al, 2003 (***double KO mice; notch1/notch3***) [78] 10. Liu et al, 2010 [90] 11. Rusanescu et al., 2014 [141]
Conventional knock-in	
R170 C	1. Wallays et al, 2011 [170] 2. Baron-Menguy, 2017 [8] 3. Li C et al., 2025 [85]
R142 C	1. Lundkvist et al, 2005 [98]
Conditional knock-in	
R1031 C	1. Arboleda-Velasquez et al, 2011 [5]
C455R	1. Arboleda-Velasquez et al, 2011 [5]
Transgenic	
R90 C	1. Oka et al, 2022 [119] 2. Monet et al, 2007 [109] 3. Ruchoux et al, 2003 [139] 4. Dubroca et al, 2005 [30] 5. Gu et al, 2012 [46] 6. Ping et al., 2021 [128]
R169 C	1. Oka et al, 2022 [119] 2. Joutel et al, 2010 [68] 3. Baron-Menguy, 2017 [8]
C428S	1. Monet-Leprêtre et al., 2009 [107]
R182 C	1. Gravesteijn et al, 2020 [44] 2. Rutten et al, 2015 [146]

NOTCH3 protein, thereby preventing the formation of larger aggregated particles [171]. These results provide promising insights into the potential of BRICHOS as a therapeutic strategy for inhibiting the aggregation of CADASIL-mutated NOTCH3 proteins. Further investigations are warranted to explore the applicability of this approach to other *NOTCH3* mutations associated with CADASIL [120, 128].

Insights from in-vivo studies

Early genetic studies of mutant Drosophila melanogaster [7, 163] gave rise to the discovery of the *NOTCH* gene. Here, this mutation produced wing 'notches', with this phenotypic presentation later contributing to the gene nomenclature. Since then, other experimental models have been employed to study *NOTCH* and *NOTCH*-related signaling, with *NOTCH* homologs next identified in nematodes and zebrafish [190]. In the 1990's, there was an influx of NOTCH-related biological and biochemical studies in mammalian systems, likely due to the association of rare mutations of *NOTCH* reception with various human diseases [163]. To date, four NOTCH family receptors have been described in mammals, with NOTCH3 identified as the third mammalian NOTCH [81]. Amongst these four proteins, NOTCH3 displays a more restricted distributed tissue distribution, and thus targeted deletion does not lead to embryonic lethality a result observed in NOTCH1 [78] and NOTCH2 [50, 101] knockout animals. In vivo murine models have been developed to examine *NOTCH3* mutations and related signaling with respect to the underlying pathological mechanisms of CADASIL and role in prospective therapeutic applications. Mouse models like R170 C incompletely mimic human CADASIL due to species differences in *Notch3* function [98]. Table 2 has listed the most common experimental models for CADASIL-related NOTCH3 dysfunctions.

NOTCH3 knock-out

The knock-out (NOTCH3$^{-/-}$) model has provided evidence for the roles of *NOTCH3*$^{-/-}$ signaling in cerebrovascular maturation, homeostasis, and integrity [27], in

particular the impact of *NOTCH3* in arterial formation. *Notch3*$^{-/-}$ mice exhibited enlarged arteries with thinner and irregular VSMC profiles, as compared to WT mice, with these features prominent from Postnatal day 28. In vitro studies suggested a direct effect of *NOTCH3* signaling on actin cytoskeletal dynamics, indicating a potential mechanism for the observed postnatal arterial maturation of VSMC. The study mentioned the role of *NOTCH3* is crucial for the postnatal maturation of VSMC to provide proper remodeling and orientation. The absence of *NOTCH3* resulted in VSMC resembling venous rather than arterial cells. In addition, it reduced expression of arterial markers like smoothelin. NOTCH3's role appears autonomous to VSMC and it can have effect on cytoskeletal dynamics and enabling VSMC to respond to mechanical stress.

