NEW INSIGHTS INTO MECHANISMS OF SMALL VESSEL DISEASE STROKE FROM GENETICS

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ABSTRACT

Cerebral small vessel disease (SVD) is a common cause of lacunar strokes, vascular cognitive impairment and vascular dementia. SVD is thought to result in reduced cerebral blood flow, impaired cerebral autoregulation and increased blood brain barrier permeability. However, the molecular mechanisms underlying SVD are incompletely understood.

Recent studies in monogenic forms of SVD, such as Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL), and 'sporadic' SVD have shed light on possible disease mechanisms in SVD. Proteomic and biochemical studies in post-mortem monogenic SVD patients, as well as in animal models of monogenic disease have suggested that disease pathways are shared between different types of monogenic disease, often involving the impairment of extracellular matrix (ECM) function.

In addition, genetic studies in 'sporadic' SVD have also shown that the disease is highly heritable, particularly among young-onset stroke patients, and that common variants in monogenic disease genes may contribute to disease processes in some SVD subtypes. Genetic studies in sporadic lacunar stroke patients have also suggested distinct genetic mechanisms between subtypes of SVD. Genome-wide association studies (GWAS) have also shed light on other potential disease mechanisms that may be shared with other diseases involving the white matter, or with pathways implicated in monogenic disease.

This review brings together recent data from studies in monogenic SVD and genetic studies in 'sporadic' SVD. It aims to show how these provide new insights into the pathogenesis of SVD, and highlights the possible convergence of disease mechanisms in monogenic and sporadic SVD.

SUMMARY STATEMENT

Recent studies in familial and 'sporadic' cerebral small vessel disease (SVD) have provided new insights into the pathogenesis of the disease. These suggest an important role for shared molecular pathways, particularly involving extracellular matrix proteins, in the mechanisms of SVD.

SHORT TITLE

Cerebral Small Vessel Disease – New Insights from Genetics

KEYWORDS

Lacunar stroke
Genetics
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INTRODUCTION

Cerebral small vessel disease

Cerebral small vessel disease (SVD) is a broad term encompassing different disease subtypes – amyloid- and non-amyloid SVD. Non-amyloid cerebral small vessel disease (SVD), which is the focus of this review, refers to several clinical and radiological features which describe disease of the small perforating blood vessels supplying the white and deep grey matter of the brain. SVD accounts for up to a fifth of all strokes, typically causing ischaemic lacunar strokes, but it is also now recognised as an important pathology underlying deep intracerebral haemorrhage (ICH). SVD is the most common pathology underlying vascular dementia and vascular cognitive impairment (VCI). (1)

SVD is characterised by a range of radiological features best seen on MRI including white matter hyperintensities (WMH) on T2/FLAIR MRI (corresponding to low signal or leukoaraiosis on CT), lacunar infarcts of presumed vascular origin, cerebral microbleeds, dilated perivascular spaces and brain atrophy.(2)

Pathogenesis of SVD – what is already known?

Despite its public health importance, the pathogenesis of SVD is incompletely understood. This has been a major limitation in developing therapies for the disease, of which there are few. Neuropathological studies show a number of abnormalities in the small perforating arteries, including both focal regions of atherosclerosis at the origin of, or in the proximal, perforating arteries, and more diffuse abnormalities affecting the small perforating vessels. These diffuse changes include thickening of the vessel wall due to the deposition of fibro-hyaline material, narrowing of the vessel lumen, and loss of smooth muscle cells in the tunica media with fibrinoid necrosis.(3)

The traditional hypothesis is that these vascular changes result in reduced cerebral blood flow and cerebral autoregulation, which in turn causes hypoperfusion. Imaging studies have confirmed both

reduced cerebral blood flow(4) and impaired cerebral autoregulation.(5) Increasing evidence supports the importance of endothelial dysfunction early in the disease and this could contribute to the impaired cerebral autoregulation.(6)

More recently it has been proposed that increased Blood Brain Barrier (BBB) permeability may play an important role.(7) Neuropathological studies have shown the presence of plasma proteins such as fibrinogen in the brain parenchyma, indicating that the BBB was open at some point.(8,9) Evidence of past BBB disruption is also provided by cerebrospinal fluid (CSF) studies showing the presence of plasma proteins in the CSF.(10) Further support is provided by more recent MRI studies demonstrating leakage of contrast agents such as gadolinium across the BBB.(11) It is likely that both hypoperfusion and increased BBB permeability interact, and endothelial dysfunction and activation could contribute to both.

Despite these advances, the molecular mechanisms underlying these processes are poorly understood, but recent data from genetic studies in both monogenic and 'sporadic' SVD are providing important novel insights. These have highlighted a number of shared molecular mechanisms that may be important in the disease, including a key role for abnormalities in the extracellular matrix (ECM).

Genetics of SVD

The majority of lacunar strokes are 'sporadic', with hypertension as the major risk factor alongside other common cardiovascular risk factors such as diabetes and smoking. However, SVD is also the stroke subtype that is most likely to present as a familial disease with the early onset of strokes.(1)

The most common familial form of SVD is Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL), caused by mutations in the NOTCH3 gene.(12) An autosomal recessive form of familial SVD has also been described in consanguineous Japanese and Chinese families, and has been attributed to mutations in the HTRA1 gene.(13) This disease is known as Cerebral Autosomal Recessive Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CARASIL), and a less severe form of the disease due to heterozygous mutations in the same gene has recently been described in Caucasian and Japanese populations.(14,15)

Mutations in a number of other genes have also been identified in familial SVD, and these are summarised in **Table 1**. Although rare, these extremes in phenotype share both clinical and radiological features with sporadic SVD, and are providing important insights into the mechanisms of the disease.

