Stroke

CLINICAL AND POPULATION SCIENCES

Cerebral Autosomal Dominant Arteriopathy With Subcortical Infarcts and Leukoencephalopathy Family Members With a Pathogenic *NOTCH3* Variant Can Have a Normal Brain Magnetic Resonance Imaging and Skin Biopsy Beyond Age 50 Years

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BACKGROUND: To determine whether extremely mild small vessel disease (SVD) phenotypes can occur in *NOTCH3* variant carriers from Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) pedigrees using clinical, genetic, neuroimaging, and skin biopsy findings.

METHODS: Individuals from CADASIL pedigrees fulfilling criteria for extremely mild *NOTCH3*-associated SVD (mSVD^{NOTCH3}) were selected from the cross-sectional Dutch CADASIL cohort (n=200), enrolled between 2017 and 2020. Brain magnetic resonance imaging were quantitatively assessed for SVD imaging markers. Immunohistochemistry and electron microscopy was used to quantitatively assess and compare NOTCH3 ectodomain (NOTCH3^{ECD}) aggregation and granular osmiophilic material deposits in the skin vasculature of mSVD^{NOTCH3} cases and symptomatic CADASIL patients.

RESULTS: Seven cases were identified that fulfilled the mSVD^{NOTCH3} criteria, with a mean age of 56.6 years (range, 50–72). All of these individuals harbored a *NOTCH3* variant located in one of EGFr domains 7-34 and had a normal brain magnetic resonance imaging, except the oldest individual, aged 72, who had beginning confluence of WMH (Fazekas score 2) and 1 cerebral microbleed. mSVD^{NOTCH3} cases had very low levels of NOTCH3^{ECD} aggregation in skin vasculature, which was significantly less than in symptomatic EGFr 7-34 CADASIL patients (P=0.01). Six mSVD^{NOTCH3} cases had absence of granular osmiophilic material deposits.

CONCLUSIONS: Our findings demonstrate that extremely mild SVD phenotypes can occur in individuals from CADASIL pedigrees harboring *NOTCH3* EGFr 7-34 variants with normal brain magnetic resonance imaging up to age 58 years. Our study has important implications for CADASIL diagnosis, disease prediction, and the counseling of individuals from EGFr 7-34 CADASIL pedigrees.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: biopsy ■ brain ■ capillaries ■ vascular dementia ■ white matter

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Supplemental Material is available at https://www.ahajournals.org/doi/suppl/10.1161/STROKEAHA.121.036307.
For Sources of Funding and Disclosures, see page 1973.

Stroke is available at www.ahajournals.org/journal/str

Nonstandard Abbreviations and Acronyms

CADASIL	Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy
DWM	deep white matter
EGFr	epidermal growth-factor-like repeat
GOM	granular osmiophilic material
LDL-C	low-density lipoprotein cholesterol
MRI	magnetic resonance imaging
mSVD^{NOTCH3}	extremely mild NOTCH3-associated small vessel disease
***NOTCH3*^{cys} variant**	cysteine altering missense NOTCH3 variant
NOTCH3^{ECD}	ectodomain of the NOTCH3 protein
SVD	cerebral small vessel disease
WMH	white matter hyperintensities

Cysteine altering variants in the *NOTCH3* gene (*NOTCH3*^{cys}) are known to be the cause of Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL), the most prevalent hereditary cerebral small vessel disease (SVD).[1] Recent studies in population databases have shown that the expression of *NOTCH3*^{cys} variants in the general population is very variable, ranging from a classical severe CADASIL to very mild SVD or even absence of SVD magnetic resonance imaging (MRI) markers up to age 70 years.[2–4] Such a mild disease expression has never been documented in CADASIL pedigrees, where the penetrance of *NOTCH3*^{cys} is consistently reported to be 100% by the age of 35 years, with brain MRI revealing the presence of periventricular and deep white matter (DWM) hyperintensities (WMH), superimposed by multiple lacunes and cerebral microbleeds later in the disease course.[1] Typically, CADASIL patients from CADASIL pedigrees present with subcortical ischemic strokes at age 45 to 50 years, followed by progressive cognitive decline and ultimately vascular dementia, but age at onset and disease progression vary.[1,5] Other symptoms associated with CADASIL are apathy, mood disturbances, and migraine with aura.[1] Modifying factors which explain part of the variability in disease severity are classical cardiovascular risk factors[6–8] and the position of the *NOTCH3*^{cys} variant within the ectodomain of the NOTCH3 protein (NOTCH3^{ECD}).[9]

Mutant NOTCH3^{ECD} has increased multimerization properties and forms aggregates in the media of small cerebral arteries,[10,11] ultimately leading to vessel wall destruction and compromised cerebral vasoreactivity and blood flow.[12–14] These pathological vessel wall changes are also present in extracerebral small arteries and capillaries, such as in the skin vasculature, although here the arteriopathy remains subclinical. In CADASIL patients and presymptomatic *NOTCH3*^{cys} variant carriers from CADASIL pedigrees, skin vessel wall changes are consistently seen from young adulthood: NOTCH3^{ECD} immunohistochemistry reveals an intense and granular staining of the media[15,16] and electron microscopy shows deposits of electron dense granular osmiophilic material (GOM) in close proximity to the basal membrane of mural cells (vascular smooth muscle cells and pericytes), which are considered pathognomonic for CADASIL.[17,18]

The presence of SVD imaging markers, ischemic events, cognitive decline, and NOTCH3 aggregation in the vessel wall have all been reported to be consistent features in CADASIL pedigrees. Recent research findings, however, are inconsistent with some of these CADASIL/*NOTCH3*^{cys} disease paradigms. Firstly, while CADASIL has an estimated minimal prevalence of 2 to 5:100 000,[19] the frequency of CADASIL-causing *NOTCH3*^{cys} variants in population databases such as UK Biobank and DiscovEHR is 100-fold higher and associated with a relatively mild and late-onset SVD in most cases.[2–4] *NOTCH3*^{cys} variants in population biobanks are almost without exception located in EGFr (epidermal growth-factor-like repeat) domains 7-34 of NOTCH3, whereas diagnosed CADASIL patients can harbor *NOTCH3*^{cys} variants in any one of the protein's 34 EGFr domains, but in the majority of CADASIL patients the *NOTCH3*^{cys} variant is located in one of EGFr domains 1-6.[9] It follows that these more N-terminal EGFr domains have recently been shown to predispose to a more severe phenotype than EGFr 7-34 variants, not only in the population,[4] but also in CADASIL cohorts.[9]