Romay et al. [137] observed similar results with the age-dependent effect of arterial detachment and disorganization observed in *Notch3*$^{-/-}$ mice from 2 weeks of age [137]. Importantly, these authors showed that the arterial organization profiles were indistinguishable between 4-week-old NOTCH3$^{-/-}$ and 2-year-old WT animals. The downstream effects of the impaired vascular integrity of *Notch3*$^{-/-}$ animals caused loss of arterial VSMC and progressive loss of vessel coverage [90], leading to intermittent leakage, arterial dilation, and aneurysm formation [55]. Interestingly, these outcomes are more prominently observed in larger diameter, rather than peripheral, arterioles. Moreover, impairment of *NOTCH3* has been shown to increase the risk of brain injury. For example, a study by Arboleda-Velasquez JF et. al. showed that *Notch3*$^{-/-}$ mice present with larger infarct area and volume, and more pronounced cerebral blood flow (CBF) deficit and greater mortality rate following over 7 days (60% in *Notch3*$^{-/-}$ *vs.* 0% in WT) following a cerebral ischemia/reperfusion protocol [6]. The loss of *NOTCH3* function in SMCs was shown to lead to downregulation of key targets Heyl and Hes1, and transcriptional changes in genes which play crucial role in muscle contraction and cell structure. *Notch3*$^{-/-}$ mice showed a higher risk of ischemic damage, such as larger ischemic lesions, neurological deficits, and increased mortality due to middle cerebral artery occlusion. Laser speckle flowmetry indicates impaired blood flow regulation in *Notch3*$^{-/-}$ animals. This experiment introduced WT *NOTCH3* in SMCs, and demonstrated recovery in stroke susceptibility due to crucial role of *NOTCH3* in maintaining vascular integrity. Overall, these studies provide evidence that *NOTCH3* expression in SMCs is necessary for maintaining arterial integrity by securing long-term functionality and survival of VSMC of CNS arteries/arterioles and plays a role in rescuing stroke susceptibility.

Further, NOTCH3 deficiency was shown to result in progressive loss of VSMCs, altering myotonic tone and increasing susceptibility to ischemic stroke [55]. The research identified a stepwise deterioration process in VSMCs, which involved gradual degradation and clearance of cellular debris. Transcriptome profiling of *NOTCH3*-deficient brain vasculature reveals significant changes in gene expression, particularly within endothelial and mural cell-associated genes. The study emphasizes the selective impact of *NOTCH3* on VSMC maintenance and function compared to pericytes. Notably, *NOTCH3* deficiency is linked to vascular pathologies such as fibrin deposition, especially in areas with defective VSMC coverage. However, the observed vascular damage and intermittent leakage do not indicate a general breakdown of endothelial junctions, as tight junctional proteins remain unchanged. In addition, pericyte morphology shows no significant changes despite downregulated pericyte markers, highlighting the specific role of *NOTCH3* in arterial VSMC maintenance.

Notch3 knock-in rodents

There have been two conventional knock-in mice assessed to date, R142 C and R170 C to study CADASIL pathogenesis.

R142C knock-in model Interestingly, R142 C did not exhibit CADASIL pathological and neuroradiological symptoms up to 20 months of age, despite corresponding to the common human mutation R141 C.

The authors ruled out the possibility that the absence of the CADASIL phenotype was due to the altered expression or processing of R142 C *NOTCH3* at the RNA or protein levels, rather suggesting that the species difference between human and murine *Notch3* could explain why mice carrying the murine gene mutated at the Arg 142 site did not express a CADASIL phenotype [98].

R170C knock-in model The mouse R170 C mutation (to mimic human R169 C mutation) presents with various CADASIL symptoms, including robust Notch3^{ECD} deposition in cerebral arteries at 4th and upregulated levels of *Notch3*, Nrip2, and Grip2 in brain arteries. HeyL expression did not experience statistically significant difference. Moreover, the passive diameter of cerebral arteries from 4-month-old Notch3 $^{R170\ C/R170\ C}$ mice was significantly decreased over a range of physiological pressures. These symptoms only occurred in a subset of the knock-in animals [8].