Furthermore, increasing evidence suggests that genetic susceptibility is also important in 'sporadic' SVD. This includes both epidemiological data showing that family history of stroke is a risk factor for SVD,(16) and recent genome-wide association study (GWAS) data demonstrating a significant heritability for 'sporadic' SVD of the predominant lacunar ischaemic stroke sub-phenotype.(17)

115 **Table 1: Monogenic forms of SVD**

Disease	Gene(s)	Gene function(s)	Mutations	Purported role in disease	Key clinical features
CADASIL	NOTCH3	Notch3 transmembrane receptor has roles in angiogenesis, vascular smooth muscle cell remodelling (18)	Cysteine-changing mutations in epidermal growth factor-like repeat region (EGFr) in exons 2 – 24 (19)	Accumulation of NOTCH3 ectodomain cleaved from mutant protein in extracellular spaces of small vessels.(20)	• Migraine with aura • Subcortical lacunar infarcts • Vascular dementia • Psychiatric disturbances • Encephalopathy
CARASIL (Autosomal dominant HTRA1-related CSVD has also been described) (14,15)	HTRA1	High temperature requirement serine protease A1 (HtrA1) switches off transforming growth factor β pathway(21)	Missense, nonsense and splice site mutations (13–15)	• Decreased protease activity (14) • Impaired activation of wild-type HtrA1 trimer subunits • Inhibition of HtrA1 trimer formation and stabilisation (13)	• Subcortical lacunar infarcts • Non-neurological features – alopecia, spondylosis • Vascular dementia
COL4-related SVD	COL4A1 COL4A2	COL4A1/A2 encode α1 and α2 collagen chains, which are the most abundant components of the extracellular matrix (22)	Missense mutations - most of which affect glycine residue in highly conserved Gly-X-Y repeat regions (23,24)	• Disrupted conformation of α1 or α2 chains (25), or impaired secretion of α1 and α2 chains , preventing formation of collagen helix (26), and resulting in basement membrane abnormalities (27) • Intracellular accumulation of non-secreted α1 and α2 may contribute to disease via endoplasmic reticulum stress (28,29)	• Porencephaly • Infantile hemiparesis • Intracerebral haemorrhage • Axenfeld-Rieger anomaly • Nephropathy • Muscle cramps
Retinal Vasculopathy with Cerebral Leukodystrophy	TREX1	TREX1 encodes DNase III (Three prime repair exonuclease), which has roles in DNA repair (30)	Frameshift mutations in C-terminus (31)	Impaired cellular localization of DNase III in endoplasmic reticulum (32)	• Retinal vasculopathy • Subcortical lacunar infarcts, WMH, pseudotumours • Migraine

and Systemic Manifestations (RVCL-S)					• Cognitive impairment • Psychiatric disturbances • Seizures • Multi-organ involvement: Raynaud's phenomenon, hepatic cirrhosis, renal dysfunction, osteonecrosis
FOXC1/PITX2-related SVD	FOXC1 PITX2	• Forkhead box transcription factor C1 (Foxc1) has roles in blood vessel development (33) • PITX2 encodes Paired-like homeodomain transcription factor 2, which determines left-right asymmetry of internal organs (34)	• Deletions or duplications of 6p25 (35) • Mutations in Foxc1 (36)	• FOXC1 interacts with PITX2 (36) • FOXC1 involved in pericyte and endothelial cell proliferation • Impaired blood brain barrier function (37)	• Axenfeld-Rieger anomaly • WMH • Cerebellar malformations • Hydrocephalus • Periventricular heterotopia

New insights from studies in monogenic SVD - the ECM and the matrisome

Recent studies in in monogenic SVD have provided evidence for the involvement of key extracellular matrix (ECM) or 'matrisome' proteins in the pathogenesis of the disease. The ECM is the non-cellular component of tissues made up of water, proteins and polysaccharides. It provides scaffolding for cellular components by producing fibrous proteins such as collagen, laminin and elastin, and is also biochemically active, providing signals which contribute to tissue function and homeostasis. The ECM also binds and serves as a reservoir for many other biochemically active molecules.(38) The matrisome is thus defined as the ensemble of nearly 300 proteins which make up the ECM (core matrisome), or are associated with the ECM (matrisome-associated proteins), and have been characterised by bioinformatics and proteomic methods. (39)

In the blood vessels, the ECM interacts with other vascular cells to influence vascular development and remodelling. The blood vessels have ECM components in each of its three layers. The innermost layer (tunica intima) is lined with endothelial cells on a basement membrane comprising of matrisome proteins such as type IV collagen; the tunica media contains sheets of smooth muscle cells separated by ECM, while the outermost tunica adventitia contains myofibroblast cells and is rich in type I and III collagen in addition to many other matrisome proteins.(40) The reader is directed to a recent review by Joutel et al for an in-depth discussion on the role of the matrisome in the small vessels, and the evidence for the alteration of matrisome function in SVD.(41)

Matrisome involvement in CADASIL

The involvement of the matrisome in different monogenic forms of SVD suggests that the ECM may be the basis of shared molecular pathways in SVD. This has been illustrated in CADASIL, where the basis of ECM involvement has now been characterised at several stages - from histopathological studies in post-mortem analysis,(42) to a direct link to cerebral vasoreactivity in animal models of CADASIL. (43–45)

Post-mortem studies in patients with CADASIL have shown a possible aggregation cascade of matrisome proteins. The basis of this arose from the fact that a pathognomonic feature of CADASIL is the deposition of granular osmiophilic material (GOM) in the extracellular space of the small blood vessels systemically,(46) and the accumulation of deposits of the NOTCH3 ectodomain ($NOTCH3^{ECD}$) cleaved from the mutant NOTCH3 receptor.(20) In transgenic mice expressing the human NOTCH3 R90C mutation, $NOTCH3^{ECD}$ accumulation and GOM deposits are often the earliest pathological features of the disease. (47) This is followed by a potassium channelopathy which precedes and results in the onset of impaired cerebral vasoreactivity,(48) eventually leading to the development of white matter lesions. (49)

Studies in post-mortem specimens from CADASIL patients and transgenic CADASIL mouse models suggest that increased levels of $NOTCH3^{ECD}$ may promote the formation of disulphide cross-linked aggregates in a protein aggregation cascade. These aggregates sequester key matrisome proteins which have roles in maintaining the integrity and function of the ECM in the walls of the blood vessels.(42,50) A summary of these proteins and their functions is provided in **Table 2**.

This protein aggregation cascade demonstrated by proteomic studies in CADASIL shows parallels with the progression of features in animal models of CADASIL, suggesting that each protein's involvement may contribute to different features of the disease pathway. Decreased baseline cerebral blood flow and cerebrovascular reactivity have been demonstrated in CADASIL patients, with decreased cerebrovascular reactivity showing an association with the progression of white matter lesions.(51) A transgenic mouse model of CADASIL has recapitulated these features, showing dysfunctional cerebral vasoreactivity early in disease. This was characterised by the impaired cerebral blood flow autoregulation in response to vasodilator stimuli (hypercapnia and acetazolamide), and increased vessel resistance in the context of hypertension. (52)

The impaired cerebral vasoreactivity in a transgenic CADASIL mouse model was later shown to be due to an increase in the number of voltage-gated potassium channels in the membranes of the

smooth muscle cells. These channels oppose depolarisation due to pressure, and downregulation of these channels restores normal myogenic responses to pressure. (48)

In biochemical and proteomic studies of cerebral vessels from CADASIL patients, $NOTCH3^{ECD}$ aggregation was found to induce the co-aggregation of Tissue inhibitor of metalloproteinase 3 (TIMP3), which then promotes the sequestration of another matrisome protein, vitronectin, in these aggregates.

