Early-Onset Vascular Leukoencephalopathy Caused by Bi-Allelic *NOTCH3* Variants

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Abstract

Objective Heterozygous *NOTCH3* variants are known to cause cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), with patients typically presenting in adulthood. We describe three patients presenting at an early age with a vascular leukoencephalopathy. Genome sequencing revealed bi-allelic variants in the *NOTCH3* gene.

Methods Clinical records and available MRI and CT scans of three patients from two unrelated families were retrospectively reviewed.

Results The patients presented at 9 to 14 months of age with developmental delay, seizures, or both. The disease course was characterized by cognitive impairment and variably recurrent strokes, migraine attacks, and seizures. MRI findings pointed at a small vessel disease, with extensive cerebral white matter abnormalities, atrophy, lacunes in the basal ganglia, microbleeds, and microcalcifications. The anterior temporal lobes were spared. Bi-allelic cysteine-sparing *NOTCH3* variants in exons 1, 32, and 33 were found.

Interpretation This study indicates that bi-allelic loss-of-function *NOTCH3* variants may cause a vascular leukoencephalopathy, distinct from CADASIL.

Keywords
- *NOTCH3*
- leukoencephalopathy
- small vessel disease

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Introduction

Leukodystrophies are a large group of genetic disorders of the central nervous system white matter. They may present at all ages. In individual patients, the diagnostic process may be difficult, despite advanced diagnostic methodologies. Most leukodystrophies are associated with distinct patterns of magnetic resonance imaging (MRI) abnormalities, making MRI pattern recognition a central tool in the diagnosis.[1] MRI pattern recognition has also been pivotal in the definition of novel diseases or new variants of disease. Next generation sequencing (NGS) techniques are of great value in associating such new diseases or new disease forms with genes and gene variants.[2]

A recently proposed classification of leukodystrophies is based on underlying pathology.[2] One class comprises vascular leukoencephalopathies, pointing at small vessel disease (SVD) at the basis of the leukodystrophy. Vascular leukoencephalopathies often have an onset in adulthood (e.g., cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy [CADASIL, MIM 125310], cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy [CARASIL, MIM 600142], Cathepsin A-related arteriopathy with strokes and leukoencephalopathy [CARASAL, MIM 613111], and cerebral amyloid angiopathy [CAA, MIM 605714]). Conversely, *COL4A1*- and *COL4A2*-related disorders may present at any age and often include familial porencephaly. Some vascular leukoencephalopathies have rather specific clinical or imaging features (e.g., porencephaly in defects in collagen IVA1 and IVA2, T_1-hyperintense pulvinar and multiorgan involvement in Fabry disease [MIM 301500], or cysts in leukoencephalopathy with calcification and cysts [LCC, MIM 614561]).[3–8]

We describe three patients from two families with early-onset vascular leukoencephalopathy, in whom NGS revealed bi-allelic loss-of-function variants in *NOTCH3*.

Methods

Patients

We retrospectively analyzed three patients from two unrelated families, who presented at a young age with an MRI appearance most consistent with a vascular leukoencephalopathy. Written informed consent was obtained from parents. Patient records were reviewed for clinical information and results of laboratory studies.

Neuroimaging

Available MRIs were scored according to a previously published protocol[1] and computed tomography (CT) scans were evaluated for the presence or absence of calcifications. Clinical MRIs were obtained in different centers, and typically included T_2-weighted, T_1-weighted, and fluid-attenuated inversion recovery (FLAIR) images. Several MRIs also included susceptibility-weighted imaging, diffusion-weighted imaging, and spectroscopy.

Electron Microscopy

Ultrastructural analysis was performed on skin biopsies according to routine methods.

Genome Sequencing

In patients 1 and 2, we performed trio genome sequencing using 2×150-nucleotide paired-end reads on an Illumina X10 (Illumina Cambridge Ltd). Read alignment was performed using BWA-mem; variant calling was performed using GATK HaplotypeCaller v3.7. SnpEff v4.3m was used for variant annotation and a custom script for variant filtration and prioritization.