Recent studies on the R170 C knock-in model have revealed impaired glymphatic influx and efflux, driven by reduced aquaporin-4 (AQP4) expression in astrocytic end feet. The process is regulated by the Notch3-RUNX1-CMYB signalling axis. This disruption impairs waste clearance, accelerating brain senescence, as evidenced by increased perivascular spaces and brain atrophy. This impairment is associated with the *Notch3* mutation, which disrupts AQP4 expression and subsequently affects glymphatic clearance. Furthermore, the research indicates that restoring AQP4 using adeno-associated virus (AAV) vectors can enhance glymphatic function and potentially mitigate the aging processes associated with brain senescence in CADASIL. However, the precise timing of GOM formation and the full characterization of clinical phenotypes in these models remain incomplete [85]. Impaired glymphatic influx and efflux in R170 C mice hinder waste clearance, contributing to WMH and increased ischemic susceptibility, as hallmarks of CADASIL, also observed in human patients with brain atrophy and enlarged perivascular spaces [85, 183].This dysfunction, driven by reduced AQP4 expression in astrocytic end feet via the Notch3-RUNX1-CMYB axis, may exacerbate cognitive decline by impairing clearance of neurotoxic metabolites [74–76, 183].

Baron-Menguy et al. investigated whether the R169 C mice mutation in the *Notch3* gene contributes to specific alterations [8]. Researchers analyzed mice and found an increase in deposition of Notch3^{ECD} in cerebral arteries, and elevated expression of *Notch3* target genes. Additionally, the mice exhibited decreased cerebral artery diameter and impaired dilator capacity. The increases in *Notch3* signaling activity in cerebral arteries is also mentioned as well as the influence of mutation context or overexpression on *Notch3* activity which can result in a reduced lumen diameter, affecting vasodilator capacity. Notably, the observed impact on vascular structure is comparable to that seen in cases of chronic hypertension. The majority of CADASIL-associated *Notch3* mutations with an odd number of cysteine residues in *Notch3*^{ECD} support the idea that other CADASIL mutations may increase *Notch3* activity, but the reason behind this issue is unclear [8].

Conditional knock-ins (C455R and R1031C) The C455R and R1031 C mutations in the *Notch3* gene are associated with CADASIL [5]. The C455R mutation, located in the ligand-binding domain (EGFR11), leads to early ischemic events. It results in stronger loss-of-function mechanism, as shown in in vitro experiments of using mouse embryonic fibroblasts [5]. In comparison, the R1031 C mutation in EGFR26 is linked to a typical onset in the fourth decade of life. Both mutations have exhibited an age-dependent hypomorphic phenotype [4, 5].

Transgenic models

Potential CADASIL biomarkers have been identified from transgenic mice with these mutations, including increased plasma levels of COL18 A1, endostatin, and HTRA1, with proteomic analysis showing their presence in GOMs of CADASIL-affected arteries [4, 5]. Transgenic mouse models have been developed which express various *Notch3* mutations, including R90 C, R169 C, C428S, and R182 C [100]. These models exhibit distinct CADASIL-related pathological features, including VSMC degeneration, impaired cerebral vasoreactivity [80], the lack of myogenic response [9], increased white matter lesions [68], GOM accumulation and modified Notch3 activity [108]. For instance, in a transgenic mouse model, TgNotch3$^{R90\ C}$ is defined as an archetypal CADASIL mutation located in the EGFR2 and its role is increasing a cysteine residue [30, 139].

The identified signs were VSMCs degeneration, such as cytoskeleton changes and defective anchorage to extracellular matrix and cells in order to Notch3^{ECD} deposition and GOM accumulation [46, 109]. Degeneration of VSMCs to the surrounding microenvironment led to an impaired myogenic response the impacts of stress, while agonist or receptor-induced tone remains unchanged. Furthermore, the increased actin polymerization in VSMCs results in higher myogenic tone of arteries. The altered flow-mediated dilation can occur due to an effect on endothelial cells indirectly. In addition, functional effects on cerebral vasoreactivity includes increased resistance of cerebral arteries [100, 108].

