How common are single gene mutations as a cause for lacunar stroke?
A targeted gene panel study

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Abstract

Objectives
To determine the frequency of rare and pertinent disease-causing variants in small vessel disease (SVD)-associated genes (such as NOTCH3, HTRA1, COL4A1, COL4A2, FOXC1, TREX1, and GLA) in cerebral SVD, we performed targeted gene sequencing in 950 patients with younger-onset apparently sporadic SVD stroke using a targeted sequencing panel.

Methods
We designed a high-throughput sequencing panel to identify variants in 15 genes (7 known SVD genes, 8 SVD-related disorder genes). The panel was used to screen a population of 950 patients with younger-onset (<70 years) MRI-confirmed SVD stroke, recruited from stroke centers across the United Kingdom. Variants were filtered according to their frequency in control databases, predicted effect, presence in curated variant lists, and combined annotation dependent depletion scores. Whole genome sequencing and genotyping were performed on a subset of patients to provide a direct comparison of techniques. The frequency of known disease-causing and pertinent variants of uncertain significance was calculated.

Results
We identified previously reported variants in 14 patients (8 cysteine-changing NOTCH3 variants in 11 patients, 2 HTRA1 variants in 2 patients, and 1 missense COL4A1 variant in 1 patient). In addition, we identified 29 variants of uncertain significance in 32 patients.

Conclusion
Rare monogenic variants account for about 1.5% of younger onset lacunar stroke. Most are cerebral autosomal dominant arteriopathy with subcortical infarcts and leukencephalopathy variants, but the second most common gene affected is HTRA1. A high-throughput sequencing technology platform is an efficient, reliable method to screen for such mutations.
Cerebral small vessel disease (SVD) accounts for around 25% of strokes in the form of lacunar strokes and deep intracerebral hemorrhages (ICH), and is the primary pathology underlying vascular cognitive impairment. In the majority of cases, it is a sporadic disease of aging related to hypertension and subsequent arteriosclerosis, but a minority of cases are due to rare genetic variants. The most common inherited form of SVD is cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) due to NOTCH3 variants. More recently, other genes have been reported to cause similar phenotypes, including HTRA1, COL4A1, COL4A2, TREX1, GLA, and FOXC1. However, the frequency of these variants in populations with presumed sporadic SVD is unknown.

Identification of disease-causing variants currently largely relies on Sanger sequencing of the gene of interest. Often for cost reasons this involves sequencing a subset of exons, such as in CADASIL, where exons 3 and 4 are most frequently affected, and therefore preferentially screened. As the spectrum of monogenic SVD expands, testing on a gene-by-gene basis is not cost- or time-effective. High-throughput sequencing (HTS) panels using next-generation sequencing technologies allow simultaneous testing in multiple genes underlying a single disease phenotype in a more cost-effective manner and are increasingly being used in clinical practice.

In this study, we developed a HTS panel comprising 15 genes linked to the SVD phenotype. We evaluated the platform for disease diagnosis and to determine the frequency of monogenic disease-causing variants in a well-defined population with MRI-confirmed younger-onset lacunar stroke. This study evaluates both known disease-causing mutations and novel, potentially disease-causing variants.

**Methods**

**Platform design**

The gene panel was developed to include 7 genes known to be causal of SVD (NOTCH3, HTRA1, FOXC1, COL4A1, COL4A2, TREX1, GLA) as well as 8 genes associated with disorders with SVD-related phenotypes. These include familial cerebral amyloid angiopathy (APP, CST3, ITM2B), familial hemiplegic migraine (ATP1A2, CACNA1A, SCN1A), and connective tissue disorders (ABCC6, COL3A1). These disorders share clinical manifestations with monogenic forms of SVD (for example, lacunar stroke, MRI white matter hyperintensities [WMH], dementia, migraine with aura, and encephalopathy) and could therefore present similarly. For each gene, the transcript on which to report variants was selected based on size, RefSeq information, and previously reported variants, and submitted to the Locus Reference Genomic database. The capture design has previously been described by Simeoni et al.

**Study population**

The study population consisted of patients from the UK DNA Lacunar Stroke Study. A total of 72 specialist centers across the United Kingdom recruited unrelated patients of European ancestry with MRI-confirmed lacunar stroke occurring at or before the age of 70. The study was approved by the Multi-Centre Research Ethics Committee for Scotland (04/MRE00/36) and informed consent was obtained from participants. Stored DNA was available for 950 patients, all of whom were included in this study.

Lacunar stroke was defined as a clinical lacunar syndrome, with an anatomically compatible lesion on MRI (subcortical infarct ≤15 mm in diameter). All patients underwent full stroke investigations including brain MRI, carotid artery imaging, and ECG. Echocardiography was performed when appropriate. Patients were excluded if the cause of stroke was not SVD, including stenosis >50% in the extracranial or intracranial vessels; previous carotid endarterectomy; cardioembolic source of stroke defined according to Trial of Org 10172 in Acute Stroke Treatment criteria as high or moderate probability; cortical infarct on MRI; subcortical infarct >15 mm in diameter, as these can be caused by embolic mechanisms (striatocapsular infarcts); and any other specific cause of stroke (e.g., lupus anticoagulant, vasculitis, dissection, known monogenic cause).

