Rare neurovascular genetic and imaging markers across neurodegenerative diseases

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RESEARCH ARTICLE

Rare neurovascular genetic and imaging markers across neurodegenerative diseases

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Abstract

Introduction: Cerebral small vessel disease (SVD) is common in patients with cognitive impairment and neurodegenerative diseases such as Alzheimer's and Parkinson's. This study investigated the burden of magnetic resonance imaging (MRI)-based markers of SVD in patients with neurodegenerative diseases as a function of rare genetic variant carrier status.

Methods: The Ontario Neurodegenerative Disease Research Initiative study included 520 participants, recruited from 14 tertiary care centers, diagnosed with various neurodegenerative diseases and determined the carrier status of rare non-synonymous variants in five genes (*ABCC6*, *COL4A1/COL4A2*, *NOTCH3/HTRA1*).

Results: *NOTCH3/HTRA1* were found to significantly influence SVD neuroimaging outcomes; however, the mechanisms by which these variants contribute to disease progression or worsen clinical correlates are not yet understood.

Discussion: Further studies are needed to develop genetic and imaging neurovascular markers to enhance our understanding of their potential contribution to neurodegenerative diseases.

KEYWORDS
HTRA1, neurodegeneration, NOTCH3, ONDRI, small vessel disease, WMH

1 | INTRODUCTION

Cerebral small vessel disease (SVD) represents a group of pathologies with heterogeneous etiology that affect the small arteries, veins, and capillaries of the brain's vascular system.[1] Notably, SVD most commonly leads to vascular cognitive impairment and dementia in an aging population. Additionally, SVD is associated with several markers that are quantifiable on structural magnetic resonance imaging (MRI), including white matter hyperintensities (WMH), MRI-visible perivascular spaces (PVS), and lacunes.[2] These observable neuroimaging features have been reported in various neurodegenerative diseases, including Alzheimer's disease (AD), mild cognitive impairment (MCI), amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), and frontotemporal dementia (FTD), as well as in cerebrovascular disease (CVD),[3–10] all of which are represented in the Ontario Neurodegenerative Disease Research Initiative (ONDRI). These diagnoses were selected based on their high prevalence within the aging population as well as the high rates of neuropsychological dysfunction reported in these patient populations. As the cases of dementia increase worldwide, one of the key objectives of ONDRI is to accurately identify the individuals at risk of developing dementia, with and without comorbidities with other neurodegenerative and/or neurovascular disorders, at an earlier stage. Therefore, these diagnoses were selected to align with this objective.

Previously, several rare nonsynonymous variants had been associated with vascular neuroimaging markers in various neurodegenerative diseases via linkage analyses and candidate gene sequencing of patient cohorts.[11–17] Specifically, five genes that were previously associated with SVD and vasculopathy include ATP Binding Cassette Subfamily C Member 6 (*ABCC6*), Collagen Type IV Alpha-I (*COL4A1*), Collagen Type IV Alpha-II (*COL4A2*), High-Temperature Requirement A Serine Peptidase 1 (*HTRA1*), and Notch Receptor 3 (*NOTCH3*). Under normal conditions, *ABCC6* encodes a protein that aids in the transportation of molecules across the cell membrane; however, when mutated, it can cause arterial calcification and pseudoxanthoma elasticum, an autosomal recessive disease primarily affecting the connective tissue in the skin, retina, and cardiovascular system, and more recently the gene has been associated with increased ischemic strokes.[18,19] *COL4A1* and *COL4A2* encode for collagen chain proteins that combine and together play a critical role in supporting the basement membranes of human vasculature.[20] Autosomal dominant *COL4A1* mutations cause *COL4A1*-related SVD, which presents as early strokes and brain cysts,[21] whereas autosomal dominant *COL4A2* mutations cause intracerebral hemorrhage and SVD, which result in neural degeneration.[22] *NOTCH3* encodes a transmembrane protein receptor critical for the survival and function of vascular smooth muscle cells; however, when mutated it is associated with a monogenic form of SVD commonly known as cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL).[17] Similarly, *HTRA1* encodes a protein involved with cell signaling, musculoskeletal development, vascular maturation, and protein degradation; however, when mutated it is associated with a phenotype similar to CADASIL, known as cerebral autosomal *recessive* arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL).[23] Although the compendium of SVD-associated genes continues to grow, these five largely remain a consensus of the most well-established genes of interest.[24]