Individuals above 50 years of age with *NOTCH3*^{cys} variants and a normal brain MRI have recently been reported in the general population.[2] However, in CADASIL pedigrees, with the exception of one case-report,[20] all affected family members are reported to have WMH by the age of 35 years,[1] regardless of *NOTCH3*^{cys} variant position. Our hypothesis is that these extremely mild SVD phenotypes can occur in CADASIL pedigrees with a *NOTCH3*^{cys} variant located in EGFr domains 7-34. To test this hypothesis, we queried the cross-sectional Dutch CADASIL cohort for individuals aged 50 years or older with no history of stroke or cognitive impairment, and no or only minimal SVD imaging markers on MRI. We describe the clinical, genetic, neuroimaging, and skin biopsy findings in these extremely mild cases and compare them with their affected first-degree relatives and the rest of the patients in the Dutch CADASIL cohort.

METHODS

Data Availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Identification of Extremely Mild *NOTCH3*^{cys} SVD Cases From the Dutch CADASIL Cohort

Individuals fulfilling the criteria for extremely mild *NOTCH3*^{cys} SVD (mSVD^{NOTCH3}) were selected from the cross-sectional Dutch CADASIL cohort which includes 200 fully characterized patients and presymptomatic family members with a genetically confirmed *NOTCH3*^{cys} variant, enrolled between November 2017 and December 2020. This study was approved by the medical ethics committee of the Leiden University Medical Center (P18.164). All participants gave written informed consent.

Criteria for mSVD^{NOTCH3} were (1) age 50 years or older (2) no history of stroke and no prior diagnosis of mild cognitive impairment or dementia (3) a recent brain MRI showing no lacunes of presumed vascular origin and no or only minimal WMH (Fazekas DWM <2) in cases between 50 and 65 years of age, and nonconfluent WMH (Fazekas DWM <3) in cases older than 65 years of age.

Clinical Characterization

Stroke was defined as rapidly evolving focal symptoms lasting >24 hours with no apparent cause other than of vascular origin. Mild cognitive impairment (minor neurocognitive disorder) and dementia (major neurocognitive disorder) were defined according to the Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM-V). All patients underwent an extensive cognitive examination on the same day as the brain MRI and skin biopsy (Supplemental Material). Neuropsychiatric symptoms were assessed by interviewing relatives using the Dutch version of the Neuropsychiatric Inventory.[21] Apathy was defined as a previous diagnosis, or a Neuropsychiatric Inventory score of apathy ≥1 with symptoms fulfilling the diagnostic criteria proposed by Starkstein.[22] Depression was defined as a previous diagnosis, or a Neuropsychiatric Inventory score of depression ≥1 with symptoms fulfilling the diagnostic criteria of DSM-V. Migraine with and without aura were defined according the third edition of the International Classification of Headache Disorders. Disability was assessed using the modified Rankin Scale.

Hypertension was defined as a previous diagnosis of hypertension (>140 mm Hg systolic or >90 mm Hg diastolic) or use of an antihypertensive agent. Hypercholesterolemia was defined as a previous diagnosis of hypercholesterolemia or nonfasting level of LDL-C (low-density lipoprotein cholesterol) >3.5 mmol/L or total cholesterol > 6.5 mmol/L. Diabetes was defined as a previous diagnosis of diabetes type 1 or 2 or use of a hypoglycemic agent.

Evaluation and Quantification of SVD Imaging Markers on Brain MRI

Brain MRIs of mSVD^{NOTCH3} cases were quantitively assessed for SVD imaging markers and compared with brain MRIs of their first-degree relatives with a *NOTCH3*^{cys} variant as well as the 89 patients with an EGFr 7-34 variant and a brain MRI from the Dutch CADASIL cohort. The latter group was selected for comparison with mSVD^{NOTCH3} cases, as these turned out to all have *NOTCH3*^{cys} EGFr 7-34 variants.

Brain MRIs were made on a 3 Tesla MR system and evaluated according to consensus criteria.[23] Acquisition parameters are presented in the Supplemental Material. The WMH burden in the DWM was evaluated according to the simplified Fazekas scale.[24] Cerebral atrophy was qualitatively assessed on FLAIR images using the global cortical atrophy scale.[25] The following SVD imaging markers were quantitatively assessed: WMH volume, volume of lacunes of presumed vascular origin, number of cerebral microbleeds, and brain parenchymal fraction. Brain parenchymal fraction was defined as the ratio of brain parenchymal volume to the intracranial volume expressed as a percentage. WMH and lacune volume were normalized to the intracranial volume: (normalized volume=[unnormalized volume/intracranial volume]×100). Details on the quantification of SVD imaging markers are presented in the Supplemental Material.[26]

Quantitative Analysis of NOTCH3 Aggregation in Skin Vasculature: NOTCH3 Score and GOM Count

To assess levels of vascular NOTCH3 aggregation,[27] the immunohistochemical NOTCH3 score and electron microscopic GOM count were determined in skin vessels of mSVD^{NOTCH3} cases and compared with first-degree relatives with symptomatic CADASIL of whom skin tissue samples were available, age-matched unrelated symptomatic EFr 7-34 CADASIL patients and 5 age-matched healthy controls. Symptomatic CADASIL was defined as modified Rankin Scale score ≥2 with confluent deep WMH (Fazekas DWM 3) and >1 lacune on brain MRI. Skin biopsies from the lateral upper arm were taken using 4 mm skin punch biopsy. The NOTCH3 score and GOM count was assessed by 2 independent and blinded observers (Drs Gravesteijn and Cerfontaine).