All MRI scans and clinical histories were reviewed centrally by one physician (H.S.M.). The presence and extent of WMH was graded on T2-weighted or fluid-attenuated inversion recovery (FLAIR) scans using the Fazekas scale: 0 = none, 1 = mild, 2 = early confluent, 3 = severe confluent, as previously described. Lacunar infarcts were identified as a high signal lesion on acute diffusion-weighted imaging performed within 3 weeks of acute stroke, or as a cavitated hypodense lesion on T1 or FLAIR sequences. Cerebral microbleeds were identified

### Glossary

- **CADASIL** = cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
- **CARASIL** = cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy
- **CI** = confidence interval
- **CNV** = copy number variant
- **DHPLC** = denaturing high-performance liquid chromatography
- **FLAIR** = fluid-attenuated inversion recovery
- **gnomAD** = genome aggregation database
- **HTS** = high-throughput sequencing
- **ICH** = intracerebral hemorrhage
- **WMH** = white matter hyperintensities
- **WES** = whole-exome sequencing
- **WGS** = whole-genome sequencing

- **APP** = amyloid-β precursor protein
- **CST3** = cytokine signaling 3
- **ITM2B** = integral membrane protein 2B
- **ATP1A2** = ATPase, Na/K-transporting, subfamily A, member 2
- **CACNA1A** = voltage-gated calcium channel, subfamily A, member 1A
- **SCN1A** = sodium channel, voltage-gated, type I, alpha subunit
- **ABCC6** = ATP-binding cassette, subfamily C (MDM2 and MAD2L2-like), member 6
- **COL3A1** = collagen, type III, alpha 1
on gradient echo sequences. Family history was collected for first-degree relatives (table 1).

**Sample processing**
DNA samples were processed as previously described.8

**Clinical bioinformatics**
Sequence reads were processed as previously described.8 The filtering step was adapted to SVD: single nucleotide variants and indels were prioritized in the following: (1) minor allele frequencies in the genome aggregation database (gnomAD)12 <0.0001; (2) predicted impact according to SnPEff15 is high, moderate, or splice region; (3) presence in HGMD Pro (2017.2) or in curated locus-specific databases (in particular the Leiden Open-Source Variation Database [May 2017 version])13; (4) degree of deleteriousness according to Combined Annotation Dependent Depletion score14 ≥15. NOTCH3 variants that resulted in the gain or loss of a cysteine residue in the EGF-like repeats were automatically prioritized, as they are known to cause CADASIL.15 The resulting variants were assessed according to the American College of Medical Genetics and Genomics guidelines16 and retained if classified as pathogenic, likely pathogenic, or of unknown significance.

Large copy number variants (CNVs) were called using a custom pipeline based on ExomeDepth 1.1.1019 as previously described.17

**Panel validation**
The panel was assessed by comparing the results with those obtained by 2 independent methods: whole-genome sequencing (WGS) and NOTCH3 and GLA sequencing.

Thirty-four samples sequenced using the panel were also sequenced using WGS, as part of the National Institute for Health Research (NIHR) BioResource–Rare Disease study. The NIHR BioResource projects were approved by Research Ethics Committees in the United Kingdom and appropriate national ethics authorities in non–United Kingdom enrollment centers. WGS was performed by Illumina (San Diego, CA) on HiSeqXTen generating 150 bp paired-end reads per lane with minimum coverage of 15X for at least 95% of the genome (30X on average). Reads were aligned to the GRCh37 build of the human genome reference using the Isaac Aligner, and variants were called using the Isaac VariantCaller.18 Variants in the 15 genes were analyzed following the same criteria as for the HTS panel.

Samples from all 950 patients had been previously screened for disease-causing NOTCH3 and GLA variants.9 Exons in 3, 4, 5, 6, 11, 18, 19, and 22 of NOTCH3 were screened using denaturing high-performance liquid chromatography (DHPLC), and in addition, exons 3 and 4 were screened using Sanger sequencing.9 Five patients with typical CADASIL-causing variants were identified. GLA was screened using high-resolution melt-curve analysis, covering all exons and intron/exon junctions, and one deep intronic region containing a known pathogenic variant. No Fabry-causing variants were identified.9

**Statistical analysis**
Comparisons of variant frequency between patients with and without a family history of stroke and with and without confluent WMH were performed using the Fisher exact test. Analyses were performed using R statistical software (version 3.5.1).

**Data availability**
Anonymized data will be made available upon reasonable request.

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### Table 1 Study population

|                        | Values       |
|------------------------|--------------|
| **Age at first stroke, y** |              |
| Mean (SD)              | 56.3 (8.7)   |
| Median                 | 57.4         |
| **Male sex, n (%)**    | 674 (70.9)   |
| **BMI, mean (SD)**     | 28.7 (6.2)   |
| **Number (%) of patients with the following:** |     |
| Hypertension           | 679 (71.5)   |
| Diabetes               | 157 (16.5)   |
| Hyperlipidemia         | 643 (67.7)   |
| Smoker (current or previous) | 663 (69.8)   |
| Alcohol excess (≥20 units/wk) | 272 (28.6)   |
| Migraine               | 189 (19.8)   |
| Migraine with aura     | 106 (11.1)   |
| Myocardial infarction/coronary artery bypass graft or angioplasty | 31 (3.3)     |
| Peripheral vascular disease | 26 (2.7)     |
| Previous or recurrent strokes | 70 (7.4)     |
| White matter hyperintensities (Fazekas grade ≥2) | 309 (32.5)   |
| Microbleeds present*   | 58 (18.1)    |

Abbreviation: BMI = body mass index.