In patient 3, clinical exome sequencing was performed by targeting DNA with the Agilent Clinical Research Exome kit, and sequencing on an Illumina HiSeq2000 sequencing system with 100bp paired-end reads. Bi-directional sequence with analysis based on human genome build GRCh37/UCSC Hg19; variant calling was performed by GeneDX using Xome Analyzer.

Sanger Sequencing

We performed Sanger sequencing in patients 1 and 2 to confirm the candidate variant in *NOTCH3* (NM_000435.2) identified with genome sequencing and its segregation with the disease within the family.

Results

Patients

Patients 1 and 2 are siblings. They are the first and fourth child of consanguineous parents (full cousins) and have two healthy siblings. Their mother suffers from frequent migraines with unilateral sensorimotor complaints and is diagnosed with relapsing remitting multiple sclerosis. This diagnosis was established after a myelopathy and optic neuritis and supported by oligoclonal bands in the cerebrospinal fluid and typical lesions on MRI, fulfilling the McDonald criteria. The father has no relevant medical history.

Patient 1 presented at 14 months of age due to developmental delay and seizures. Pregnancy and delivery at term were uncomplicated; birth weight was borderline—low (2,500 g). She could walk without support from 21 months of age. At the age of 5 years, she acutely developed a transient mild left-sided hemiplegia. MRI showed ipsilateral restricted diffusion in the head of the caudate nucleus, the anterior limb of the internal capsule, and the anterior part of the putamen (►**Fig. 1d**). Throughout her childhood, she had behavioral difficulties. At the latest examination, at the age of 26 years, she was clumsy. She had bilateral Babinski signs, but normal deep tendon reflexes and no spasticity. She was severely cognitively impaired and could speak only a few words. She never displayed livedo reticularis.

Patient 2 presented with a status epilepticus at 10 months. She was born at term after a normal pregnancy with caesarean section due to breech position. Birth weight was borderline—low (2,700 g) and microcephaly was noted (head circumference 31.5 cm, −2.5 SD). Neonatal screening