Altered cerebral blood flow regulation and increased hypotension susceptibility in a condition lead to heightened risk of ischemic events. In TgNotch3$^{R90\ C}$ mice, pericytes show mitochondrial injury and autophagic degeneration, with unaffected *Notch3* activity and no inhibition of WT *Notch3* function by Notch3^{ECD} aggregates [8, 119].

TgNotch3$^{R182\ C}$ mice, consist of the human *Notch3* gene. These mice depict gradual increasing age- and *Notch3* RNA expression level-dependent vascular accumulation of NOTCH3 and GOM deposition [44, 146], but do not result in brain parenchymal lesions. This finding illustrated the importance of the "NOTCH3 score" as quantitative biomarker for CADASIL, as a proper model for pre-clinical testing of therapeutic approaches [44]. Using a longitudinal mouse model which overexpressed human NOTCH3 protein [44], the study classified the GOM into five stages based on size, morphology, and electron density. There was a temporal increase in GOM size and density, yet the mice did not show other typical CADASIL-related pathologies, such as changes in smooth muscle actin staining, BBB leakage, and cognitive and motor dysfunctions. GOM count, size, and the

percentage of GOM-positive vessels increased over time. Notably, GOM deposits were predominantly located on the abluminal side of mural cells. In CADASIL patients, GOM deposits were observed in 96% of microvessels, with stage IV being the most frequent. Patients' microvessels also contained large confluent patches of GOM (stage V) not observed in mice. The electron density of GOM in patients was less homogeneous than in mice.

In TgNotch3$^{R169\ C}$ mice, the Notch3$^{R169\ C}$ mutation model of CADASIL exhibits early WM lesions, hypoperfusion, and altered myogenic response due to abnormal hyperpolarization in arterioles and VSMCs [68]. The mice show impaired *Notch3* function in hippocampal precursor cells, which leads to a decrease in neurogenesis, and the R169 C *Notch3* mutation resulted in cognitive decline and vascular phenotypic changes. Additionally, the model confirms the involvement of endoplasmic reticulum stress and RhoA/Rho kinase in CADASIL pathogenesis, along with implications for BBB disruption and reduced pericyte coverage in cortical vessels [8, 30, 40, 119].

C428S mutation in the *NOTCH3* human gene expressed under the control of the murine SM22α promoter (TgNOTCH3^{C428S}) resulted in a loss of WT *NOTCH3* activity and a mild dominant negative effect. NOTCH^{ECD} accumulation was shown to induce the abnormal recruitment of extracellular matrix proteins, including tissue inhibitor of TIMP3 and vitronectin, whose dysregulation contributes to the toxicity of these aggregates on small vessels [107, 108].

A transgenic mouse model investigated the impact of CADASIL *NOTCH3* mutations (R90 C and R169 C) on ischemic stroke outcomes, aiming to understand the underlying mechanisms and processes [119]. CADASIL mutations did not seem to influence residual tissue perfusion, instead, the research suggested that the brain tissue of the transgenic mice required a higher amount of blood flow to survive, indicating an increased sensitivity to ischemia. There was also an elevated susceptibility to spreading depolarizations in CADASIL mutant mice, contributing to a more severe stroke phenotype. Specifically, the mutations were linked to abnormal extracellular ion homeostasis, particularly potassium, which impacted the brain's response to ischemic injury. These findings suggested that the observed vulnerability to ischemic injury in CADASIL might be associated with an impaired ability to handle extracellular potassium and an increased susceptibility to spreading depolarization. The study also noted that a vascular defect, particularly in pericytes and SMCs which express *Notch3,* can cause abnormal potassium ion buffering in the brain. Therefore, therapeutic implications targeting SDs and improving potassium homeostasis may mitigate the impact of CADASIL mutations on ischemic outcomes, independent of vasomotor dysfunction.