*A total of 321 patients had gradient echo sequences performed to evaluate the presence of cerebral microbleeds.
Results

Overall frequency of potentially disease-causing variants

Previously reported disease-causing variants and novel rare variants are shown in Table 2.

In the 7 known SVD genes, known disease-causing variants were identified in 14 individuals (1.5%); this represented 11 different mutations. The proportion of patients with a reported family history of stroke found to have mutations was higher (8 of 372 patients [2.2%]) than in patients without a reported family history of stroke (6 of 578 patients [1.0%]), although this difference was not significant ($p = 0.18$) (figure 1A).

In addition, we identified 31 novel rare variants in the 7 known SVD genes. These were identified in 35 individuals. Excluding 2 COL4A1 variants in 3 patients that were predicted to be benign in ClinVar (table 3), the overall frequency

| Table 2 Known pathogenic NOTCH3 (ENST00000263388), HTRA1 (ENST00000368984), and COL4A1 (ENST00000375820) variants |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Gene            | Sex  | Age, y | Risk factors | Imaging features | FHx  | Variant       | MAF in gnomAD | References/remarks |
| NOTCH3          | M    | 61     | HTN, HLD, DM, S, BMI 34 | WMH FS 3, bilateral ATP involvement, no GE | N    | c.227G>A, p.Cys76Tyr | —             | 41 p.Cys76Arg42 and p.Cys76Trp43,44 also reported Not identified on DHPLC/Sanger sequencing Also detected on WGS |
|                 | M    | 46     | HLD, S, BMI 30, MA | WMH FS 3, no GE | Y    | c.505C>T, p.Arg169Cys | —             | 6,45 Not identified on DHPLC/Sanger sequencing Also detected on WGS |
|                 | M    | 47     | HLD, ETOH, BMI 30 | WMH FS 3, minor left ATP involvement, no GE | Y    | c.619C>T, p.Arg207Cys | 8.1 × 10^{-6} | 42 Identified on DHPLC and Sanger sequencing |
|                 | M    | 58     | HTN, HLD, S | WWMH FS 3, no GE | Y    | c.967T>A, p.Cys323Ser | —             | Internal CADASIL database Identified on DHPLC |
|                 | M    | 35     | S, ETOH | WWMH FS 2, no microbleeds on GE | N    | c.1162T>C, p.Cys388Arg | 3.6 × 10^{-5} | 45,47 Identified on DHPLC |
|                 | F    | 67     | HTN, HLD, ETOH, BMI 45, Dep, MA, TIA | WWMH FS 3, bilateral ATP involvement | Y    | c.1590C>T, p.Arg530Arg | —             | 46 Not sequenced on DHPLC/Sanger |
|                 | F    | 65     | HTN, HLD, S | WWMH FS 3, no microbleeds on GE | N    | c.3356G>A, p.Cys1119Tyr | 8.1 × 10^{-6} | Internal CADASIL database Not sequenced on DHPLC/Sanger |
|                 | M    | 53     | BMI 35 | ILI, F 1, no microbleeds | Y    | c.3664T>G, p.Cys1222Gly | 1.1 × 10^{-4} | 48 Not identified on DHPLC20,21 |
|                 | M    | 50     | HLD, S | MLI, F 1, no microbleeds | Y    | c.904C>T, p.Arg302Ter | 1.1 × 10^{-5} | 48 Identified on DHPLC |
|                 | M    | 55     | HTN, HLD, S, TIA | WWMH FS 3, microbleeds | N    | c.1348G>A, p.Asp450His | 7.2 × 10^{-5} | Variant in PDZ domain previously reported in autosomal dominant disease21 |
| HTRA1           | M    | 43     | HTN, HLD, DM, S, BMI 30, Dep | MLI, F 0, no microbleeds | Y    | c.1055G>T, p.Pro352Leu | 6.5 × 10^{-6} | Previously associated with ICH,24 impair COL4A1 secretion24 |
| COL4A1          | F    | 70     | HTN, MA | MLI, F 1, no GE | Y    | c.227G>A, p.Cys76Tyr | —             | 41 p.Cys76Arg42 and p.Cys76Trp43,44 also reported Not identified on DHPLC/Sanger sequencing Also detected on WGS |

Abbreviations: ATP = anterior temporal pole; BMI = body mass index; CADASIL = cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy; Dep = depression history; DHPLC = denaturing high-performance liquid chromatography; DM = diabetes mellitus; ETOH = alcohol excess; FHx = family history of stroke; FS = Fazekas score; GE = gradient echo imaging for microbleeds; gnomAD = genome aggregation database; HLD = hyperlipidemia; HTN = hypertension; ICH = intracerebral hemorrhage; ILI = isolated lacunar infarcts; MA = migraine with aura; MAF = minor allele frequencies; MLI = multiple lacunar infarcts; S = smoking history; WGS = whole-genome sequencing; WMH = white matter hyperintensities.

FHx was collected for first-degree relatives (parents, siblings, offspring).
of novel variants was 3.4% (32 of 950 patients). There was no difference in the proportion of novel rare variants among patients with and without a family history of stroke (11 of 364 vs 21 of 572, respectively; \( p = 0.71 \)) (figure 1A).