A potential mechanistic link between increased TIMP3 activity and impaired cerebral blood flow regulation has recently been demonstrated. Increased TIMP3 expression in transgenic mice were shown to promote the upregulation of potassium channel current density in the cerebral arterial myocytes, and thus the reduction of myogenic tone and cerebral autoregulation.(44,45) This process is thought to be mediated by TIMP3/ a disintegrin and metalloproteinase 17 (ADAM17) interactions.

ADAM proteases cleave off the extracellular domains in the activation of membrane-bound proteins. In particular, ligands of the EGFR family, such as heparin-binding EGF-like growth factor are substrates of ADAM17.(53) The ADAM17/Heparin-binding EGF-like growth factor (HB-EGF)/EGFR (ErbB1/ErbB4) signalling axis regulates cerebral arterial tone and cerebral blood flow. (54) TIMP3 inhibits this signalling axis, and restoration of this axis with the delivery of exogenous ADAM17 or HB-EGF restores cerebral blood flow autoregulation in transgenic mice.(45)

TIMP3 is associated with potassium channelopathy and impaired cerebrovascular reactivity but not white matter lesion load, while the subsequent involvement of vitronectin is associated with the presence of white matter lesions but not cerebral vasoreactivity impairment. (42) The stepwise involvement of each protein in the cascade thus shows direct parallels with each stage of disease progression in the animal model. (**Fig.1**)

The cascade of sequential recruitment and aggregation of matrisome proteins triggered by an altered $NOTCH3^{ECD}$ is also reminiscent of the ‘prion hypothesis’ in other neurodegenerative diseases

such as Alzheimer's Disease and Parkinson's Disease, where a misfolded protein acts as a 'seed' and triggers further misfolding and protein aggregation.(55) In these diseases, proteins such as Aβ, tau and α-synuclein adopt β-sheet-rich conformations and self-propagate.(56) Although mutant proteins in the aggregatory process in CADASIL may not necessarily act in a prion-like manner – but instead promote the aggregation of different proteins - the similarities between these processes may eventually point toward common targetable pathways.

Table 2: Matrisome proteins found to co-aggregate with NOTCH3 ^{ECD}.

Matrisome protein	**Function in ECM**	**Involvement in CADASIL / CARASIL**	**Models studied**
Thrombospondin-2 (TSP2)	• Interacts with NOTCH3(57) • Regulates ECM assembly processes, such as collagen fibrillogenesis • Regulates angiogenesis(58,59)	• NOTCH3^{ECD} deposits found to co-aggregate with thrombospondin-2. (60)	Post-mortem human CADASIL specimens
Latent TGFβ-binding protein (LTBP-1)	• TGFβ is secreted as an inactive complex with LTBP-1 and latency associated peptide (LAP) • LTBP-1 regulates bioavailability of active TGFβ in the ECM(61)	• NOTCH3^{ECD} deposits found to co-aggregate with LTBP-1.(50) • CARASIL mutations preclude physiological cleavage of LTBP-1 by HtrA1(62)	Mouse brain tissue, embryonic and patient skin fibroblasts
Tissue inhibitor of metalloproteinase 3 (TIMP3)	• Regulatory function in ECM remodelling – inhibits a disintegrin and metalloproteinase 17 (ADAM17), a metalloprotease which degrades ECM. (63)	• NOTCH3^{ECD} forms complexes with TIMP3(42) • Increase in TIMP3 activity contributes to vessel fibrosis, dysfunctional cerebral blood flow and myogenic responses to changes in neural activity, but not associated with white matter lesion load(44)	Post-mortem human CADASIL specimens and transgenic mouse models
Vitronectin	• Glycoprotein in blood plasma and ECM • Roles in cell attachment, aggregation, atherosclerosis and thrombus formation(64)	• NOTCH3^{ECD} aggregation promotes the sequestration of TIMP3, which then promotes the co-aggregation of vitronectin.(42) • Reduced vitronectin levels associated with lower white matter	Post-mortem human specimens and transgenic mouse models

		burden in mouse model but not cerebral blood flow or GOM load(45)	