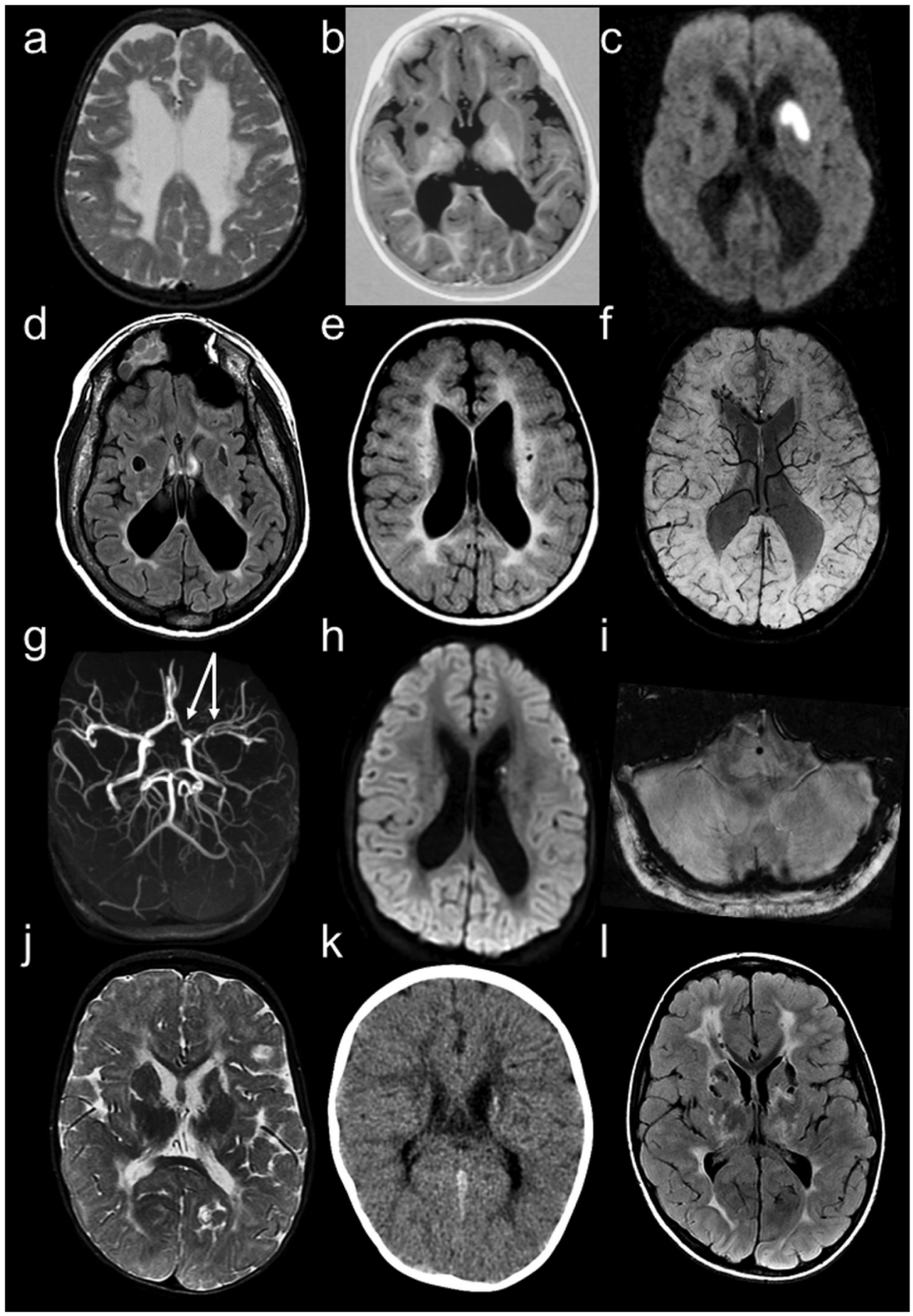

Fig. 1 MRI scans of patient 1. (**a, b**) The first MRI, at age 1 year and 7 months, shows extensive white matter signal abnormalities, a lacune in the right putamen, white matter atrophy, and a thin corpus callosum. (**c**) At the age of 5 years, a recent ischemic stroke is detected by restricted diffusion (DWI, b1000) with a low ADC (not shown) in the left putamen. (**d**) The last MRI scan at age 22 years shows severe white matter volume loss and extensive signal abnormalities of what is left of the white matter, sparing the directly subcortical white matter, as well as bilateral lacunes in the putamen. MRI scans of patient 2. (**e**) The first MRI at age 13 months shows mildly dilated lateral ventricles and extensive white matter signal abnormalities. (**f**) At age 2 years and 7 months, hemosiderin depositions along the right genu corpus callosum are seen. (**g**) MR angiography at 3 years and 3 months showed a hypoplastic left middle cerebral artery (M1- and M2-segment) and a hypoplastic left anterior cerebral artery (A1-segment); the hypoplastic areas are marked by arrows. (**h, i**) The MRI at age 7 years shows a punctate lesion with restricted diffusion in the left caudate nucleus and a microbleed at the pontomedullary junction. (**j–l**) Neuroimaging of patient 3. (**j**) The first MRI, at 1 year, shows signal abnormalities in the cerebral white matter and basal ganglia with reduced volume of both. (**k**) 7 months later, CT shows subtle hyperdensities adjacent to the left ventricle, indicative of calcium deposits. (**l**) The latest MRI, at 6 years, shows bilateral lacunes in the basal ganglia.