To determine whether variants were more common in a particular phenotype of SVD, or in more severe cases, we examined variant frequency in those with confluent WMH. Of the 309 patients with confluent WMH on MRI (Fazekas score\(^10\) \( \geq 2 \)), 9 (2.9%) had a known disease-causing variant, compared with 5 of 641 (0.8%) in those without confluent WMH (\( p = 0.018 \)). The proportions for rare novel variants of uncertain significance were 12 of 309 (3.9%) for those with WMH and 20 of 641 (3.1%) for those without (\( p = 0.57 \)) (figure 1B).

**CADASIL**

CADASIL is caused by cysteine-altering NOTCH3 mutations in the epidermal growth factor–like repeat domains encoded by exons 2 to 24. Eight different cysteine-changing variants in exons 2–24 of NOTCH3 were identified in 11 individuals (table 2). Previous screening had identified 5 disease-causing variants, and these were again identified using this platform.
Of the 6 additional individuals with cysteine-changing NOTCH3 variants, 2 variants in 2 individuals were previously missed by both DHPLC and Sanger sequencing.

The overall frequency of CADASIL-causing variants was 1.2% (95% confidence interval [CI] 0.6%–2.1%). Of patients with confluent WMH (Fazekas score ≥2) the frequency was 2.9% (9 of 309, 95% CI 1.5%–5.4%) compared to 0.3% of patients without confluent WMH (Fazekas score <2) (2 of 641, 95% CI 0.1%–1.1%) (p = 0.001).

Comparing age groups, the overall frequency of CADASIL-causing variants was 1.2% (95% CI 0.6%–2.5%) in patients ≤60 years and 1.1% (95% CI 0.4%–0.7%) in patients aged >60 years. Among patients with confluent WMH, the mutation frequency was 3.7% (95% CI 1.6%–8.3%) in patients ≤60 years and 2.3% (95% CI 0.9%–5.8%) in patients aged >60 years.

We identified 9 heterozygous missense COL4A2 variants in 9 individuals (0.9%, 95% CI 0.5%–1.8%). None of these have previously been reported (figure 2C).

Variants identified in both COL4A1 and COL4A2 were found in various regions including the Gly-X-Y regions and the C4 domain associated with disease (table 3).

Non-CADASIL monogenic small vessel arteriopathies

**HTRA1**

Missense and nonsense *HTRA1* variants have been reported in cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL). Recently, heterozygous variants have also been identified in patients with an autosomal dominant form of SVD. Eight heterozygous missense variants and 1 nonsense *HTRA1* variant were identified in 12 individuals (1.3%, 95% CI 0.7%–2.2%). Two of these have previously been reported as disease-causing; p.Arg302Ter was reported in both recessive and dominant disease, and p.Asp450His was reported in autosomal dominant disease. p.Arg302Ter has been demonstrated to result in a mutant trypsin domain, with 1 (p.Arg227Trp) identified in a separate study using Sequenom MassARRAY technology. p.Arg227Trp has been reported frameshift variant (p.Ala381_Gly382fs) (table 3). The in-frame insertion was in a low-complexity region near the forkhead box domain, and has previously been reported in ClinVar as likely pathogenic for Axenfeld-Rieger syndrome (p.Ala381GlyfsTer147). Loss-of-function variants in *FOXC1* are known to be pathogenic and the frameshift variant is predicted to lead to premature stop codon 147 residues downstream.

**FOX1**

The *FOX1* gene encodes the Forkhead box C1 transcription factor. Autosomal dominant missense, nonsense, and frameshift variants, as well as deletion or duplication of the locus (6p25), have been associated with ocular abnormalities described as the Axenfeld-Rieger syndrome. Some variants are also associated with white matter abnormalities. Two heterozygous predicted high-impact variants in *FOX1* were identified in 2 individuals (0.2%, 95% CI 0.06%–0.8%): 1 novel in-frame insertion (p.Thr68_Pro69insThrProGln) and 1 frameshift variant (p.Ala381_Gly382fs) (table 3). The in-frame insertion was in a low-complexity region near the forkhead box domain and has previously been reported in ClinVar as likely pathogenic for Axenfeld-Rieger syndrome (p.Ala381GlyfsTer147). Loss-of-function variants in *FOX1* are known to be pathogenic and the frameshift variant is predicted to lead to premature stop codon 147 residues downstream.

**TREX1**

Retinal vasculopathy with cerebral leukodystrophy and systemic manifestations arises due to frameshift variants near the C-terminus of *TREX1*. Missense *TREX1* variants are also associated with Aicardi-Goutières syndrome, a form of pediatric-onset encephalopathy, and familial chilblain lupus. Three novel missense variants (p.Gly197Ala, p.Arg229Gly, p.Lys230Asn), 1 novel in-frame deletion, and 1 previously reported frameshift variant were found in 5 individuals (0.5%, 95% CI 0.2%–1.2%). The frameshift variant (p.Ala194fs) was also identified in the same patient in a separate study using Sequenom MassARRAY technology (reported as p.Ala194fsTer21). This variant has been reported in a compound heterozygous case of Aicardi-Goutières syndrome in the DECIPHER database (patient 9303873).

**Fabry disease: GLA variants**

No pathogenic or likely pathogenic Fabry variants were identified.