For immunohistochemistry, skin tissue was processed and stained with a monoclonal antibody targeting NOTCH3^{ECD} (clone 1E4, Millipore), and full-focus microscopy images were taken, as previously described.[27] NOTCH3-staining was assessed in all visible blood vessels (median 23 blood vessels per individual, range 11–33). ImageJ was used to manually draw the inner and outer boundaries of vessel walls and Colour Threshold (Hue 0–50; Saturation 0–255; Brightness 0–175) was used to determine the NOTCH3^{ECD} positive area (NOTCH3 score), which was expressed as percentage of the vessel wall area. NOTCH3 score was defined as the average of the 10 vessels with the highest NOTCH3^{ECD} positive area. Analysis of all assessed vessels per individual did not change the results.

For electron microscopy, skin tissue was processed as previously described.[27] GOM deposits were counted in blood vessel walls in a median of 16.5 blood vessels per individual (range, 10–27). GOM count was averaged per 1000 µm blood vessel wall circumference.

Statistical Analysis

Normally distributed variables were summarized as mean±SD and compared between 2 groups using unpaired 2-sample *t* test. Non-normally distributed continuous variables were summarized as median with IQR and compared between 2 groups

 Stroke. 2022;53:1964–1974. DOI: 10.1161/STROKEAHA.121.036307

using Mann-Whitney *U* test. Statistical comparisons on binary categorical variables between 2 groups were performed using Fisher Exact Test. The following variables were transformed to obtain a normal distribution: NOTCH3 score (square root), normalized WMH volume (square root), normalized lacune volume (cube root), cerebral microbleeds count ($\log_{10}[x+1]$). General linear models were used to obtain standardized scores for SVD imaging markers, corrected for age, based on all individuals with a *NOTCH3*^{cys} EGFr 7-34 variant in the Dutch CADASIL cohort, including the mSVD^{NOTCH3} cases and their first-degree relatives. To correct for multiple testing, one-way ANOVA with Tukeys post hoc test was used to compare standardized scores of SVD imaging markers between mSVD^{NOTCH3} cases, their first-degree relatives and other EGFr 7-34 patients from the Dutch CADASIL cohort. If the assumption of homogeneity of variances was violated, as assessed by Levene test for equality of variances ($P \leq 0.05$), 1-way ANOVA with Games-Howell post hoc test was used. There were no missing values present in the data set. All statistical analyses were performed using SPSS v27.0 with 2-tailed tests and $P<0.05$ statistical significance.

RESULTS

Identification of Extremely Mild *NOTCH3*^{cys} SVD Cases

Seven individuals from 6 different CADASIL pedigrees were identified who fulfilled the criteria for mSVD^{NOTCH3} (Flow Chart is presented in Figure S1). All of these mSVD^{NOTCH3} cases had previously undergone predictive genetic testing at their own request due to a positive family history for CADASIL. All 7 mSVD^{NOTCH3} cases had a *NOTCH3*^{cys} variant in one of EGFr domains 7 to 34: 6 had a variant in EGFr domain 14 (n=5 for p.Arg578Cys, n=1 for p.Cys568Tyr), one individual had a variant in EGFr domain 17 (p.Gly667Cys).

The mean age of the mSVD^{NOTCH3} cases was 56.6±7.4 years (range, 50–72), 3 were male. Six mSVD^{NOTCH3} cases were asymptomatic, except for a history of migraine with aura in 4 individuals, with a mean age of onset of 28.5±16.9 years (range, 6–43). One individual had a history of recurrent depressive episodes since adolescence after a traumatic experience. None of the mSVD^{NOTCH3} cases had current neuropsychiatric symptoms.

On brain MRI, 6 mSVD^{NOTCH3} cases had a Fazekas DWM 1 and one case (age 72 years) had a Fazekas DWM 2. Only the 72-year-old individual had a cerebral microbleeds located in the deep periventricular white matter (Figure 1). None of the mSVD^{NOTCH3} cases had cerebral atrophy (global cortical atrophy score ≤1).

High Intrafamilial Variability of *NOTCH3*^{cys}-Associated SVD Phenotype

Five of the 7 mSVD^{NOTCH3} cases had at least one first-degree relative with symptomatic CADASIL, that is, modified Rankin Scale score ≥2 and Fazekas DWM 3 and >1 lacune (Figure 2A through 2C and Figure S2D). In the pedigrees of 3 mSVD^{NOTCH3} cases, disease expression was predominantly mild (Figure 2C and Figure S2E and S2F). One mSVD^{NOTCH3} case had a sister who also had a very mild SVD phenotype, but their brother, who was homozygous for the familial *NOTCH3*^{cys} variant, had a much more severe SVD phenotype (Figure S2F). All first-degree relatives with symptomatic CADASIL had hypertension, which was significantly more frequent than in mSVD^{NOTCH3} cases (100.0% versus 14.3%; $P=0.005$; Table). First-degree relatives with symptomatic CADASIL also more frequently had diabetes than the mSVD^{NOTCH3} cases (33.3% versus 0%; $P=0.19$). First-degree relatives of mSVD^{NOTCH3} cases did not have a significantly lower burden of SVD imaging markers than other individuals with a *NOTCH3*^{cys} EGFr 7-34 variant in the Dutch CADASIL cohort (Figure 3).

Very Mild NOTCH3 Staining and Lack of GOM in Extremely Mild *NOTCH3*^{cys} SVD Cases

For immunohistochemistry, skin tissue samples were available for all 7 mSVD^{NOTCH3} cases and 9 symptomatic CADASIL patients, of which 4 were first-degree relatives of mSVD^{NOTCH3} cases (Table S1). In the skin vasculature, NOTCH3 staining in mSVD^{NOTCH3} cases was faint and diffuse, in contrast to the typical granular NOTCH3 staining pattern which was present in most symptomatic patients with an EGFr 7-34 variant (Figure 4A). On quantitative analysis, mSVD^{NOTCH3} cases had a significantly lower NOTCH3 score than symptomatic CADASIL patients with an EGFr 7-34 variant: median 1.0 (IQR, 1.7 [range, 0.0–5.2]) versus median 10.3 (IQR, 19.4 [range, 0.0–25.7]; $P=0.010$; Figure 4B).

Ultrastructural analysis was performed in all 7 mSVD^{NOTCH3} cases and in 6 symptomatic CADASIL patients, of which one first-degree relative of a mSVD^{NOTCH3} case. GOM deposits were only seen in one (14.3%) mSVD^{NOTCH3} case, whereas GOM deposits were observed in 4 of the 6 (66.7%) symptomatic EGFr 7-34 CADASIL patients for whom EM was available ($P=0.10$). The one mSVD^{NOTCH3} case who had GOM deposits had a GOM count similar to symptomatic patients with EGFr 7-34 variants (Figure S3).