RESEARCH IN CONTEXT

1. **Systematic Review**: Burden of white matter hyperintensities, perivascular spaces, and lacunes were quantified in a study examining 520 participants diagnosed with Alzheimer's disease, mild cognitive impairment, amyotrophic lateral sclerosis, Parkinson's disease, frontotemporal dementia, or cerebrovascular disease. Carrier status of rare non-synonymous variants in *ABCC6*, *COL4A1*, *COL4A2*, *HTRA1*, and *NOTCH3* was determined for each study participant, and multiple regression models were used to estimate effects across the neuroimaging markers as a function of variant carrier status.
2. **Interpretation**: We observed a significant influence of rare variants in *HTRA1* and *NOTCH3* on neuroimaging markers of cerebral small vessel disease in a variety of neurodegenerative disease patient cohorts.
3. **Future directions**: Our study highlights the importance of genetic analysis in understanding the development of vascular injury and the potential contribution to neurodegenerative disease, yet future work must determine the biological mechanisms of these associations and their influence on downstream clinical outcomes.

The primary aim of this study was to examine the burden of MRI-based SVD markers (WMH, PVS, and lacunes) as a function of carrier status of non-synonymous, rare genetic variants of interest in a sample of patients with neurodegenerative diseases that are represented in the ONDRI study. In light of these previous findings in genetics and neuroimaging, we hypothesized that carrying rare genetic variation in SVD-associated genes described would contribute to greater burdens of SVD markers in both neurodegenerative disease and CVD patients.

2 | METHODS

2.1 | Participants

ONDRI recruited 520 participants from 14 tertiary care centers across Ontario, Canada, each of whom had passed preliminary screening. Every participant was previously diagnosed with one of five neurodegenerative diseases: (1) AD; (2) amnestic MCI; (3) ALS; (4) FTD; (5) Parkinson's disease (PD); or (6) cerebrovascular disease (CVD) with or without cognitive impairment. Detailed inclusion/exclusion criteria, demographics, and cohort characteristics of the ONDRI study were published previously.[25,26] Briefly, the prevailing consensus-based

clinical diagnostic criteria at the time of enrollment for each ONDRI-focused disease were implemented at each tertiary clinic: AD/MCI patients met National Institute on Aging–Alzheimer's Association criteria for probable or possible AD or MCI, where amnestic MCI patients were tracked annually to ensure accurate characterization of the cohort[25]; PD patients met the criteria for idiopathic PD defined by the UK's Parkinson's Disease Society Brain Bank clinical diagnostic criteria; ALS patients met El Escorial World Federation of Neurology diagnostic criteria for possible, probable, or definite familial or sporadic ALS; FTD patients included possible or probable behavioral variants of frontotemporal degeneration, agrammatic/non-fluent and semantic variants of primary progressive aphasia, and possible or probable progressive supranuclear palsy. The CVD cohort comprised of individuals who experienced mild to moderate acute ischemic stroke, transient ischemic attack, or subcortical infarction, at least 3 months prior to enrollment, in compliance with the National Institute of Neurological Disorders and Stroke and the Canadian Stroke Network vascular cognitive impairment harmonization standards. Evidence of stroke required clinical imaging confirmation; however, individuals with large vessel occlusive infarction causing severe neurological deficits were excluded. Additionally, the AD/MCI patients' MRI scans were assessed by a neuroradiologist to exclude individuals with non-AD-related causes for cognitive impairment. Ethics approval was obtained from the Research Ethics Board at each participating site. All participants provided written, informed consent.

2.2 | Gene sequencing and variant prioritization

Of the 520 participants recruited by ONDRI, 519 had a blood sample collected, and genomic DNA was extracted from them as previously described.[27] All participant DNA samples were then sequenced with the custom-designed next-generation sequencing (NGS) gene panel, ONDRISeq, which covers the exonic regions of 80 genes, each previously associated with a neurodegenerative disease or SVD phenotype.[28] Briefly, all genomic DNA samples were sequenced using ONDRISeq on the Illumina MiSeq Personal Genome Sequencer (Illumina, San Diego, CA, USA). A custom bioinformatics pipeline was subsequently used to process the FASTQ files and produce a variant calling format (VCF) file and binary alignment map (BAM) file for each participant.[27,29]