A mechanistic study on TgNotch3$^{R90\ C}$ mouse model of CADASIL depicted that stem cell factor (SCF) + granulocyte colony-stimulating factor (G-CSF) enhances brain repair and improves cognitive recovery through VEGF-A-mediated angiogenesis [129]. The treatment restores neurovascular networks, including dendrites, axons, synapses, and neurogenesis, which are positively correlated with cognitive improvements. The study emphasizes the requirement of VEGF-A-mediated angiogenesis for the enhanced brain repair and cognitive recovery in this CADASIL mouse model. The study reveals that reduced levels of cerebral VEGF/VEGF-A in TgNotch3R90 C mice are associated with decreased blood vessel density, neural structure density, synapses, and neurogenesis.

A transgenic mouse model using TgNotch3R90 C mice as a CADASIL model, explored the impacts of SCF and G-CSF treatment on cerebral capillary thrombosis and associated neuron loss [128]. The research demonstrated the distribution of capillary thrombosis in the brain, the correlation between capillary thrombosis and ischemic neuron loss, and the potential of SCF + G-CSF treatment in mitigating microvascular ischemic damage in these mice. Using bone marrow transplantation, the researchers tracked blood clots, while employing immunohistochemistry techniques to assess neuron loss in the cerebral cortex regions surrounding thrombotic capillaries. Notably, the study revealed that capillary thrombosis predominantly occurred in the cortex of the TgNotch3$^{R90\ C}$ mice, with noticeable neuron loss detected in the areas surrounding thrombotic capillaries, particularly those with bifurcations. Ultimately, the administration of SCF + G-CSF treatment demonstrated a notable reduction in neuron loss adjacent to thrombotic capillaries, indicating potential neuroprotective effects of this treatment regimen.

A study established NOTCH3^{ECD} immunotherapy as a potential therapeutic method with a mouse monoclonal antibody (5E1) [39]. 5E1 binds NOTCH3^{ECD} deposits in brain vessels and results in disease-related phenotypes. This process assessed in mouse model. It showed NOTCH3^{ECD} and GOM deposition, WM lesions, and cerebral blood flow deficits. In this study quantitative immunohistochemistry, as well as electron microscopy, and Laser-Doppler flowmeter. Ultimately, a single peripheral injection of 5E1 robustly induced NOTCH3^{ECD} deposits in the brain vessels. Long term evaluation of 5E1 demonstrated that NOTCH3^{ECD} or GOM deposition could not be lessen and perivascular microglial had not been activated. It also could not reduce development of white matter lesions. However, 5E1 treatment markedly

protected against impaired cerebral blood flow responses to neural activity and topical application of vasodilators and normalized myogenic responses of cerebral arteries.

Diagnostic challenges

CADASIL faces significant underdiagnosis due to multifaceted challenges that intersect clinical, genetic, and educational domains. Regarding its clinical overlaps, CADASIL shares phenotypic features with multiple inherited CSVDs, including CARASIL (cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy), Fabry disease, and COL4 A1/2-related disorders. For instance, Fabry disease presents with albuminuria and angiokeratomas, but its neurological manifestations such as small vessel strokes overlap with CADASIL. CARASIL and HTRA1-related CSVD also mimic CADASIL's white matter changes but differ in systemic features like alopecia and skeletal abnormalities. Hereditary angiopathy, nephropathy, aneurysms, and muscle cramps (HANAC) syndrome and RVCL-S further complicate differentiation due to shared stroke risks but distinct extracerebral involvement, including retinal vasculopathy in RVCL-S. This overlap often leads clinicians to misattribute symptoms to more common conditions resembling MS or hypertension-related CSVD [103, 162].

CADASIL also exhibits marked variability in symptom onset and severity. MA affects ~30% of patients, typically emerging at age 30, but atypical forms, such as confusional aura or encephalopathy may mimic psychiatric or epileptic disorders. Ischemic strokes occur at a mean age of 49, yet 20% present before age 40, with MRI findings (anterior temporal lobe hyperintensities, lacunar infarcts) often misinterpreted as age-related changes. Non-neurological red flags are absent despite systemic vascular pathology, unlike Fabry disease or RVCL-S [103, 160].