DISCUSSION

This study shows that individuals with *NOTCH3*^{cys} variants from CADASIL pedigrees can have an extremely mild SVD phenotype with normal brain MRIs above 50 years of age. Such an extremely mild SVD phenotype in individuals with *NOTCH3*^{cys} variants has previously only been observed in population databases.[2,3] Clearly, modifiers protecting individuals with *NOTCH3*^{cys} variants in the population from developing (severe) SVD, can also be at play in *NOTCH3*^{cys} variant carriers from CADASIL pedigrees. Identifying protective and exacerbating

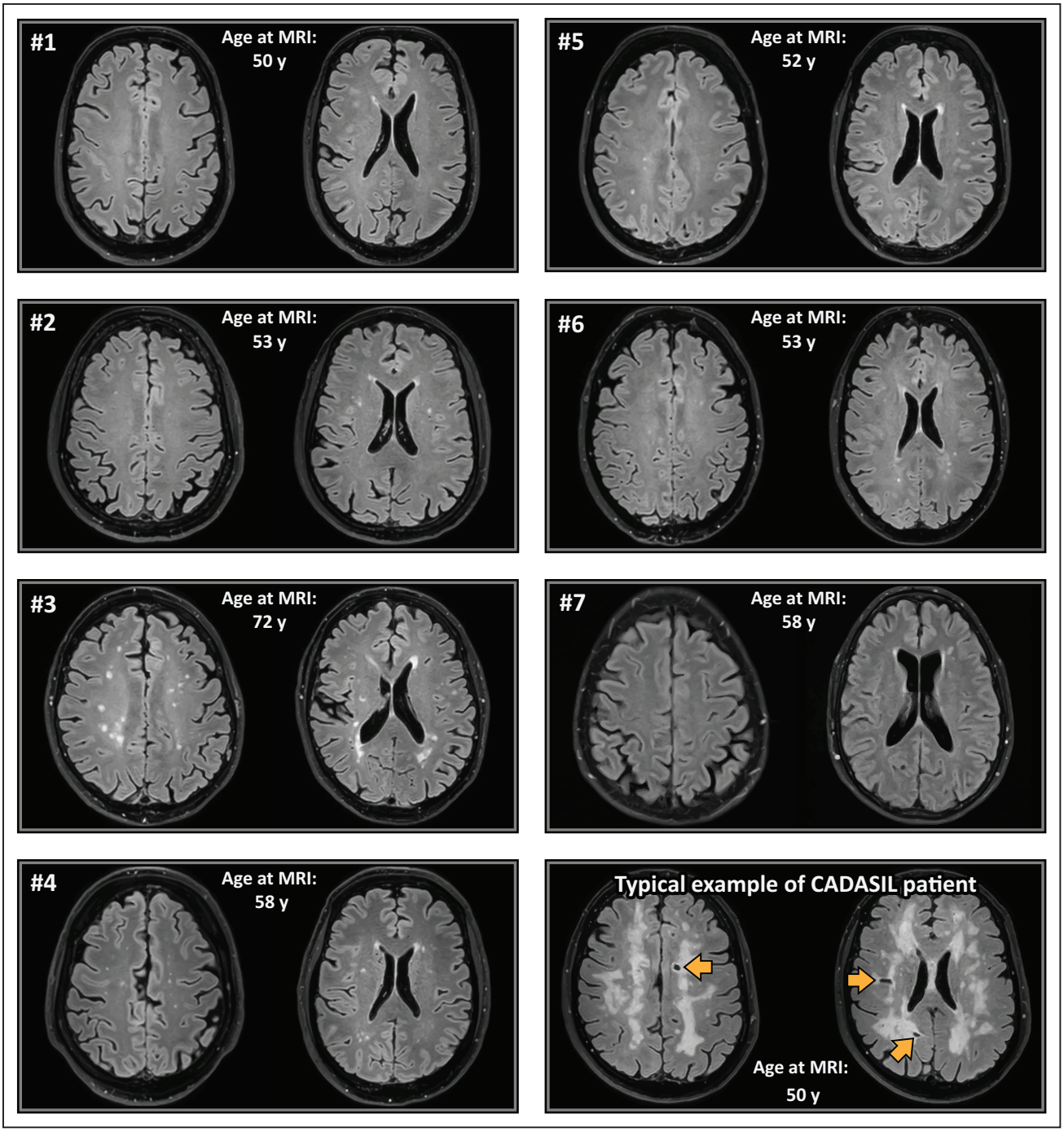

Figure 1. Brain magnetic resonance imaging (MRIs) of the 7 individuals with a *NOTCH3*^{cys} variant and an extremely mild small vessel disease (SVD) phenotype.

Brain MRI FLAIR images of 7 individuals with minimal small vessel disease (mSVD^{NOTCH3} case No. 1–7) showing absence of lacunes and only punctate foci of white matter hyperintensities (Fazekas deep white matter [DWM] 1), except for mSVD^{NOTCH3} case No. 3, who has beginning white matter hyperintensities (WMH) confluency (Fazekas DWM 2). For comparison, a brain MRI of a typical Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) patient with a *NOTCH3*^{cys} variant located in EGFr (epidermal growth-factor-like repeat) domains 7-34 is shown, who has large confluent WMH (Fazekas DWM 3) and multiple lacunes (indicated by arrows).

NOTCH3^{cys} modifiers may provide the key to future prevention and treatment of CADASIL.