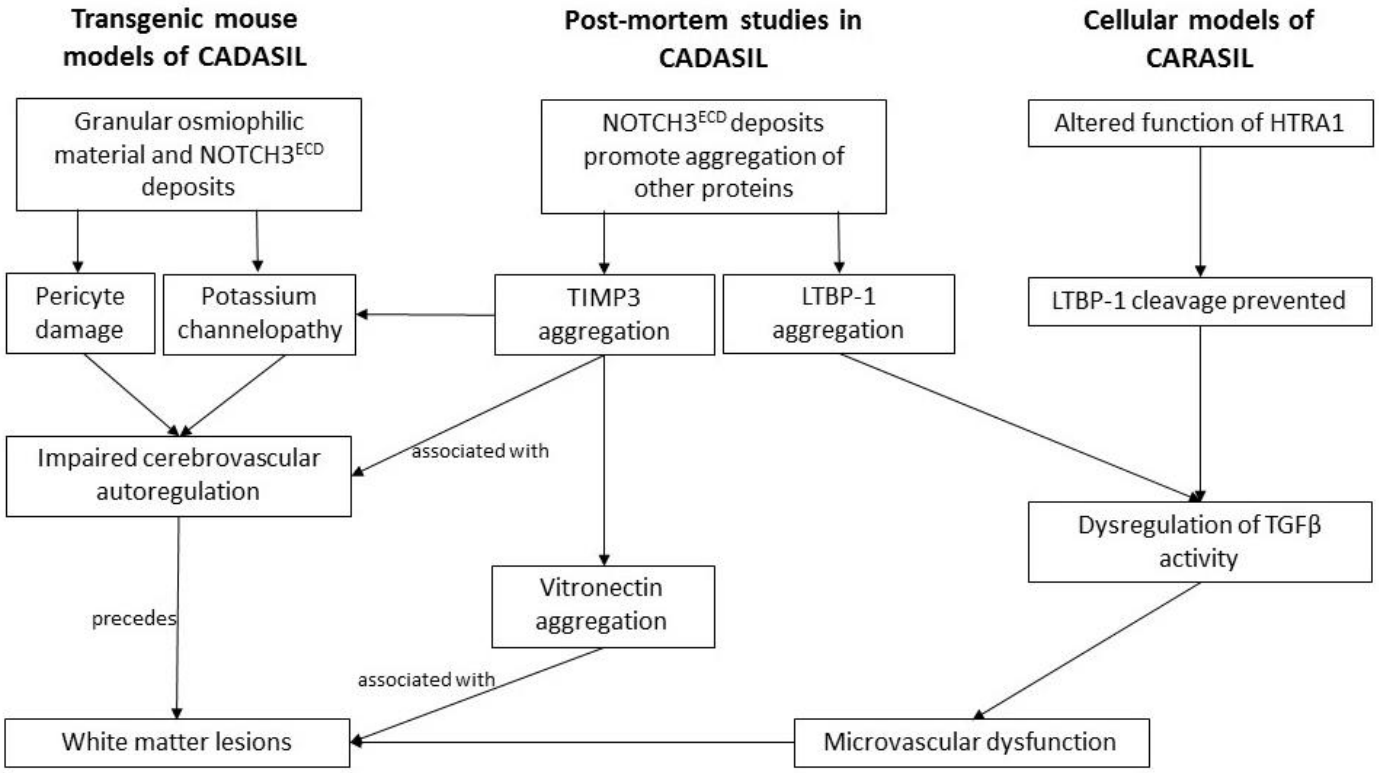
CADASIL and CARASIL: convergent disease mechanisms

One of the matrisome proteins identified in NOTCH3^{ECD} protein aggregates in CADASIL has also been identified as a key molecule in CARASIL. Latent TGFB-binding protein 1 (LTBP-1), which co-aggregates with NOTCH3^{ECD} in CADASIL,(50) was identified to be a target of the HtrA1 serine protease in a study of mouse brain tissue, as well as embryonic and patient skin fibroblasts.(62)

TGFβ is secreted as an inactive complex together with LTBP-1 and latency associated peptide (LAP). LTBP-1, through its interactions with other matrisome proteins such as fibronectin and fibrillins, regulates the bioavailability of soluble and active TGFβ in the ECM. CARASIL-causing mutations preclude the physiological cleavage of LTBP-1 by HtrA1, disrupting its binding to fibronectin and fibrillins, resulting in the dysregulation of TGFβ release from the ECM. (62)

Further evidence for the involvement of this pathway is also demonstrated with the enrichment of LAP, fibronectin and fibrillin-1 in the blood vessels of CADASIL patients. Although these did not co-aggregate with the NOTCH3^{ECD} deposits, their presence lends support to their role in downstream processes secondary to the direct involvement of LTBP-1. (50)

Figure 1: The involvement of matrisome proteins in the pathogenesis of CADASIL and CARASIL. Vitronectin and TIMP3 may serve as molecular correlates of clinical features and terminal pathways in the disease. Vitronectin levels are associated with white matter lesion load, while TIMP3 levels are associated with cerebral vasoreactivity in a transgenic CADASIL mouse model. LTBP-1, which co-aggregates with $NOTCH3^{ECD}$ in CADASIL, has also been identified as the proteolytic target of HtrA1 protease, the enzyme altered as a result of CARASIL mutations.



Relevance of CADASIL and CARASIL disease mechanisms in sporadic SVD

The molecular pathways characterised in CADASIL may be particularly relevant in our understanding of the pathogenesis of sporadic SVD. Evidence from both CADASIL and population-based genetic studies suggest that the same pathways may contribute to sporadic disease.

While GOM deposits are pathognomonic of CADASIL, other histopathological features of CADASIL recapitulate those seen in sporadic disease. These include the fibrosis of the adventitia, and the loss of endothelial and smooth muscle cells of the perforating arteries. Similar features are also seen in post-mortem studies of cerebral vasculature in CARASIL, with fibrous intimal proliferation, hyaline degeneration of the media, loss of arterial smooth muscle cells and splitting of the internal elastic lamina contributing to the narrowing of the vessel lumen. (65,66)

Genetic studies in the population also hint at the possible involvement of similar matrisome-associated proteins in the pathogenesis of sporadic SVD. In a study of 888 population-based stroke- and dementia-free individuals in the Austrian Stroke Prevention Study, the association between common single nucleotide polymorphisms (SNPs) in the NOTCH3 gene region and white matter hyperintensities and lacunes was investigated. Four common variants, rs1043994, rs10404382, rs10423702 and rs1043997, which are in strong linkage disequilibrium, were found to be significantly associated with both the presence and progression of WMH, with this effect only being present in hypertensives. This suggests that the minor alterations in Notch3 receptor function may act together with, or augment the effects of hypertension to cause this association. These results were replicated in a sample of 8545 individuals from the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE).(67) However, the association with WMH was not replicated in meta-analyses of GWAS data sets from ischaemic stroke cohorts in 3670 cases and 7397 controls, and no association was found between NOTCH3 SNPs and lacunar stroke or with WMH in stroke patients. (68) Evidence for the involvement of monogenic disease genes in sporadic SVD is summarised in **Table 3**.

Collagen gene mutations: involvement of the most abundant matrisome protein

Collagen is the most abundant protein in the ECM, and has a characteristic triple-stranded helical structure known as tropocollagen. Tropocollagen is made up of polypeptide chains with highly conserved repetitive three-residue sequences (Gly-X-Y). As glycine is the amino acid with the smallest side chain, it allows the tight assembly of each collagen strand in a helix, with glycine forming the core of each helix. Multiple tropocollagen molecules polymerise to form collagen fibrils which provide tensile strength to tissues. (22)

Type IV collagen in the basement membrane is formed with α1 and α2 collagen chains in a 2:1 ratio. These chains, encoded by the COL4A1 and COL4A2 genes respectively, are the most abundant proteins in basement membranes and surrounding smooth muscle cells in the tunica media of blood vessels.(40) The relationship between collagen mutations and vessel fragility is well described in diseases such as osteogenesis imperfecta (COL1A1 or COL1A2 mutations) and Ehlers-Danlos syndrome (COL3A1 mutations).