revealed congenital hypothyroidism, for which she received thyroid hormone therapy for 3 years. She could walk without support from the age of 16 months. At the age of 2.5 years she developed an acute right-sided hemiplegia, of which she fully recovered within days. No fresh infarction was seen on MRI. Several weeks later she had her second status epilepticus. Multiple seizures occurred over several years, often related to episodes of fever. At the age of 3 years and 3 months she had another episode of acute right-sided hemiplegia. MRI showed a punctate focus of restricted diffusion in the centrum semiovale on the right. After starting carbasalate calcium, the episodes of hemiplegia ceased to occur. From the age of 4.5 years, she suffered from frequent seizures and migraine attacks, had behavioral difficulties and temper tantrums. The migraine attacks were accompanied by nausea, vomiting, and transient right-sided paresis. At 8 years of age, her epilepsy worsened with countless seizures per day, which could not be controlled with various drugs. Her EEG was indicative of Lennox-Gastaut epilepsy. Currently, her seizures are in part controlled with rufinamide. At the last physical examination, at the age of 9 years, clumsy motor function and a mild left-sided hemiplegia were noted. She currently attends special needs education. She never had livedo reticularis.

Patient 3 presented at 9 months of age due to developmental delay, hypotonia, and microcephaly. She is the first-born child of non-consanguineous, healthy parents and was born after a 38-week pregnancy, which was remarkable for reduced fetal movements. Delivery was induced due to oligohydramnios. A low birth weight (1,870 g) was noted and she was admitted to the neonatal intensive care unit for temperature regulation and feeding difficulties. At 24 months, she could walk without support. No seizures or stroke-like episodes occurred. At the last physical examination at the age of 9 years, she was clumsy and her gait was slightly unstable and wide based. She had asymmetric bilateral spasticity, with the right side being more affected. Additionally, she was cognitively delayed and displayed anger outbursts. She never displayed livedo reticularis. Parents report no specific medical concerns.

Neuroimaging

The first MRI of patient 1, at the age of 1 year and 7 months, revealed mildly dilated lateral ventricles due to loss of white matter volume (►**Fig. 1a**). There were extensive confluent signal abnormalities in the cerebral white matter with relative sparing of the directly subcortical rim (►**Fig. 1a**). In the right putamen, a lacune was present (►**Fig. 1b**). The corpus callosum was thin and the cerebellar vermis was small. The cerebellar hemispheres and brainstem were spared. On the MRI at the age of 5 years, restricted diffusion was seen in the head of the caudate nucleus, the anterior limb of the internal capsule, and the anterior part of the putamen on the left (►**Fig. 1c**), which partly developed into a lacune on follow-up (►**Fig. 1d**). MRI at the age of 15 years revealed that the cerebral white matter abnormalities had become more extensive with the subcortical rim being affected as well. MRI at 22 years showed a para-median punctate lesion in the pons on the right, a lesion in the left middle cerebellar peduncle and several lesions in the cerebellar hemispheres. Several microbleeds were present on gradient-echo images. The CT at the age of 24 years did not show evident calcifications.

The first scan of patient 2, at the age of 13 months, revealed mildly dilated lateral ventricles with reduced volume of the white matter (►**Fig. 1e**). The cerebral white matter contained extensive confluent signal abnormalities, relatively sparing the direct subcortical rim. The corpus callosum, basal ganglia, cerebellum, and brainstem were spared. At the age of 2 years and 7 months, hemosiderin depositions were seen at the right side of the genu of the corpus callosum (►**Fig. 1f**). At the age of 3 years and 3 months, the MRI showed a small lesion with diffusion restriction in the corona radiata on the right. MR angiography showed a hypoplastic left middle cerebral artery (M1- and M2-segment) and a hypoplastic left anterior cerebral artery (A1-segment, ►**Fig. 1g**). MRI at the age of 5 years revealed bilateral signal abnormalities in the posterior limb of the internal capsules. MRI at the age of 7 years showed a punctate lesion with restricted diffusion in the left caudate nucleus (►**Fig. 1h**). The hemosiderin depositions at the corpus callosum had partially resolved. Brainstem and cerebellum were unaffected. MRI at 8 years showed a new microbleed at the pontomedullary junction (►**Fig. 1i**) and mild progression of the cerebral and cerebellar white matter atrophy. MRI at 9 years was essentially unchanged.