All mSVD^{NOTCH3} cases were members of pedigrees with a *NOTCH3*^{cys} EGFR-7-34 variant, known to predispose to a wider and milder spectrum of SVD severity than *NOTCH3*^{cys} EGFr 1-6 variants, which so far seem to always be associated with a classical severe CADASIL disease course.[2–4,9] This is underlined by the fact that none of the individuals with a *NOTCH3*^{cys} EGFr 1-6 variant in the Dutch CADASIL cohort (n=97) fulfilled the criteria for mSVD^{NOTCH3}. Other known CADASIL disease modifiers are classical cardiovascular risk factors such as

 Stroke. 2022;53:1964–1974. DOI: 10.1161/STROKEAHA.121.036307

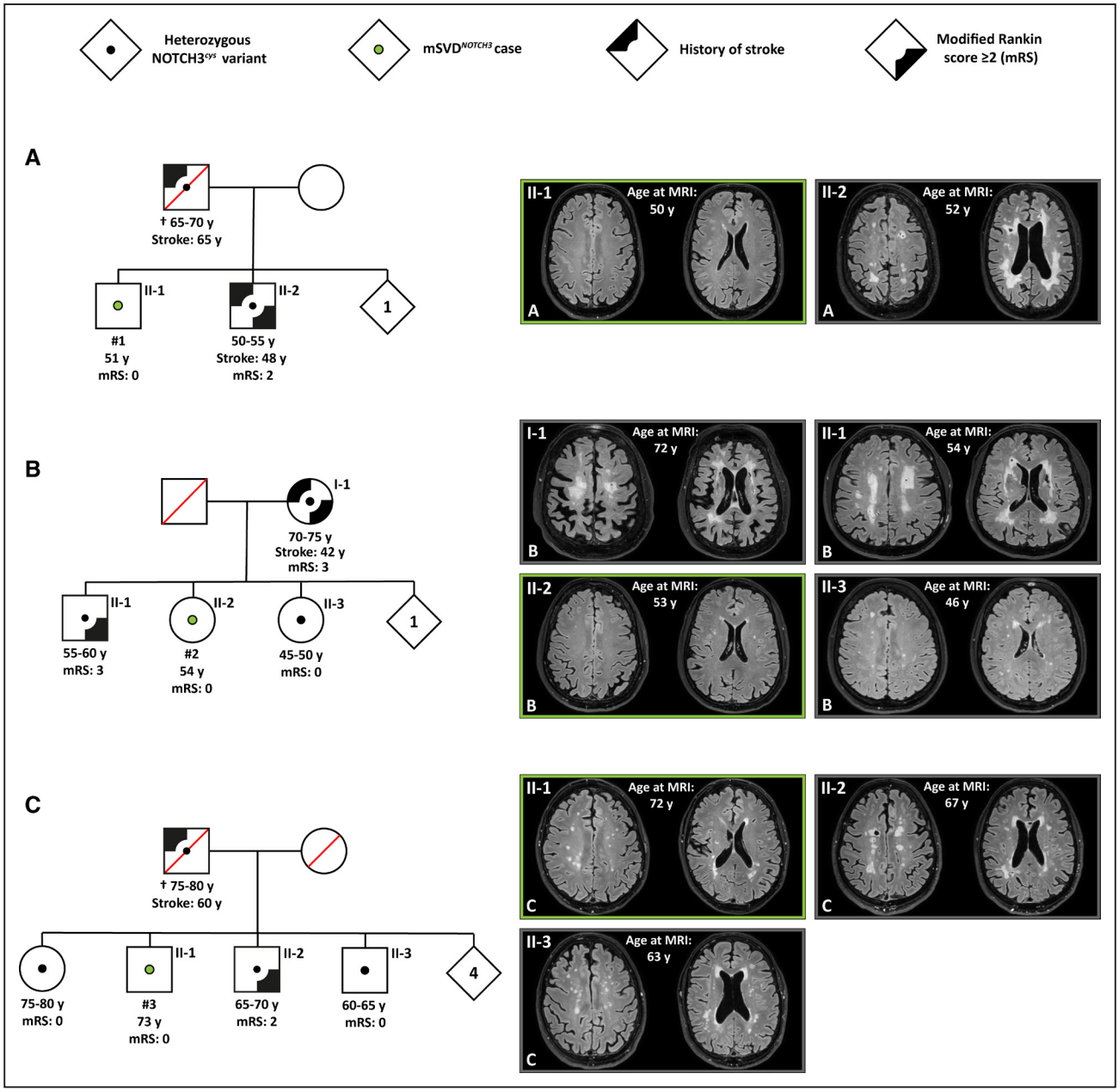

Figure 2. Pedigrees of 3 individuals with extremely mild small vessel disease.
A and **B**, High intrafamilial variability in disease expression between family members, ranging from a normal brain magnetic resonance imaging (MRI) at age 50 (mSVD*NOTCH3* case No. 1 and 2) to a classical severe Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) phenotype before age 50 y (**A**-II-2 and **B**-II-1). **A**, Brother of mSVD*NOTCH3* case No. 1 (**A**-II-2) has a classical severe CADASIL phenotype with stroke and cognitive deficits before age 50, with on brain MRI extensive confluent white matter hyperintensities (WMHs), 11 lacunes and 7 cerebral microbleed (CMB). **B**, mSVD*NOTCH3* case No. 2 (**B**-II-2) and her sister (**B**-II-3) have a much milder disease expression than their brother who has a classical severe CADASIL phenotype with severe cognitive deficits, apathy, and mood disturbances before age 50 with on brain MRI extensive confluent WMHs, 3 lacunes and 2 CMB. **C**, In another family, the disease expression is predominantly mild, most individuals are above age 60 y and asymptomatic with only minimal small vessel disease markers on brain MRI, except for one individual (**C**-II-2) aged 65–70 y, who has severe mood disturbances, history of TIA and frequent attacks of dizziness with 3 lacunes and 4 CMB, but a relatively low burden of WMH on brain MRI for a CADASIL patient of his age.

hypertension,[6–8] which in this study was also significantly more frequent in the symptomatic first-degree relatives of mSVD*NOTCH3* cases.