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**Fabry disease: GLA variants**

No pathogenic or likely pathogenic Fabry variants were identified.
Table 3 Rare, novel, or presumed benign variants identified in HTRA1 (ENST00000368984), COL4A1 (ENST00000375820), COL4A2 (ENST00000360467), FOXC1 (ENST00000380874), and TREX1 (ENST00000422277) genes

| Gene       | Sex | Age, y | Risk factors | Imaging features | Family history of stroke | Gene/variant | MAF in gnomAD | Pathogenicity                                      |
|------------|-----|--------|--------------|------------------|--------------------------|--------------|---------------|---------------------------------------------------|
| HTRA1      | F   | 68     | HLD, S       | ILL, FS 1, no microbleeds | N                         | c.521A>C, p.Asp174Ala | 4.1 × 10⁻⁶ | Novel variant*                                   |
|            | M   | 56     | HLD, S, ETOH, Dep | MLI, FS 1, no microbleeds | N                         | c.632A>C, p.Glu211Ala | —            | Novel variant*                                   |
|            | M   | 59     | HTN, HLD, DM | WMH, FS 3, no GE     | N                         | —             | —            |                                                  |
|            | M   | 62     | HTN, HLD, S, BMI 30, Mig | ILL, FS 0, no microbleeds | Y                         | c.679C>T, p.Arg227Trp | 7.9 × 10⁻⁶ | Novel variant*                                   |
|            | M   | 60     | HTN, HLD, ETOH, BMI 40 | WMH, FS 3, no GE     | N                         | —             | —            |                                                  |
|            | M   | 61     | HTN, HLD, S, ETOH | ILL, FS 0, no microbleeds | N                         | —             | —            |                                                  |
|            | M   | 50     | HTN, S       | WMH, FS 3, no GE     | Y                         | c.834C>A, p.Phe278Leu | —            | Novel variant*                                   |
|            | M   | 63     | HTN, HLD, MA | ILL, FS 1, microbleeds | N                         | c.940A>G, p.Met314Val | 8.1 × 10⁻⁶ | Novel variant*                                   |
|            | M   | 41     | None         | ILL, FS 0, no GE     | N                         | c.958G>A, p.Asp320Asn | 1.2 × 10⁻⁵ | Novel variant*                                   |
|            | F   | 62     | HLD, BMI 36  | WMH, FS 2, no GE     | Y                         | c.1103A>G, p.His368Arg | —            | Novel variant found between trypsin and PDZ domains |
| COL4A1     | F   | 53     | HTN, HLD, S, ETOH, D | WMH, FS 2, no GE     | Y                         | c.994G>C, p.Gly332Arg | 4.3 × 10⁻⁵ | ClinVar: likely benign variant; ncbi.nlm.nih.gov/clinvar/variation/311067/ |
|            | F   | 45     | BMI 37       | ILL, FS 0, no GE     | N                         | c.1246C>G, p.Pro416Ala | 4.9 × 10⁻⁵ | Novel variant*                                   |
|            | F   | 63     | HTN, HLD, S, BMI 32, Dep, MA | WMH, FS 2, no GE     | N                         | c.2093A>G, p.Lys698Arg | 5.9 × 10⁻⁵ | Novel variant*                                   |
|            | F   | 55     | HTN, HLD, S, BMI 32 | ILL, FS 0, no GE     | NK                        | c.2174C>T, p.Pro725Leu | —            | Novel variant*                                   |
|            | M   | 42     | ETOH, BMI 33 | ILL, FS 0, no GE     | N                         | c.4010C>T, p.Pro1337Leu | 7.9 × 10⁻⁵ | ClinVar: likely benign variant in context of COL4A1-related SVD*; ncbi.nlm.nih.gov/clinvar/variation/311030/ |
|            | F   | 68     | HLD          | ILL, FS 0, no microbleeds | N                         | —             | —            |                                                  |
|            | M   | 38     | HTN, S, BMI 30 | ILL, FS 0, no GE     | NK                        | c.4423T>C, p.Tyr1475His | 1.6 × 10⁻⁵ | Novel variant*                                   |
|            | M   | 65     | S            | WMH, FS 3, no GE     | Y                         | c.4678G>A, p.Val1560Met | 1.2 × 10⁻⁵ | Novel variant*                                   |
|            | M   | 69     | S, ETOH      | WMH, FS 2, no microbleeds | N                         | c.4970C>T, p.Thr1657Met | 2.4 × 10⁻⁵ | Novel variant*                                   |
| COL4A2     | M   | 59     | HTN, HLD, S, ETOH, BMI 37 | WMH, FS 2, no GE     | Y                         | c.661C>A, p.Pro221Thr | 1.5 × 10⁻⁵ | Novel variant*                                   |
|            | M   | 58     | HLD          | WMH, FS 3, no microbleeds | N                         | c.965G>A, p.Arg322Gln | 3.6 × 10⁻⁵ | Novel variant*                                   |
|            | M   | 65     | HTN, HLD, S | ILL, FS 0, no GE     | NK                        | c.1396G>A, p.Gly466Ser | 5.4 × 10⁻⁵ | Novel variant*                                   |

Continued
After excluding CNVs with a Bayes factor lower than 20, only 1 CNV was detected in the 7 known cerebral SVD genes. This was a large duplication of both \textit{COL4A1} and \textit{COL4A2} in a single individual.