In patient 3, MRI at the age of 13 months showed mild external cerebral atrophy due to reduced cerebral white matter volume; the ventricles had a normal size (►**Fig. 1j**). Confluent cerebral white matter abnormalities were present, relatively sparing the immediate subcortical white matter. Focal signal abnormalities were present in the basal ganglia and thalami, with also volume loss (►**Fig. 1j**). A microbleed was present on the medial side of the right thalamus. No restricted diffusion or cysts were seen. CT scan at the age of 16 months showed micro-calcifications, confined to the left and right caudate head (►**Fig. 1k**). The latest MRI, at 6 years, showed bilateral lacunes in the basal ganglia (►**Fig. 1l**).

Genetic Analysis

Genome sequencing in patients 1 and 2 revealed a homozygous *NOTCH3* frameshift variant, NM_000435.2: c.29_53del; p.Arg10Hisfs*16, classified as pathogenic per the ACMG/AMP variant classification criteria (►**Fig. 2a**). This variant is absent from population allele frequency databases. It causes a premature stop codon in exon 1 that is expected to result in nonsense-mediated decay. Segregation analysis revealed that both parents are heterozygous carriers and none of the healthy siblings are homozygous for the variant.

In patient 3, exome sequencing revealed compound heterozygous *NOTCH3* variants, p.His1944Tyr and p.Leu1976Profs*11 (►**Fig. 2a**). The first variant, NM_000435.2: c.5830C > T; p.His1944Tyr, is a variant of uncertain significance with likely pathogenicity and was found to be maternally inherited. The p.His1944 residue is totally conserved across all vertebrates and is the histidine in a ALHWAAAVNN motif that is conserved across all NOTCH proteins back to drosophila