The fact that *NOTCH3*^{cys} EGFr 7-34 variants can be associated with such an extremely mild phenotype has important implications for clinical practice, not only when such a variant is ascertained as a chance finding in for example whole exome sequencing,[2] but also when such a variant is ascertained after predictive DNA-testing in family members of CADASIL patients. Clearly, in such cases the physician should be cautious about diagnosing CADASIL and should be aware that the presence of a

Table. Cardiovascular Risk Factors in Individuals With an Extremely Mild SVD Phenotype and First-Degree Relatives With Symptomatic CADASIL

n	mSVD^{NOTCH3} cases* 7	First-degree relatives with symptomatic CADASIL† 6	*P* value
Demographic characteristics			
Age at last visit, mean (SD)	56.6 (7.4)	61.3 (7.9)	0.29
Sex, n (%)	3 (42.9)	5 (83.3)	0.27
Vascular risk factors			
Hypertension, n (%)	1 (14.3%)	6 (100.0)	0.005‡
Hypercholesterolemia, n (%)	1 (14.3)	2 (33.3)	0.56
Diabetes, n (%)	0	2 (33.3)	0.19
Current or past smoking, n (%)	6 (85.7)	4 (66.7)	0.56
Packyears, median (IQR)	11.3 (28.6)	5 (38.2)	0.45

CADASIL indicates Cerebral Autosomal Dominant Arteriopathy With Subcortical Infarcts and Leukoencephalopathy; DWM, deep white matter; MRI, magnetic resonance imaging; mRS, modified Rankin Scale; mSVD^{NOTCH3}, extremely mild NOTCH3-associated small vessel disease; *NOTCH3*^{cys} variant, cysteine altering missense *NOTCH3* variant; and SVD, small vessel disease.

*mSVD^{NOTCH3}=extremely mild *NOTCH3*^{cys} small vessel disease.

†Symptomatic CADASIL is defined as mRS score of ≥2 and confluent deep white matter hyperintensities (Fazekas DWM 3) and >1 lacune on brain MRI.

NOTCH3^{cys} EGFr 7-34 variant is not necessarily predictive of early onset cerebral SVD.

We found that mSVD^{NOTCH3} cases had a very low NOTCH3 aggregation burden in skin vasculature, that is, very low NOTCH3 scores and absence of GOM. This is in line with our recently published study which shows that CADASIL patients with a *NOTCH3*^{cys} EGFr 7-34 variant have a significantly lower vascular NOTCH3 aggregation burden than patients with EGFr 1-6 variants.[27] The lack of apparent NOTCH3 aggregation in mSVD^{NOTCH3} cases also has implications for clinical practice, as the presence of GOM and granular NOTCH3 staining in skin vessels is sometimes still used to diagnose or exclude CADASIL. Clearly, in some individuals, the absence of GOM or granular NOTCH3 staining does not rule out the presence of a *NOTCH3*^{cys} variant. Whether levels of NOTCH3 aggregation in skin vessels may serve as a marker for disease severity and even be a predictor of disease course remains to be established.

Six of the 7 mSVD^{NOTCH3} cases had a *NOTCH3*^{cys} variant located in EGFr domain 14, of which 5 had the p.Arg578Cys variant, which is a founder variant in the Netherlands (present in 22.5% of Dutch CADASIL patients). Whether the p.Arg578Cys variant or *NOTCH3*^{cys} variants in EGFr domain 14 are more frequently associated with a milder SVD phenotype than other *NOTCH3*^{cys} EGFr 7-34 variants, will be addressed in our future studies.

The extreme intrafamilial variability in *NOTCH3*^{cys} SVD severity in our pedigrees illustrates yet again the presence of strong disease modifiers, particularly in cases with a *NOTCH3*^{cys} variant in EGFr domains 7-34. Next to cardiovascular risk factor burden other, likely genetic, modifiers must be involved. The significant difference in NOTCH3 aggregation between mSVD^{NOTCH3} cases and symptomatic EGFr 7-34 CADASIL patients suggests that these disease modifiers have an effect on vascular NOTCH3 aggregation. The nature of these modifiers is unknown, but these may include other variants in *NOTCH3*, variants in genes of matrisome proteins known to be involved in NOTCH3 aggregation or in genes known to be implicated in other Mendelian SVDs.[28–30] Although reports to date show conflicting results,[31–33] bi-allelic *NOTCH3*^{cys} variants may predispose to a more severe CADASIL phenotype, as the individual with a homozygous *NOTCH3*^{cys} EGFr 7-34 variant in our study had a much more severe SVD phenotype than his heterozygous family members. Unfortunately, we could not assess NOTCH3 aggregation in this homozygous individual, as skin biopsy was not available.

More than half of the mSVD^{NOTCH3} cases had a history of migraine with aura, which is similar to the frequency of migraine with aura observed in the Dutch CADASIL cohort (44.5%), and in other CADASIL populations.[34,35] This suggests that migraine with aura has a distinct pathomechanism to the subcortical ischemic events and cognitive decline in CADASIL patients, which is the result of a reduced cerebral blood flow and vasoreactivity.[12–14] This hypothesis is also supported by the occurrence of migraine with aura in young otherwise presymptomatic individuals with *NOTCH3*^{cys} variants, even before the appearance of WMH on brain MRI.[1] How *NOTCH3*^{cys} variants lead to cortical spreading depolarization, which underlies migraine with aura,[36] needs to be further elucidated.

The prevalence of mSVD^{NOTCH3} in CADASIL pedigrees is likely underestimated due to selection bias inherent to the study design, as asymptomatic individuals are less inclined to genetically test for a *NOTCH3*^{cys} variant. Another limitation of this study is the relatively small sample size for immunohistochemistry and electron microscopy. Strengths of this study are the prospectively collected data, extensive phenotypic characterization of mSVD^{NOTCH3} cases and their first-degree relatives, and the high number of blood vessels examined per individual.

In conclusion, our study shows that extremely mild SVD phenotypes can occur in individuals from CADASIL pedigrees with *NOTCH3*^{cys} EGFr 7-34 variants, which has important implications for clinical practice. Physicians should be aware that the presence of the familial *NOTCH3*^{cys} EGFr 7-34 variant in a CADASIL family member does not necessarily mean this individual will go on to develop CADASIL and likewise that a normal brain MRI in the sixth decade and the absence