The majority of reported mutations in COL4A1/A2-related SVD tend to affect the glycine residue, disrupting the three-dimensional conformation of each α1 or α2 strand and thus impairing the formation of the resulting tropocollagen molecule. (69) This is thought to result in the impaired synthesis of the basement membrane, culminating in blood vessel fragility. (27) Both human and mouse mutations in COL4A2 have been shown to cause the impaired secretion of both α1 and α2 chains, thus resulting in the retention of mutant α1 and α2 chains in the endoplasmic reticulum (26). The accumulation of mutant α1 or α2 chains results in endoplasmic reticulum stress, which may also contribute to disease.(28,29)

In a phenotypic subtype of COL4A1-related SVD known as Hereditary Angiopathy, Nephropathy, Aneurysms and Cramps (HANAC syndrome), mutations were found to aggregate in the 31-residue CB3[IV] region of the COL4A1 gene, which is a critical integrin binding site, suggesting that abnormal interactions between type IV collagen and cells may result in a systemic form of the disease (25,70).

While a precise molecular pathway in COL4A1 and A2-associated SVD has not yet been identified, it is likely that the impaired function of collagen in the ECM contributes to the disease process.

Relevance of collagen genes in sporadic SVD

While COL4A1/A2 mutations can cause familial SVD, recent evidence suggests common variants in the same genes are associated with sporadic SVD and ICH. A meta-analysis of genotype data from large GWAS studies in stroke in individuals of European ancestry identified three common variants (rs9521732, rs9521733, rs9515199) in intronic regions in COL4A2 which were significantly associated with deep ICH. There was a similar trend for lacunar stroke and WMH although the associations did not achieve the stringent significance levels set to account for the multiple comparison made.(71) (**Table 3**)

A multi-ethnic genome-wide meta-analysis of dementia- and stroke-free cohorts found a SNP located in an intron of the COL4A2 gene, rs9515201 which was associated with WMH in community populations; this SNP (72) is in strong linkage disequilibrium with SNPs that were previously identified to be associated with sporadic ICH.(71) (**Table 3**)

Other possible mechanisms of disease – insights from genetics

Blood Brain Barrier development and integrity

Mutations and copy number variations in the FOXC1 gene were initially identified as a cause for Axenfeld-Rieger Syndrome (ARS) and cerebellar malformations.(73) In multiple case reports of patients with 6p25 deletions, individuals with ARS and other developmental abnormalities were also found to have WMH from as early as 18 months of age.(35) A meta-analysis and study of expression quantitative trait loci in GWAS data from the CHARGE consortium later demonstrated that 3 SNPs associated with WMH strongly influenced FOXC1 transcript levels, and that 18 out of 18 patients with FOXC1-related ARS also showed MRI evidence of SVD.(36) (**Table 3**)

The FOXC1 gene codes for the forkhead box transcription factor C1 (Foxc1), critical in the development of blood vessels.(74) Foxc1 originates from the neural crest and is expressed by brain pericytes, which are integral components of the BBB, and regulates vascular morphogenesis and maturation during embryological brain development. While Foxc1 deletion does not preclude angiogenesis and may not affect BBB formation and permeability, it results in altered brain pericyte and endothelial cell proliferation, impairing blood vessel stability and thus predisposing these vessels to haemorrhage. (74) Hence, although the precise mechanisms behind FOXC1-associated SVD are not known, the theoretical basis of this disease suggests an impairment of the BBB.

FOXC1 interacts with Paired-like homeodomain transcription factor 2 or Pituitary Homeobox 2 (PITX2), a developmental transcription factor expressed in the neural crest. Mutant forms of PITX2 also cause ARS.(73) ARS patients with PITX2 mutations also had features of SVD on brain imaging.(36) The similar phenotype seen with PITX2 mutations lends further support for the involvement of the FOXC1 pathway in the development of SVD.

Studies in FOXC1 knockout models have led to speculation that matrisome proteins may mediate disease mechanisms in FOXC1-related SVD.(41) The expression of matrix metalloproteinases (MMPs), which regulate the ECM, is increased in the cornea of global and neural crest-deleted $Foxc1^{-/-}$ mice. These MMPs regulate the bioavailability of vascular endothelial growth factors sequestered in the ECM. Upregulation of MMP expression leads to disorganisation of the ECM and excessive growth of vessels in the cornea of mutant mice.(33) Suppression of Foxc1 in zebrafish also reduced expression of platelet-derived growth factor (PDGF), a matrisome-associated protein integral to the development of vasculature. Consistent with evidence in humans that alterations in Foxc1 dosage were associated with SVD, zebrafish with either Foxc1 knockdown or overexpression also exhibited cerebral haemorrhage.(36)

Adjacent to the FOXC1 gene on chromosome 6p25 is FOXF2, a gene that encodes the Foxf2 transcription factor. Foxf2 is expressed specifically in CNS pericytes and is required for pericyte

differentiation and BBB development. (75) FOXF2 knockout mouse embryos develop defects in the BBB, and FOXF2 inactivation in adult mice lead to BBB breakdown, cerebral infarction and microhaemorrhage. (76)

FOXF2 mutations and copy number variations have been implicated in Anterior Segment Dysgenesis, an ocular condition which also occurs in ARS. (77) In ARS, patients with FOXC1 and FOXF2 both deleted have more extensive WMH than those with deletion of only FOXC1, suggesting that the loss of interactions between FOXC1 and FOXF2 contribute to a shared disease pathway. (78)

The same forkhead box protein loci have also been implicated in sporadic SVD. (**Table 3**) A meta-analysis of GWA data of the FOXC1 and PITX2 gene locus identified 10 WMH-associated SNPs which lie in an intron of the GDP-mannose 4,6-dehydratase gene (GMDS) adjacent to FOXC1. Three of these SNPs have effects on FOXC1 transcript levels. (36) In the PITX2 gene locus, nine SNPs were found to be significantly associated with WMH. (36) Another recent large-scale GWAS meta-analysis in ischaemic stroke identified a novel locus close to FOXF2. (75) The same SNP was also associated with WMH, suggesting that the mechanism by which disease risk is conferred is though SVD. These converging results from sporadic and monogenic disease lend support to the possible roles of the FOXC1-PITX2-FOXF2 interactions and their roles in maintaining BBB integrity via proteins in the matrisome.