### SVD-related genes

Forty-five heterozygous variants in 8 genes associated with SVD-related disorders were identified in 47 individuals (Table 4). Of note, 2 novel variants were identified in cystatin 3 (\textit{CST3}), where variants are known to cause familial...
Validation of panel

In the 34 individuals sequenced by WGS, 2 were found to harbor CADASIL-causing NOTCH3 variants. These were also detected by the HTS platform (table 2). No other exonic variants passing the filters could be identified in the remaining 32 individuals, either by WGS or by targeted sequencing. In addition, 5 cysteine-changing NOTCH3 variants had been detected on prior screening, all of which were also identified by the platform.

Discussion

We developed an HTS panel for 7 genes known to be implicated in monogenic SVD and 8 genes associated with related phenotypes that can enter in the differential diagnosis of monogenic SVD. Validation against other techniques found the panel to be as sensitive, if not more sensitive than conventional screening methods, and detected all previously identified NOTCH3 variants, including some missed on previous screening.

NOTCH3 variants, which underlie CADASIL, the most common cause of monogenic SVD, were identified in about 1% of patients with apparently sporadic SVD aged 70 or under. The yield rose to 3.7% if analysis was limited to younger (age ≤60) patients who also had confluent WMH.
Table 4 Rare variants identified in genes associated with phenotypes related to small vessel disease: COL3A1 (ENST00000304636), APP (ENST00000346798), CST3 (ENST00000376925), ITM2B (ENST00000378565), ATP1A2 (ENST00000361216), and CACNA1A (ENST00000361216), SCN1A (ENST00000375405)

| Related phenotype | Gene       | Variant                           | dbSNP ID   | No. of individuals | MAF in gnomAD | Remarks                                                                 | Reference |
|-------------------|------------|-----------------------------------|------------|--------------------|---------------|--------------------------------------------------------------------------|-----------|
| Ehlers-Danlos (IV) | COL3A1     | c.74A>G, p.Gln25Arg               | —          | 1                  | —             | In signal peptide                                                        | —         |
|                   |            | c.1856C>T, p.Pro619Leu             | rs373838193| 1                  | 4.3 × 10^{-5} | In low complexity region VUS on ClinVar for aortic aneurysm/dissection and Ehlers-Danlos type IV | ncbi.nlm.nih.gov/clinvar/variation/190718/ |
|                   |            | c.1864C>T, p.Pro622Ser             | rs772638774| 2                  | 3.3 × 10^{-5} | In low complexity region                                                 | —         |
|                   |            | c.226A>G, p.Asn76Asp               | rs142045411| 1                  | 6.2 × 10^{-5} | In von Willebrand factor type C domain                                   | —         |
|                   |            | c.1165A>T, p.Asn389Tyr             | rs200394946| 1                  | 9.7 × 10^{-5} | In collagen triple helix repeat domain VUS on ClinVar for aortic aneurysm/dissection and Ehlers-Danlos type IV | ncbi.nlm.nih.gov/clinvar/variation/190718/ |
|                   |            | c.1859C>T, p.Pro620Leu             | rs759926843| 1                  | 1.6 × 10^{-5} | In low complexity region                                                 | —         |
|                   |            | c.1909+5G>A, p.Glu93Ter            | rs200271509| 1                  | 1.5 × 10^{-5} | In coiled coil domain                                                    | —         |
|                   |            | c.2148C>T, p.Ile716Ile             | rs145564988| 1                  | 6.9 × 10^{-5} | In C-terminus domain; Ile716Thr reported on ClinVar (no clinical interpretation available); Ile716Val associated with familial Alzheimer disease | ncbi.nlm.nih.gov/clinvar/variation/98240/50   |
|                   | CST3       | c.277G>T, p.Glu93Ter               | —          | 1                  | 4.1 × 10^{-5} | p.Leu94Gln reported in familial Icelandic dementia                       | 51        |
|                   |            | c.277_296delGAGCT. GGGCCGAACCACGTG, p.Glu93fs | rs765138253| 1                  | 6.1 × 10^{-5} | Low complexity region                                                    | —         |
| Cerebral amyloid angiopathy | APP       | c.373G>A, p.Asp125Asn              | —          | 1                  | —             | In amyloid A4 N-terminal heparin binding domain                           | —         |
|                   |            | c.682G>A, p.Val228Ile              | rs755841034| 2                  | 4.1 × 10^{-5} | In low complexity region, near p.Val225Ala, a VUS associated with familial Alzheimer disease | —         |
|                   |            | c.727G>A, p.Asp243Asn              | rs750279232| 1                  | 3.3 × 10^{-5} | In coiled coil domain                                                    | —         |
|                   |            | c.736G>A, p.Glu246Lys              | rs147485129| 1                  | 1.8 × 10^{-5} | In coiled coil domain                                                    | —         |
|                   |            | c.1859C>T, p.Pro620Leu             | rs759926843| 1                  | 1.6 × 10^{-5} | In low complexity region                                                 | —         |
|                   |            | c.1909+5G>A, p.Glu93Ter            | rs200271509| 1                  | 1.5 × 10^{-5} | In coiled coil domain                                                    | —         |
|                   |            | c.2148C>T, p.Ile716Ile             | rs145564988| 1                  | 6.9 × 10^{-5} | In C-terminus domain; Ile716Thr reported on ClinVar (no clinical interpretation available); Ile716Val associated with familial Alzheimer disease | ncbi.nlm.nih.gov/clinvar/variation/98240/50   |
| Cerebral amyloid angiopathy | CST3       | c.277G>T, p.Glu93Ter               | —          | 1                  | 4.1 × 10^{-5} | p.Leu94Gln reported in familial Icelandic dementia                       | 51        |
|                   |            | c.277_296delGAGCT. GGGCCGAACCACGTG, p.Glu93fs | rs765138253| 1                  | 6.1 × 10^{-5} | Low complexity region                                                    | —         |
|                   |            | c.92C>G, p.Pro31Arg                | rs150336652| 1                  | 8.9 × 10^{-5} | Low complexity region                                                    | —         |
| FHM               | ATP1A2     | c.1262G>A, p.Arg421Gln             | rs139499540| 1                  | 5.5 × 10^{-5} | Between E1-E2 and Cation ATPase domains Reported as VUS for FHM on ClinVar | ncbi.nlm.nih.gov/clinvar/variation/406194/ |
|                   |            | c.2146G>T, p.Val716Leu             | —          | 1                  | —             | Near metal ion binding sites at residues 714 and 718 p.Asp718Asn reported as pathogenic in FHM | 52        |
|                   | CACNA1A    | c.130G>A, p.Ala44Thr               | rs201398669| 1                  | 2.5 × 10^{-5} | Low complexity region                                                    | —         |