 Stroke. 2022;53:1964–1974. DOI: 10.1161/STROKEAHA.121.036307

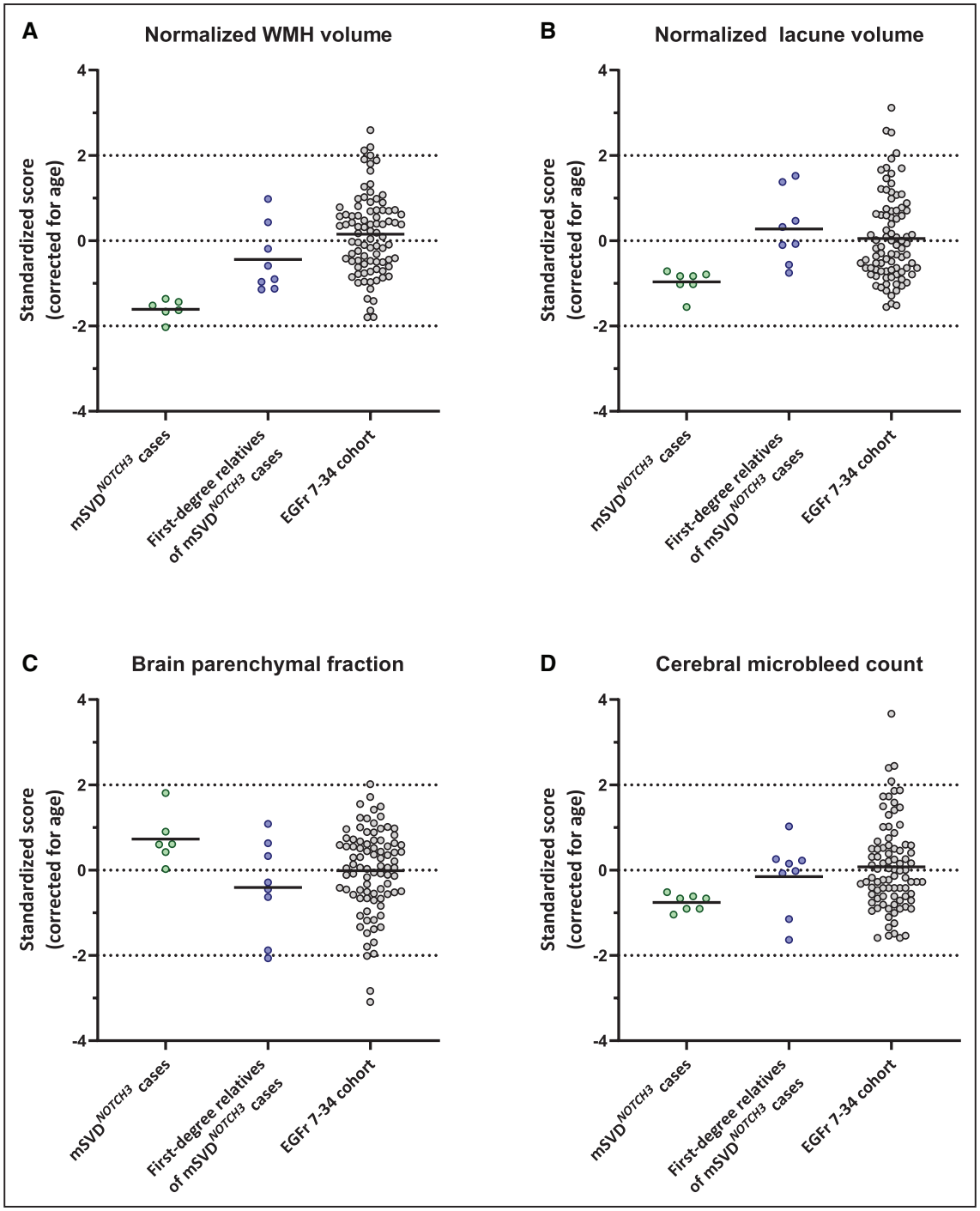

Figure 3. Magnetic resonance imaging (MRI) small vessel disease (SVD) burden in extremely mild small vessel disease cases, compared with their first-degree relatives with a *NOTCH3*^{cys} variant and other individuals in the Dutch Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) cohort with a *NOTCH3*^{cys} EGFr (epidermal growth-factor-like repeat) 7-34 variant.

A–D, Scatter plots showing standardized scores (corrected for age) of SVD imaging markers, based on all individuals with a *NOTCH3*^{cys} EGFr 7-34 variant in the Dutch CADASIL cohort, including mSVD^{NOTCH3} cases* and their first-degree relatives†: mSVD^{NOTCH3} cases have a lower burden of SVD imaging markers than their first-degree relatives (nWMH volume P=0.009; nLacune volume P=0.009; brain parenchymal fraction [BPF] P=0.09; cerebral microbleed [CMB] count P=0.18). The fact that the mSVD^{NOTCH3} cases did not always have the lowest standardized scores is due to age differences. There was a high variability in burden of SVD imaging markers in symptomatic first-degree relatives, which was similar to the variability seen in the CADASIL EGFr 7-34 cohort. There was no significant difference in standardized scores of SVD imaging markers between first-degree relatives and the complete CADASIL EGFr 7-34 cohort: nWMH volume (P=0.09); nLacune volume (P=0.55); BPF (P=0.29); and CMB count (P=0.55). * The nWMH volume and BPF could not be quantified for mSVD^{NOTCH3} case No. 7, because 3D-FLAIR and 3D-T1 MRI sequences were not available. †Only first-degree relatives harboring a heterozygous cysteine altering missense NOTCH3 variant (NOTCH3^{cys}) variant with a brain MRI performed on our 3T MRI scanner were included.