Evidence for the involvement of the BBB in SVD is also seen in CADASIL. Histopathological examinations of cerebral vessels from CADASIL patients and transgenic mouse models of CADASIL have shown damaged pericytes. (79–81) The TGFβ pathway, which has been implicated in both CADASIL and CARASIL, may provide a possible explanation for this process. In vitro studies of TGFβ1, which is the most extensively studied form of TGFβ, have shown that TGFβ1 reduces pericyte proliferation and elevates the expression of MMPs and other proinflammatory cytokines which may disrupt BBB function. (82)

Impairment of DNA Damage Response

Retinal Vasculopathy with Cerebral Leukodystrophy and Systemic Manifestations (RVCL-S) is an autosomal dominant form of SVD caused by mutations in the Table 1) The TREX1 gene codes for the most abundant DNA exonuclease in mammals, known as DNase III or Three prime Repair Exonuclease.(31)

DNase III has a role in the repair of DNA damage, being translocated from the endoplasmic reticulum to the nucleus during oxidative DNA damage.(32) DNase III enzymatically digests cytosolic single-stranded DNA to prevent the cell from responding to immunostimulatory DNA, such as those arising from pathogenic viruses.

Dysfunctional DNase III arising from TREX1 mutations may thus result in the erroneous recognition and clearance of self-nucleic acids, resulting in autoimmune and inflammatory diseases such as systemic lupus erythematosus (SLE), an inherited form of SLE known as familial chilblain lupus, and an inflammatory early-onset encephalopathy known as Aicardi-Goutiéres Syndrome. (15)

RVCL mutations lead to the expression of DNase III with a truncated C-terminus, disrupting the transmembrane domain and impairing the cellular localisation of DNase III in the endoplasmic reticulum.(31) The nuclear target of DNase III was recently identified as poly(ADP-ribose) polymerase-1 (PARP1), an enzyme which repairs single stranded DNA breaks through a process of base excision repair, and is integral to the cell's DNA damage response. (83) The disrupted localisation of DNase III may theoretically have a toxic gain-of-function effect, or attenuate normal DNA damage responses, however the precise mechanisms underlying RVCL remain to be characterised.

Further insights from GWAS– common and distinct mechanisms across the SVD spectrum

A number of insights into the underlying genetic architecture of sporadic SVD have also come from recent GWA studies.

An important question is whether SVD pathology is homogeneous across all individuals, or whether there are distinct pathological pathways leading to SVD in different groups. In a population of stroke patients, (84) genetic factors underlying WMH were distinct in hypertensive individuals compared to non-hypertensives, with only a very low correlation between the genetic components (r^2=0.15). (84) This points to distinct disease pathways leading to SVD in the two groups.

A recent investigation of the genetic component of lacunar ischaemic stroke using GWAS data from a young onset population with MRI-confirmed lacunar stroke showed that genetic factors are an important contributor to risk in this population, (17) with higher heritability than in previous populations where most phenotyping was done using CT. (85) Much of the heritability arose from regions of the genome influencing expression of genes, or in DNase I Hypersensitivity sites, suggesting that the genetic risk of sporadic SVD is conferred through subtle changes to gene expression and regulation. In addition, when dividing lacunar cases into those with extensive WMH and those without, analysis suggested that distinct but different rare genetic variants contributed to disease in the two groups, again highlighting that multiple distinct pathways lead to different manifestations SVD in different groups of patients. (17)

GWAS studies in MRI-determined WMH have identified 13 loci robustly associated with the trait, as summarised in **Table 4** (72,86,87) Four of the loci arise from an extended region containing *NEURL1, PDCD11,* and *SH3PXD2A*. Of these genes, *NEURL* - a highly conserved E3 ubiquitin ligase - is of particular interest as it inhibits the Notch pathway through decreasing expression of the Notch ligand, JAG1. (88,89) Interestingly, 5 of the associated loci fall in genes which have been implicated

in malignant brain tumours of the white matter involving glial cells, highlighting the importance of these cells in pathogenesis of SVD. As well as influencing WMH in both community and stroke patient populations, (86) 12 of the identified WMH loci also confer risk of lacunar stroke, (90) and one of the loci, on 1q22, is also associated with ICH. (91)

Taken together, GWAS to date emphasise that there are likely multiple pathways leading to SVD. Some of these pathways are shared across manifestations of disease, but also some are likely to be specific to disease groups; and in some cases are likely to act through interactions with risk factors such as hypertension.

Concluding remarks

Studies in both monogenic forms of SVD and the genetics of 'sporadic' SVD are now beginning to fill in the blank edges in the map of the disease processes in SVD. (**Figure 2**) Shared pathways affecting the integrity and function of the ECM appear to play an integral role in these disease pathways. It is likely that there are multiple shared pathways, each being involved to different degrees in different manifestations or subtypes of SVD. These genetic mechanisms, as well as their interactions with environmental factors, may provide explanations as to why different patients in the sporadic disease population exhibit each feature of SVD to different extents.

In addition, there is now accumulating evidence of a protein aggregation cascade seen in CADASIL, suggesting that the convergence of pathways may extend beyond SVD, and there may be a convergence of pathogenic pathways seen in neurodegenerative diseases in general.

Elucidating the disease pathways in SVD may allow us to identify therapeutic targets. An example is seen in a monogenic large vessel vasculopathy, Marfan syndrome, which can be caused by mutations in the fibrillin-1 gene. Fibrillin-1 is a key ECM component and binds to the latent TGFβ complex. Antagonists of TGFβ signalling such as losartan have now been shown to reduce the

development of aortic aneurysms in a mouse model of Marfan syndrome. (92) The involvement of the TGFβ pathway in SVD may lead us towards the use of TGFβ antagonists to halt disease progression, while the protein aggregation cascade may suggest the potential utility of drugs being developed in the treatment of other neurodegenerative diseases with similar mechanisms.

Further genetic studies in SVD will likely provide more conclusive evidence of overlap of disease pathways involved in both monogenic and sporadic disease. While understanding the processes in each disease, whether a monogenic form of SVD or sporadic disease, may aid the development of treatment options for the specific disease, it is possible that the distinction between each of the diseases are blurred and the same few convergent processes will eventually serve as therapeutic targets.