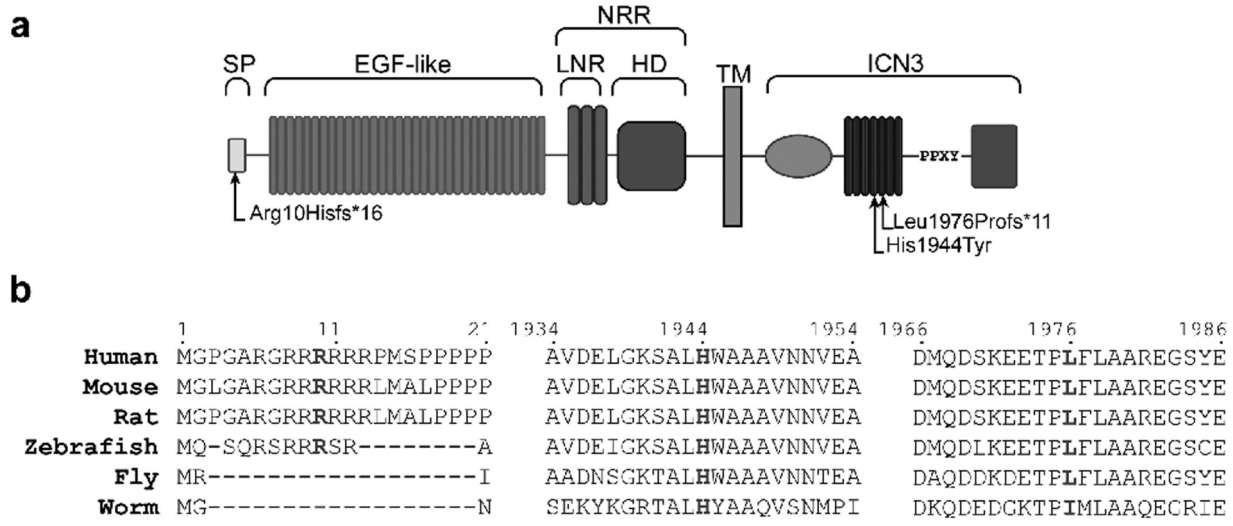

Fig. 2 (**a**) A schematic presentation of the *NOTCH3* gene with the locations of the three variants. (**b**) Sequence alignment of the regions of the three variants. The residues affected by the variants are printed in bold. EGF, epidermal-growth factor; HD, heterodimer region; LNR, lin12 repeats; NRR, negative regulatory region; SP, signal peptide; TM, transmembrane.

(►**Fig. 2b**).[9] The second variant, NM_000435.2:c.5926dupC; p.Leu1976Profs*11 is classified as pathogenic per the ACMG/AMP criteria. This variant was not observed in the mother but DNA from the father was not available for testing. As the variant is in the final exon of NOTCH3, this frameshift is expected to escape nonsense-mediated decay and result in the generation of a truncated protein product lacking the final 345 residues of wild-type NOTCH3. We cannot speculate on the specific impact a truncation of this nature might have on protein folding, stability, or function. However, ClinVar contains six frameshift variants in NOTCH3 that are classified as pathogenic or likely pathogenic and all six are located downstream of p.Leu1976Profs. Both variants are absent from population allele frequency databases and both fall within an Ankyrin (ANK) domain of the Notch intracellular domain (NICD). This domain is responsible for mediating protein–protein interactions with the cytosolic NICD that is responsible for target activation.[9] No other (possibly) causative variants were found.

Other investigations

Skin biopsies were performed in patients 1 and 2. Electron microscopy did not reveal deposits of granular osmiophilic material (GOM). The capillaries had a normal vessel wall. No signs of reduplication of the vascular basal membrane, abnormal density, or thickness of collagen bundles were seen.

Discussion

We describe three patients with a clinical and neuroimaging presentation consistent with a vascular leukoencephalopathy and bi-allelic *NOTCH3* variants. All patients had a low birth weight and presented during infancy with developmental delay, clumsiness, and mild to severe cognitive impairment. Patients 1 and 2 experienced transient ischemic attacks and strokes, and patient 2 also had complicated migraines and severe epilepsy. Patient 3 never suffered from clinically manifest strokes. In all three patients, neuroimaging revealed the picture consistent with a leukoencephalopathy caused by SVD with extensive periventricular and deep cerebral white matter abnormalities, strokes, microbleeds, and calcifications. In one patient MR angiography also revealed abnormalities in larger blood vessels.

Until now, three patients with a vascular leukoencephalopathy caused by bi-allelic loss-of-function *NOTCH3* variants have been reported.[10,11] These patients had their first ischemic strokes between the ages of 4 months and 7 years, and the MRI findings were similar to those of our patients with extensive cerebral white matter T_2-hyperintensities, relatively sparing the subcortical rim, microbleeds, and lacunar infarcts.[11,12] Unlike our patients, all previously described patients had signs of Sneddon Syndrome (MIM 182410), a noninflammatory arteriopathy characterized by livedo reticularis and cerebrovascular disease in early adulthood.[13] One patient had aneurysms and in the skin biopsy multilayering and shedding of the vascular basement membrane, which were not present in our patients.[10]