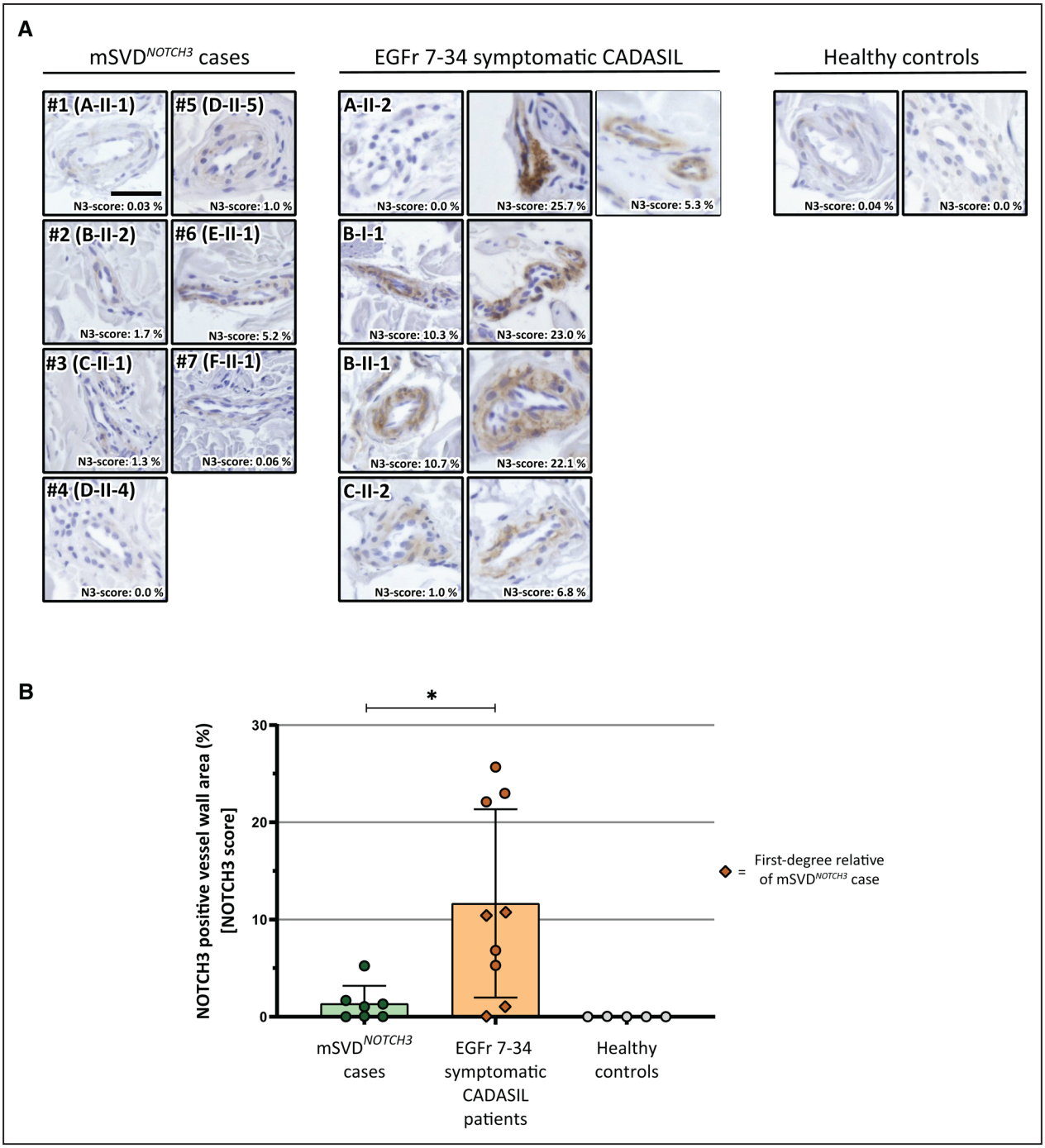

Figure 4. NOTCH3-immunohistochemistry of skin vessels in extremely mild small vessel disease cases compared with symptomatic Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) patients with an EGFr (epidermal growth-factor-like repeat) 7-34 variant and healthy controls.
A, Representative images of NOTCH3-immunohistochemistry of skin biopsies of 7 extremely mild NOTCH3-associated small vessel disease (mSVD^{NOTCH3}) cases and 9 symptomatic CADASIL patients with a cysteine altering missense NOTCH3 (*NOTCH3*^{cys}) variant located in EGFr 7-34, of which 4 individuals are first-degree relatives of the mSVD^{NOTCH3} cases (**A**-II-2, **B**-I-1, **B**-II-1, and **C**-II-2), and 2 healthy controls. NOTCH3 staining of mSVD^{NOTCH3} cases did not show the intense granular NOTCH3 staining typically seen in CADASIL, except for in mSVD^{NOTCH3} case No. 6. **B**, The NOTCH3 score was significantly lower in mSVD^{NOTCH3} cases than in symptomatic CADASIL patients: median 1.0 (interquartile range [IQR], 1.7 [range, 0.0–5.2]) vs median 10.3 (IQR, 19.4 [range, 0.0–25.7]; P=0.010). Bar represents 50 µm. *P<0.05.

of NOTCH3 aggregation and GOM in skin vessels, does not exclude the presence of the familial *NOTCH3*^{cys} variant. The modifiers protecting some CADASIL family members from developing CADASIL may be the same as those associated with the mild *NOTCH3*^{cys} SVD phenotype observed in the population. Identifying these modifiers may provide new clues for prevention and treatment of CADASIL.

 Stroke. 2022;53:1964–1974. DOI: 10.1161/STROKEAHA.121.036307

ARTICLE INFORMATION

Received June 17, 2021; final revision received November 16, 2021; accepted December 15, 2021.

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Acknowledgments

Dr Hack designed and conceptualized the study, acquisition of data, analyzed and interpreted the data, and drafted the article. Dr Gravesteijn designed and conceptualized the study, acquisition of data, analyzed and interpreted the data, critical revision of the article for important intellectual content. Dr Cerfontaine acquisition of data, analyzed and interpreted the data, critical revision of the article for important intellectual content. I.M. Hegeman and A.A. Mulder involved in acquisition of data, critical revision of the article for important intellectual content. Drs Lesnik Oberstein and Rutten involved in designed and conceptualized the study, acquisition of data, analyzed and interpreted the data, drafted the article, critical revision of the article for important intellectual content, and supervision. We wish to acknowledge all the CADASIL patients who participated in our studies and would like to thank Peter Neeskens for the processing of skin biopsies.

Sources of Funding

This work was funded by Netherlands Organization for Health Research and Development (ZonMW 91717325).

Disclosures

Dr Hack is funded by the Netherlands Organization for Health Research and Development (ZonMW 91717325). Dr Lesnik Oberstein has financial research support from the Netherlands Organization for Health Research and Development (ZonMW 91717325) and the Netherlands Brain Foundation (HA2016-02-03) and receives institutional support from Leiden University Medical Center. Dr Rutten is funded by the Netherlands Organization for Health Research and Development (ZonMW 91717325) and the Netherlands Brain Foundation (HA2016-02-03). The other authors report no conflicts.

Supplemental Material

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