Table 3: Common variants, found in monogenic disease genes, are associated with features of SVD

Monogenic disease gene	Phenotype and populations studied	Approach	No. of patients/ controls	Variants identified (intronic, I/ exonic, E, downstream, DS, intergenic (IG)	Strength of evidence	References
NOTCH3	WMH in community-based population	Direct sequencing of all 33 exons, promoter and 3'-untranslated region of NOTCH3	• Sequenced:195 community-based Caucasians, 82 controls with no WMH • Genotyped: 888 participants from Austrian Stroke Prevention Study	4 SNPs associated with WMH presence and progression in hypertensives • rs1043994 (I) • rs10404382 (I) • rs10423702 (I) • rs1043997 (E)	• Only candidate gene studied • Replication of rs10404382 in GWAS data from hypertensive stroke-free elderly individuals in CHARGE consortium (n=8545)	(67)
	Ischaemic stroke	Direct sequencing of all 33 exons	269 Caucasians with ischaemic stroke, 95 controls	1 SNP associated with ischaemic strokes • rs785101403	• Only candidate gene studied • Insufficient power to study demonstrate association with stroke subtypes	(93)
	Symptomatic lacunar stroke or WMH in stroke patients	Meta-analysis of GWAS data sets	1350 European patients with MRI-confirmed lacunar stroke, 3670 patients with ischaemic stroke and WMH, 7397 controls	No association between NOTCH3 variants and lacunar stroke or WMH volume	Only candidate gene studied	(68)
	Leukoaraiosis (Fazekas scale 3)	Screen of exons 3, 4, 5, 6 of NOTCH3 gene by polymerase chain reaction	218 patients with lacunar stroke (48 with leukoaraiosis)	No association identified between common polymorphisms and leukoaraiosis	Limited screen of NOTCH3 gene only	(94)

Monogenic disease gene	Phenotype and populations studied	Approach	No. of patients/ controls	Variants identified (intronic, I/ exonic, E, downstream, DS, intergenic (IG)	Strength of evidence	References
		(PCR)-single-stranded conformational polymorphism analysis				
	Symptomatic ischaemic cerebrovascular disease	PCR analysis of T6746C polymorphism	235 Japanese patients with CT/MRI defined ischaemic stroke/TIA (142 with lacunar stroke), 315 controls	No association found between T6746C and cerebrovascular disease or lacunar stroke	Only one polymorphism studied in NOTCH3 gene	(95)
	Ischaemic stroke and dementia	Novel diagnostic array for known mutations and polymorphisms in exons 3 and 4 of NOTCH3	70 patients with CT/MRI-confirmed ischaemic stroke and 77 patients with dementia, 117 controls	No association between known polymorphisms and stroke or dementia	Only 5 previously identified polymorphisms in 2 exons studied	(96)
HTRA1	None reported					
COL4A1	Presumed sporadic ICH	Direct sequencing of coding regions of COL4A1, including flanking intronic regions	• 48 patients with presumed hypertension-related deep ICH • 48 with probable cerebral amyloid angiopathy-related ICH	2 rare coding variants associated with ICH: • c.C1055T (p.P352L) (E) • c.C1612G (p.R538G) (E)	• Only candidate gene studied • Only rare variants analysed for pathogenicity – common variants not studied • Cellular assay of variants demonstrated impaired secretion of α1 chain.	(97)

Monogenic disease gene	Phenotype and populations studied	Approach	No. of patients/ controls	Variants identified (intronic, I/ exonic, E, downstream, DS, intergenic (IG)	Strength of evidence	References
			• 145 controls			
COL4A2	• Intracerebral haemorrhage (ICH)(deep/lobar) • Ischaemic stroke (cardioembolic, large vessel, SVD) • WMH (ischaemic stroke and population-based)	Meta-analysis of GWAS data sets	• 1545 patients with ICH, 1485 controls • 1854 patients with lacunar stroke, 2733 with ischaemic stroke and WMH, and 9361 controls	3 SNPs associated with deep ICH: • rs9521732 (I) • rs9521733 (I) • rs9515199 (I)	• Only candidate genes studied • No significant eQTLs with 3 SNPs or 5 other SNPs in high LD with these 3. • SNPs located in regions with possible regulatory roles • SNPs did not reach significance threshold for association with lacunar stroke or with WMH volume	(71)
	WMH in stroke patients	Meta-analysis of GWAS data	3670 stroke patients	4 novel SNPs associated with WMH, one of which is in COL4A2 • rs9515201 (I)	SNP in strong linkage disequilibrium (LD) with those previously identified (above) SNP may have regulatory function	(72)
COL4A2	Presumed sporadic ICH	Direct sequencing of coding regions of COL4A2, including flanking intronic regions	• 48 patients with presumed hypertension-related deep ICH • 48 with probable cerebral amyloid angiopathy-related ICH • 145 controls	3 rare coding variants associated with ICH: • c.3368A>G (p.E1123G) (E) • c.3448C>A (p.Q1150K) (E) • c.5068G>A (p.A1690T) (E)	• Only candidate gene studied • Only rare variants analysed for pathogenicity – common variants not studied • Cellular assay of variants demonstrated impaired secretion of α1 and α2 chains.	(26)

Monogenic disease gene	Phenotype and populations studied	Approach	No. of patients/ controls	Variants identified (intronic, I/ exonic, E, downstream, DS, intergenic (IG)	Strength of evidence	References
TREX1	None reported					

FOXC1 / PITX2	WMH in community-based dementia- and stroke-free populations	Meta-analysis of GWAS data and study of patients with FOXC1-related Axenfeld-Rieger Syndrome (ARS)	9361 patients in GWAS, 18 patients with FOXC1-related ARS	10 SNPs located in GMDS gene (lies adjacent to FOXC1)
				• rs12206258 (I)
				• rs12203614 (I)
				• rs12199578 (I)
				• rs12193217 (I)
				• rs10458129 (I)
				• rs12206340 (I)
				• rs12189662 (I)
				• rs6936881 (I)
				• rs7765461 (I)
				• rs7765344 (I)
					• Only candidate gene region studied
					• 3 SNPs strongly modify FOXC1 transcript levels:
					  ○ rs12206258
					  ○ rs6936881
					  ○ rs7765344
					• 18 of 18 patients with FOXC1-related ARS have features of SVD
						(36)

				9 SNPs near PITX2 • rs2129979 (DS) • rs11931959 (DS) • rs13121924 (DS) • rs3866831 (IG) • rs6533531 (IG) • rs3866832 (IG) • rs13141190 (IG) • rs6533530 (IG) • rs7697491(IG) • rs723363 (IG)	• Only candidate gene regions studied	
	Stroke and stroke subtypes (ischaemic,	Meta-analysis of GWAS data	84961 European participants (4348 with stroke, of which 1770	rs12204590 near FOXF2 associated with all-stroke and WMH	• rs12204590 replicated in validation samples (stroke patients), associated with risk	(75)