Continued
Table 4 Rare variants identified in genes associated with phenotypes related to small vessel disease: COL3A1 (ENST00000304636), APP (ENST00000346798), CST3 (ENST00000376925), ITM2B (ENST00000378565), ATP1A2 (ENST00000361216), and CACNA1A (ENST00000361216), SCN1A (ENST00000375405) (continued)

| Related phenotype | Gene | Variant | dbSNP ID    | No. of individuals | MAF in gnomAD   | Remarks                                                                 | Reference                                                                 |
|-------------------|------|---------|-------------|--------------------|-----------------|-----------------------------------------------------------------------|--------------------------------------------------------------------------|
|                   |      |         |             |                    |                 | Ion transport domain p.Ser211Asn reported as VUS for unspecified phenotype | ncbi.nlm.nih.gov/clinvar/variation/426949/                               |
|                   |      |         |             |                    |                 | Intron variant                                                         |                                                                          |
|                   |      |         |             |                    |                 | Between ion transport domains; p.Ala841Ser reported as VUS for unspecified phenotype | ncbi.nlm.nih.gov/clinvar/variation/385371/                               |
|                   |      |         | rs771423362 | 1                  | 4.1 × 10⁻⁵      | Between ion transport domains; reported as VUS for unspecified phenotype |                                                                           |
|                   |      |         |             |                    |                 | Low complexity region                                                  |                                                                          |
|                   |      |         |             |                    |                 | Low complexity region; p.Arg987Pro reported as VUS for unspecified phenotype | ncbi.nlm.nih.gov/clinvar/variation/387025/                               |
|                   |      |         | rs554091859 | 1                  | 8.6 × 10⁻⁵      | Between ion transport domains; reported as VUS for unspecified phenotype |                                                                           |
|                   |      |         | rs201311000 | 1                  | 3.3 × 10⁻⁵      | Between ion transport domains; Gly1890Cys reported as VUS for unspecified phenotype |                                                                           |
|                   |      |         | rs376365775 | 1                  | 1.6 × 10⁻⁵      | Between ion transport domains; reported as VUS for unspecified phenotype |                                                                           |
|                   |      |         | rs759263620 | 1                  | 4.1 × 10⁻⁵      | Between ion transport domains                                           |                                                                           |
|                   |      |         | rs376815942 | 1                  | 1.2 × 10⁻⁵      | GPHH (voltage-dependent L type calcium channel) domain                 |                                                                           |
|                   |      |         | rs774657158 | 1                  | 8.3 × 10⁻⁵      | Outside of Ca channel IQ domain                                        |                                                                           |
|                   |      |         | rs121908235 | 1                  | 6.2 × 10⁻⁵      | Outside of Ca channel IQ domain VUS for unspecified phenotype and associated with episodic ataxia type 2 (impact uncertain) | ncbi.nlm.nih.gov/clinvar/variation/68440                                |
|                   |      |         |             |                    |                 | Low complexity region                                                  |                                                                          |
|                   |      |         |             |                    |                 | Patient also has a deletion in ABCC6 outside of Ca channel IQ domain   |                                                                           |
|                   |      |         |             |                    |                 | Outside of Ca channel IQ domain                                        |                                                                           |
|                   |      |         |             |                    |                 | Outside of Ca channel IQ domain                                        |                                                                           |
|                   |      |         |             |                    |                 | Outside of Ca channel IQ domain                                        |                                                                           |
|                   |      |         |             |                    |                 | Outside of Ca channel IQ domain                                        |                                                                           |
|                   |      |         |             |                    |                 | Outside of Ca channel IQ domain                                        |                                                                           |

Continued
A number of other monogenic forms of SVD have been reported, and although they appear to be rarer than CADASIL, their frequency in cases of lacunar stroke is unknown. Our study provides data on their prevalence in cases of symptomatic lacunar stroke.