Heterozygous *NOTCH3* variants are known to cause CADASIL. The function of NOTCH3 has been studied extensively.[14–17] NOTCH signaling is important in cell–cell communication and plays a major role in the development of blood vessels. The NOTCH3 receptor is mainly expressed in vascular smooth muscle cells.[18] In CADASIL, the *NOTCH3* variants have a dominant inheritance and most often cause an odd number of cysteine residues within the epidermal growth factor domain of the NOTCH3 receptor.[14] Accumulation and aggregation of the NOTCH3 extracellular domain lead to GOM in the walls of arterial vessels, the pathological hallmark of CADASIL.[19] Most studies suggest that CADASIL is caused by toxic effects of the mutant protein, with the GOM in vessel walls being central in its pathophysiology, but the

contribution of loss of function effects of *NOTCH3* variants is a matter of debate and it is unclear whether decreased NOTCH3 receptor activity also plays a central role.[20]

Several disease characteristics of CADASIL are different from those in our patients. The age of onset is much earlier in our patients than in CADASIL, where patients most often present in the fourth decade. In contrast to CADASIL, skin biopsies in patients 1 and 2 did not reveal GOM. There are, however, also striking similarities. Clinically, both CADASIL patients and our patients present with transient ischemic attacks, strokes and migraines. Both display the typical MRI features of an SVD with progressive cerebral white matter abnormalities predominantly affecting the periventricular and deep cerebral white matter and relatively sparing the directly subcortical white matter, in combination with ischemic events (typically small lacunar infarcts), most often involving the basal nuclei, thalami, and brain stem. As in our patients, microbleeds are seen in one- to two-thirds of CADASIL patients.[19]

A *Notch3* knockout mouse is often used to study the function of NOTCH3. It is not a true model for CADASIL, but may better reflect the disease in our patients. *Notch3* knockout mice are viable and fertile.[16] Both the structure and function of vasculature are abnormal with altered shape, size, and organization of vascular smooth muscle cells. Interestingly, MR angiography also revealed hypoplasia of some cerebral arteries in one of our patients. Also, there is an impaired reaction of cerebral blood flow in *Notch3* knockout mice.[16,17] The mice do not have GOM. The findings in these mice are relevant to our patients. As expected, the skin biopsies performed in our patients and the previously described patients did not show GOM. A limitation of the study is that we do not have neuropathological confirmation of altered small vessels within the brain.

The neuroradiological findings in our patients are typical for an SVD. An SVD with an onset in infancy or early-childhood is likely to have a genetic etiology. Aicardi-Goutières syndrome (AGS, MIM 225750), collagen IVA-related disorders, leukoencephalopathy with calcifications and cysts (LCC), and Coats' plus syndrome (MIM 612199) are examples of these. Distinction based on MRI alone can be challenging. In LCC, AGS, and Coats' plus syndrome, the calcifications tend to be more extensive than observed in our patients.[5,21,22] In collagen IVA-related syndromes, porencephaly and parenchymal hemorrhages are more common, although not obligatory.[22,23] Clinically, ophthalmic (e.g., in Coats' plus syndrome and collagen IVA-related syndromes), dermatologic (e.g., chilblains in AGS) and other systemic signs and symptoms may point to a certain diagnosis.[24] In LCC, systemic signs and symptoms are typically lacking.[5] As presented in the previously described patients with homozygous *NOTCH3* variants, Sneddon syndrome may co-occur.[10–12] The disorders mentioned in this paragraph are rare to ultrarare, but the cumulative incidence of SVD with onset in infancy or early-childhood is probably higher than expected, making the pediatric neurologists likely meet patients and need to be aware of the differential diagnosis.

The description of multiple patients with a vascular leukoencephalopathy and bi-allelic loss-of-function *NOTCH3* variants indicates that it represents a syndrome characterized by onset in infancy or early childhood, recurrent strokes and extensive cerebral white matter changes. Livedo reticularis, indicative of Sneddon syndrome, may occur, but is not obligatory. Realizing biallelic NOTCH3 variants may also cause pathology is important, as they may otherwise be missed due to incorrect variant curation. Although distinct from CADASIL, it is likely that a similar pathway is affected, hindering optimal cerebral blood flow. This case series will help in the diagnosis of future cases.

Authors' Disclosures
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Conflict of Interest
None declared.

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