Generated VCF files for each ONDRI participant were annotated using VarSeq® (Golden Helix, Bozeman, MT, USA). Annotations included sequence ontologies, minor allele frequencies (MAFs) from the Genome Aggregation Database (gnomAD; version 3.1), in silico prediction scores from Combined Annotation Dependent Depletion (CADD, version 1.3), and ClinVar pathogenicity classifications. The variant data were then filtered to include only those within genes on ONDRISeq that had been previously associated with SVD or neurovasculopathy, namely, *ABCC6*, *COL4A1*, *COL4A2*, *HTRA1*, *NOTCH3*, *SAMHD1*, and *TREX1*. Of the variants in these genes, we identified those considered non-synonymous, including missense variants, frameshift insertions or deletions, non-frameshift insertions or deletions, nonsense variants, and splicing variants. Variants were further prioritized to identify those most likely to be deleterious, first by identifying those considered rare in the general population (MAF < 0.01, gnomAD version 3.1), then by selecting variants with a CADD score $\geq$ 20 (top 1% of deleterious variants in the human genome)[30] or variants that had been classified as pathogenic or likely pathogenic in ClinVar. All prioritized variants are hereafter referred to as "non-synonymous, rare variants of interest." The genes *SAMHD1* and *TREX1* were eliminated from further analysis, as no non-synonymous, rare variants of interest were identified in *SAMHD1* and only two non-synonymous, rare variants of interest were identified in *TREX1*, each carried by only a single ONDRI participant.

Based on the similar molecular pathways and disease associations of the genes under study and to preserve statistical power due to our modest sample size, rare, non-synonymous variants of interest identified in *COL4A1* or *COL4A2* were binned, as were variants of interest identified in *NOTCH3* or *HTRA1*. Participants carrying a non-synonymous, rare variant of interest in at least one of *ABCC6*, *COL4A1/COL4A2*, or *NOTCH3/HTRA1* were considered variant positive, while those not carrying a variant in at least one a gene bin were considered variant negative.

All ONDRI participants were also assessed for the *APOE* genotype using the ONDRISeq data by extracting calls for the *APOE* variants rs429358(CT):p.Cys130Arg and rs7412(CT):p.Arg176Cys and mapping to the respective genotype, as previously described.[31] Any participant carrying at least one copy of either the $\varepsilon 2$ or $\varepsilon 4$ genotype were considered *APOE* variant positive, as both variants have been previously shown to increase the presence of SVD markers, including WMH burden.[32–34]

2.3 | Neuroimaging

Of the 520 ONDRI study participants, 513 had usable 3 Tesla MRI that included the following sequences: three-dimensional (3D) T1-weighted (T1), interleaved proton density and T2-weighted (T2), and T2-fluid attenuated inversion recovery (FLAIR). Imaging protocol details were published previously.[35] All MRI images were evaluated to ensure excellent imaging quality by a medical biophysicist (R.B.) and for clinical incidental findings by a licensed neuroradiologist (S.S.). Neuroimaging-based markers for cerebral SVD (WMH, PVS, lacunes) were quantified using ONDRI's standardized image-processing pipeline[36] in compliance with the STRIVE criteria.[2] Following initial segmentation, PVS and lacunar counts were generated using a 3D, six-connected voxel connectivity contour segmentation algorithm.[37]

2.4 | Statistical analyses

Of the 513 ONDRI participants with usable 3 Tesla MRI and 519 ONDRI participants that underwent genetic testing, 512 had both imaging and genetics data available. However, two participants' imaging segmentation failed quality control of the aforementioned

TABLE 1 Participants in ONDRI carrying non-synonymous, rare variants of interest in CVD-associated genes.

Cohort	Sample size	*ABCC6* variant positive	*COL4A1/COL4A2* variant positive	*HTRA1/NOTCH3* variant positive
ONDRI	510	27 (5.3%)	28 (5.5%)	42 (8.2%)
AD/MCI	126	5 (4.0%)	8 (6.3%)	8 (6.3%)
ALS	38	1 (2.6%)	2 (5.3%)	3 (7.9%)
FTD	52	1 (1.9%)	3 (5.8%)	3 (5.8%)
PD	139	6 (4.3%)	10 (7.2%)	15 (10.8%)
CVD	155	14 (9.0%)	5 (3.2%)	13 (7.9%)

Abbreviations: AD/MCI, Alzheimer's disease/mild cognitive impairment; ALS, amyotrophic lateral sclerosis; CVD, cerebrovascular disease with or without cognitive impairment; FTD, frontotemporal dementia; ONDRI, Ontario Neurodegenerative Disease Research Initiative; PD, Parkinson's disease.

image-processing pipeline, resulting in a final ONDRI total cohort size of 510 used in our analyses.