Another challenge in this context is limited clinician awareness and diagnostic delays. CADASIL affects 2–5/100,000 individuals, but less than 2% of younger-onset lacunar strokes are linked to genetically confirmed cases. While neurologists may recognize stroke patterns, psychiatrists often overlook CADASIL in patients presenting with depression, apathy, or vascular dementia. Only 12% of CADASIL-associated strokes occur without vascular risk factors, further masking its genetic etiology. Furthermore, although *NOTCH3* mutation testing is available, interpretation is complicated by variants of uncertain significance, such as cysteine-sparing mutations [103, 160, 162]. Upon systemic challenges in diagnosis, pathognomonic anterior temporal lobe lesions are present in MRI findings of 90% of cases but require targeted imaging protocols. Family history of early-onset stroke or migraine is frequently overlooked, delaying cascade testing [162].

Consequently, delayed diagnosis exacerbates morbidity, as 75% develop cognitive impairment by their fourth decade, and recurrent strokes lead to stepwise dementia. While no disease-modifying therapies exist, early diagnosis enables risk factor management (e.g., avoiding anticoagulants due to microbleed risks) and familial screening.

To overcome these challenges, CADASIL can be integrated into neurology and psychiatry training, emphasizing red flags such as MA in young adults. Combining neuroimaging expertise with genetic counseling can also be beneficial to address diagnostic complexities. Last, prioritizing studies on *NOTCH3* signaling pathways and immune dysregulation to identify therapeutic targets.

Therapeutic implications

Understanding the intricate interplay between genetic mutations, signaling pathways, and disease-modifying genes will assist in the development of therapeutic options for CADASIL (Table 3). The identification of disease-modifying genes, such as *RNF213*, has underscored the significant genetic landscape contributing to CADASIL pathophysiology [186]. These findings emphasize the need for targeted therapeutic approaches directed at modulating specific components of the disease pathway to alleviate the disease's burden and enhance patient outcomes [14, 108]. Adrenomedullin has an effect on oligodendrocyte precursor cells which could potentially compensate for the death of oligodendrocytes in CADASIL, through the resulting increase in the phosphorylated Akt cell survival signal [152]. Adrenomedullin may also have a role in promoting angiogenesis and inhibiting microglial activation and inflammation, which are observed features in CADASIL [61].

There is evidence that both hypomorphic and hypermorphic *NOTCH3* activities can be associated with *NOTCH3* mutation models, implying that maintaining an optimal range of *NOTCH3* signaling is essential for vascular health [150]. The therapeutic implications utilizing an A13 NOTCH3 agonist antibody in CADASIL mice, the study demonstrates prevention of mural cell loss in small-caliber vessels, as evidenced by smooth muscle actin staining in retinal vasculatures [99]. The A13 antibody treatment also leads to a reversal of plasma biomarker changes, including $NOTCH3^{ECD}$, endostatin, IGFBP- 1, and HTRA1. Study found the therapeutic potential of the A13 NOTCH3 agonist antibody in the context of CADASIL [99].

New treatments targeting *NOTCH3* signaling have gained attention because they might affect the root causes of diseases like CADASIL. One promising approach involves the use of the A13 *NOTCH3* agonist antibody, which has shown efficacy in preclinical models by preventing mural cell loss and normalizing plasma

Table 3 Therapeutic implications of CADASIL-related signaling pathways

Therapeutic target or Signaling pathway	Therapeutic strategy (gene therapy, drug, etc.)	Study model	Monitoring method	Is it clinically applicable?	Reference (s)
NOTCH3 Signaling Pathway	BRICHOS molecular chaperone	In vitro (cell lines)	Turbidity assays, TEM imaging	Potential for in vivo testing in CADASIL mouse model	[171]
NOTCH3 Signaling Pathway	NOTCH3-knockout	Mouse models	Structural arterial defects, BBB leakage, BOLD brain MRI scans	Complex effects on cerebrovascular integrity, may increase susceptibility to ischemic strokes	[3, 9, 29, 55, 56]
TGF-β Signaling Pathway	Modulation of TGF-β signaling	Not specified	Molecular evaluations	Not specified	[72, 156]
Disease-Modifying Genes	Modulation of TIMP3 and vascular fibronectin	Not specified	Not specified	Not specified	[14, 108, 186]
Adrenomedullin (AM)	Potential benefits in addressing CADASIL	Not specified	Not specified	Not specified	[61]
Stem Cell Factor (SCF) and Granulocyte Colony-Stimulating Factor (G-CSF)	Treatment for cerebral capillary thrombosis	TgNOTCH3R90 C mice	Bone marrow transplantation, immunohistochemistry	Potential neuroprotective effects observed	[128]