We found no homozygous CARASIL-causing variants, but up to 1.3% of cases had potentially disease-causing heterozygous missense and nonsense variants, suggesting that HTRA1-associated autosomal dominant SVD is the second most common cause of familial SVD. These findings are consistent with previous smaller studies in which about 5% of patients with NOTCH3-negative familial SVD carry missense and nonsense HTRA1 variants.21,33,34 In addition, we identified several rare variants predicted to be damaging in COL4A1, COL4A2, and FOXC1. One individual had a COL4A1 variant (c.1055C>T, p.Pro352Leu), which has been described in an individual with sporadic ICH.24 However, without functional support and evidence of segregation with disease, the pathogenicity of this variant remains uncertain.

We identified a frameshift FOXC1 variant (c.1141_1142insG, p.Ala381_Gly382fs). Given that FOXC1 has 1 exon, the transcript containing this predicted premature stop codon may escape nonsense-mediated decay. Functional studies are required to confirm pathogenicity of this high-impact variant.

Fabry disease has been suggested as an underreported cause of young stroke,35 and has been associated with MRI features of SVD,36 but we found no Fabry variants in this cohort. This is consistent with recent data suggesting that its importance as a cause of early-onset cryptogenic stroke may have been overestimated.37,38

Our results highlight a major challenge in the use of HTS panels in clinical practice; namely, determining whether a variant is pathogenic or benign. In CADASIL, variant interpretation is aided by the knowledge that all known disease-causing variants are cysteine-altering. Assigning pathogenicity of variants in genes such as HTRA1, however, is challenging. Variant interpretation guidelines highlight the use of allele frequency in large public databases. This presents particular challenges in late-onset diseases such as SVD. Databases such as gnomAD,39 which are used to infer population-level frequencies of potentially disease-causing variants, include affected populations from diseases such as dementia, which might include patients with

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**Table 4** Rare variants identified in genes associated with phenotypes related to small vessel disease: COL3A1 (ENST00000304636), APP (ENST00000346798), CST3 (ENST00000376925), ITM2B (ENST00000378565), ATP1A2 (ENST00000361216), and CACNA1A (ENST00000361216), SCN1A (ENST00000375405) (continued)

| Related phenotype | Gene          | Variant                        | dbSNP ID | No. of individuals | MAF in gnomAD | Remarks                                                                 | Reference                                                   |
|-------------------|---------------|--------------------------------|----------|--------------------|---------------|-------------------------------------------------------------------------|-------------------------------------------------------------|
| Collagen type III | COL3A1        | c.7166G>A, p.Arg2389Gln         | —        | 1                  | —             | Outside of Ca channel IQ domain                                         |                                                             |
| Collagen type III | SCN1A         | c.136G>A, p.Glu46Lys            | rs769582667 | 1                  | 3.2 × 10⁻⁵    | Low complexity region                                                   |                                                             |
| Collagen type III | SCN1A         | c.1457C>G, p.Ala486Gly          | rs777120925 | 1                  | 5.8 × 10⁻⁵    | Low complexity region                                                   |                                                             |
| Collagen type III | SCN1A         | c.1738C>G, p.Arg580Gly          | —        | 1                  | —             | Cytoplasmic domain of voltage-gated Na ion channel                      | ncbi.nlm.nih.gov/clinvar/variation/167646/53                |
| Collagen type III | ATP1A2        | c.1843G>A, p.Gly615Arg          | —        | 1                  | 4.1 × 10⁻⁶    | Cytoplasmic domain of voltage-gated Na ion channel                      |                                                             |
| Collagen type III | ATP1A2        | c.3394G>A, p.Glu1152Lys         | —        | 1                  | —             | Sodium ion transport-associated domain                                 |                                                             |
| Collagen type III | ATP1A2        | c.4690C>T, p.Arg1564Cys         | rs121918807 | 1                  | 7.7 × 10⁻⁵    | Ion transport domain                                                    |                                                             |
| Collagen type III | ATP1A2        | c.5750G>A, p.Arg1971His         | —        | 1                  | —             | Outside of ion transport domain                                         |                                                             |

Abbreviations: FHM = familial hemiplegic migraine; gnomAD = genome aggregation database; MAF = minor allele frequency; VUS = variants of unknown significance.

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SVD due to misclassification. Determining whether variants segregate with disease in families is important but often family members are unavailable.

The use of targeted HTS panels presents several advantages. While WGS, whole-exome sequencing (WES), and HTS panels can all provide clinically acceptable coverage, the higher read depth of HTS panels allows for the detection of high-confidence CNVs. HTS panels also allow for savings in terms of cost and analysis time, compared to WGS and WES.

Our study has limitations. Our analyses did not include sequencing a cohort of MRI-phenotyped unaffected individuals. Previous studies have used age- and sex-matched controls unscreened for disease as comparison. However, the value of such controls is questionable. Much of SVD is subclinical prior to cerebrovascular events and ruling out subclinical disease in such controls is not possible.

This study was performed in patients of European ancestry. It would not be possible to extrapolate these results to populations of other ancestries as variant frequencies may vary significantly in different populations. Similar studies should be performed in other populations, particularly as population databases expand to better represent these ethnicities.

Our results demonstrate that monogenic mutations account for about 1.5% of SVD stroke. Furthermore, they demonstrate the utility of targeted HTS platforms in the diagnosis of monogenic forms of SVD. We showed they could detect CADASIL-causing variants, suggesting they could replace commonly used Sanger sequencing techniques, with the added benefit of screening for other genes, such as HTRA1, shown in this study to be the second most common cause of monogenic SVD.

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| Matthew Traylor     | University of Cambridge | Author               | Data analysis, drafting of manuscript            |
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