Monogenic disease gene	Phenotype and populations studied	Approach	No. of patients/ controls	Variants identified (intronic, I/ exonic, E, downstream, DS, intergenic (IG)	Strength of evidence	References
	cardioembolic, non-cardioembolic) WMH in stroke-free adults		were non-cardioembolic ischaemic strokes)	burden in stroke-free adults	of all-stroke • rs12200309, in complete LD with rs12204590, associated with small vessel ischaemic stroke in validation samples • Region includes enhancers, with 2 SNPs in high LD with rs12204590 having probable roles in regulating gene expression	

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434 **Table 4: SNPs associated with WMH in community and stroke populations**

SNP	Chromosome	Nearest Gene	Phenotype Association	References
rs7214628	17	TRIM65	WMH in community and stroke populations	(72,86)
rs72848980	10	NEURL	WMH in community populations	(72,86)
rs7894407	10	PDCD11	WMH in community populations	(72,86)
rs12357919	10	SH3PXD2A	WMH in community populations	(72,86)
rs7909791	10	SH3PXD2A	WMH in community populations	(72,86)
rs78857879	2	EFEMP1	WMH in community and stroke populations	(72,86)
rs2984613	1	PMF1-BGLAP	WMH in community populations, intracerebral haemorrhage	(72,86,91)
rs11679640	2	HAAO	WMH in community populations	(72,86)
rs72934505	2	NBEAL1	WMH in community and stroke populations	(72,86)
rs941898	14	EVL	WMH in community and stroke populations	(72,86)
rs962888	17	C1QL1	WMH in community and stroke populations	(72,86)
rs9515201	13	COL4A2	WMH in community and stroke populations, intracerebral haemorrhage	(71,72,86)
rs12445022	16	ZCCHC14	Small vessel stroke, WMH in stroke populations	(87)

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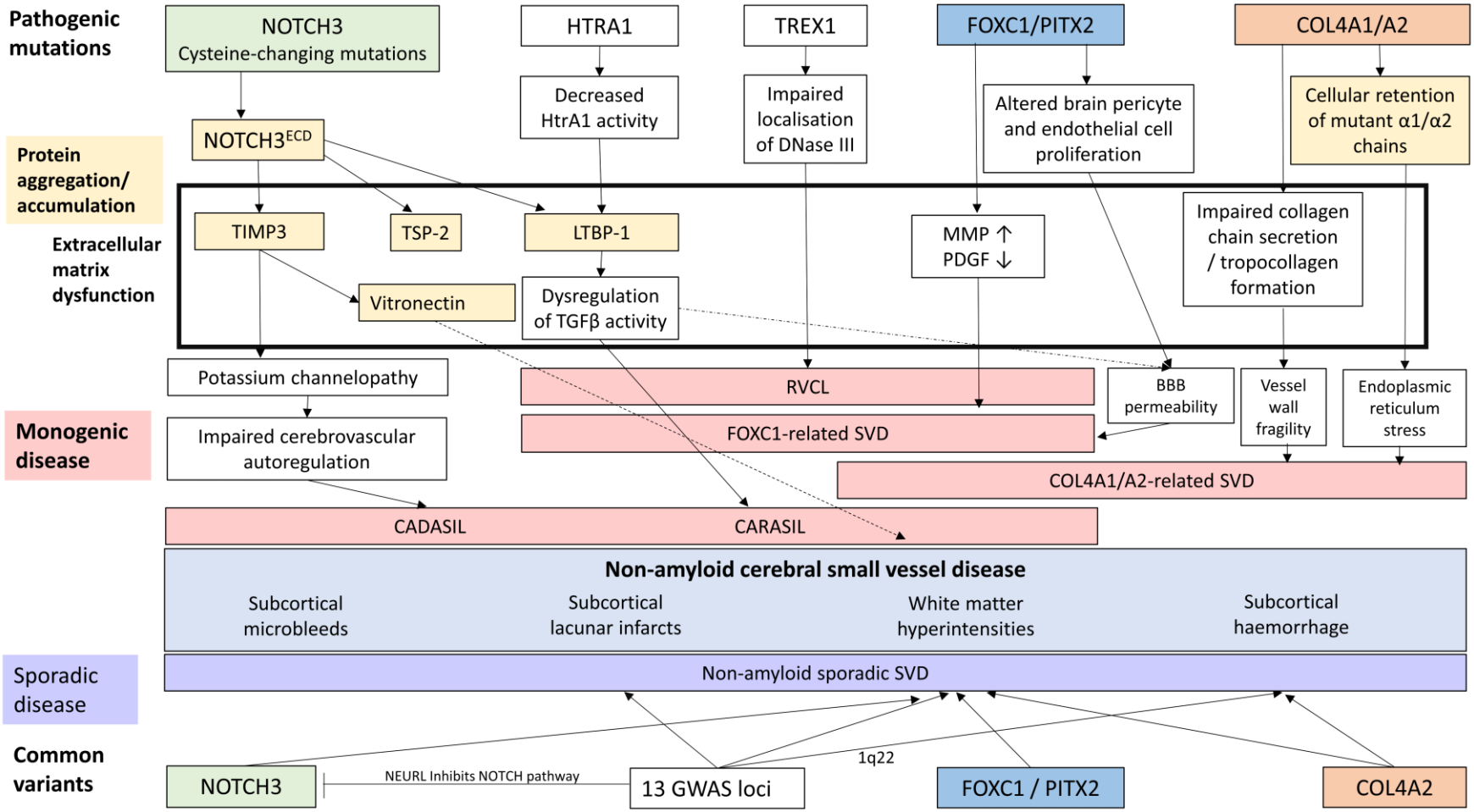
Figure 2: The convergence of disease pathways, particularly in the extracellular matrix (ECM), in the mechanisms underlying monogenic SVD. These pathways may also be biological correlates for clinical and other disease features identified in post-mortem and transgenic animal studies, as seen in the example of CADASIL. Pathological and clinical features are also shared between monogenic and sporadic disease, lending support to the possibility of these shared pathways also being involved in sporadic SVD.



DECLARATIONS OF INTEREST

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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