biomarkers like $NOTCH3^{ECD}$ and HTRA1 [34]. This suggests that the A13 antibody could be a viable strategy for restoring *NOTCH3* signaling balance, thereby improving vascular health and function [178]. Other possible treatments are also being studied. One example is controlling the TGF-β pathway, which could reduce fibrosis (stiffening) of blood vessels and improve the function of VSMCs. These new methods highlight how important it is to focus on *NOTCH3* signaling when developing treatments for vascular diseases [34, 41].

Conclusions

The *NOTCH3* signaling pathway plays a critical role in the integrity of vascular walls and the function of vascular smooth muscle cells. Key insights from recent studies have highlighted the decline in *NOTCH3* signaling as a biomarker for vascular aging and neurodegeneration, which is particularly relevant in the context of CADASIL. Mutations in the *NOTCH3* gene disrupt normal vascular function, leading to protein deposits and vascular injuries that are characteristics of CADASIL. In addition to the *NOTCH3* pathway, the TGF-β signaling pathway also plays a significant role in the pathogenesis of CADASIL. The deregulation of TGF-β signaling is closely associated with the recruitment of LTBP-1 into $NOTCH^{ECD}$ deposits and the overexpression of LAP within the affected vessels, altering TGF-β bioavailability and contributing to the disease's progression.

Currently, there is no cure or specific therapy for CADASIL. However, supportive care, including practical help, emotional support, and counseling, is recommended for affected individuals and their families. Migraines, a common symptom, should be treated symptomatically and with preventative methods. Future studies in this context should focus on larger patient cohorts and longer follow-up periods to better predict risks and define outcomes that matter to patients, which will aid in designing therapeutic trials. Moreover, novel measurements and more precise CADASIL models should be employed to compare with the progressive loss of *NOTCH3* function observed in the aging process.

Underdiagnosis of CADASIL could be addressed by wider use of genetic screening and advanced imaging like 7 T-MRI. National and international collaborations will also help advancing research into vascular contributions to cognitive decline.

Abbreviations

ADAM	A Disintegrin and Metalloprotease
BBB	Blood Brain Barrier
CADASIL	Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy
CARASIL	Cerebral Autosomal Recessive Arteriopathy with Subcortical Infarcts and Leukoencephalopathy
CBF1-Su(H)-Lag1	CSL (CBF1-Su(H)-Lag1)
CM	Cerebral Microbleeds
cSVD	Cerebral Small Vessel Disease
DSL	Delta/Serrate/LAG- 2
EGFr	Epidermal Growth Factor-like Repeats
ExAC	Exome Aggregation Consortium
G-CSF	Granulocyte Colony-Stimulating Factor
HANAC syndrome	Hereditary Angiopathy, Nephropathy, Aneurysms, and Muscle Cramps
HGMD	Human Gene Mutation Database
iPSCs	Induced Pluripotent Stem Cells
LTBP- 1	Latent TGF-β–Binding Protein 1
MA	Migraine with Aura
MS	Multiple Sclerosis
$NOTCH^{ICD}$	NOTCH Intracellular Domain
$NOTCH3^{ECD}$	NOTCH3 Extracellular Domain
$NOTCH3^{ICD}$	NOTCH3 Intracellular Domain
$NOTCH3^{TMIC}$	NOTCH Transmembrane Intracellular Domain
OPCs	Oligodendrocyte Precursor Cells

TIMP	Tissue Inhibitor of Metalloproteinases
TGF-β	Transforming Growth Factor Beta
VSMCs	Vascular Smooth Muscle Cells
WMHs	White Matter Hyperintensities

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