Multiple regression models were used to estimate effects across the three neuroimaging-based markers of SVD (PVS, lacunes, and WMH) as a function of carrier status of rare, non-synonymous variants of interest in each of the gene bins (*ABCC6*, *COL4A1/COL4A2*, and *HTRA1/NOTCH3*) and of disease cohort (AD/MCI, ALS, FTD, PD, CVD), while also accounting for interactions between the gene bins and disease cohort.

The individual coefficient estimates were used to determine the extent to which predictor variables contributed to each neuroimaging metric. We applied negative binomial generalized linear models for the outcome variables that were counts, namely, PVS counts and lacune counts, whereas we applied Gamma log distribution generalized linear models for the continuous variable of WMH volume. All multiple regression models were adjusted for age, sex, supratentorial total intracranial volume (head size), smoking history, and *APOE* carrier status.

To maximize statistical power based on our modest sample sizes, participants diagnosed with AD and participants diagnosed with MCI were binned into a single AD/MCI cohort. Participant disease cohorts were transformed using a weighted effect coding with the wec R package version 0.4-1,[38] which adjusts the point of reference for the dataset to be the sample mean of all participants, regardless of cohort. However, one cohort needed to be excluded from the model coding to avoid statistical redundancy. As the ALS cohort was reported to have the least amount of SVD pathology anecdotally, it was chosen as the remainder cohort. The model was rerun using FTD as the omitted cohort to obtain coefficient estimates for the ALS cohort that could be included in the visualizations of the results.[39]

To adjust for multiple comparisons, corrected *p* values were calculated using the Benjamini–Hochberg false discovery rate (FDR) method.[40] Significance for the multiple regression models was then measured at an alpha level of 0.05.

Statistical estimates were calculated using R statistical software version 3.6.0[41] in R Studio 1.1.463, and data visualization was performed using the ggplot2 R package version 3.3.s.[42]

3 | RESULTS

3.1 | Study participants and variant prioritization

In total, after quality control evaluation, genomic and neuroimaging data were available for 510 ONDRI participants (AD/MCI = 126, ALS = 38, FTD = 52, PD = 139, CVD = 155), of which 104 carried a non-synonymous, rare variant of interest in at least one of the genes: *ABCC6*, *COL4A1*, *COL4A2*, *HTRA2*, *NOTCH3* (Table 1). Of the genes examined in this study, 14 unique variants were found in *ABCC6*, 11 unique variants were found in *COL4A1*, nine unique variants were found in *COL4A2*, six unique variants were found in *HTRA1*, and 21 unique variants were found in *NOTCH3* (Supplemental Table S1). All variants identified were of heterozygous zygosity. *HTRA1/NOTCH3* non-synonymous, rare variants of interest were found at the greatest frequency in the full ONDRI cohort, with the highest proportion of carriers in the PD cohort. Four participants were found to carry rare variants in multiple gene groupings, including two participants diagnosed with CVD carrying rare variants in *ABCC6* and *NOTCH3*, one participant diagnosed with PD carrying rare variants in *ABCC6* and *HTRA1*, and one participant diagnosed with FTD carrying rare variants in *COL4A2* and *NOTCH3*. Demographic and whole-brain volumetric comparisons of the ONDRI participants carrying a non-synonymous, rare variant of interest in a SVD-associated gene to non-carriers are displayed in Table 2.

Using multiple regression models, we assessed the contribution of variant carrier status in *ABCC6*, *COL4A1/COL4A2*, or *NOTCH3/HTRA1*; disease cohort; and the two predictor variables to the neuroimaging volumetrics PVS count, lacune count, and WMH volume. Including an interaction term between disease cohort and variant carrier status within the model allowed us to determine the influence of carrying a non-synonymous, rare variant of interest on participant neuroimaging volumetrics in each individual ONDRI disease cohort. Supplementary Figure S1 displays the magnitude of difference in the neuroimaging volumetrics of participants carrying non-synonymous, rare variants of interest in *ABCC6*, *COL4A1/COL4A2*, and *NOTCH3/HTRA1* in comparison to non-carriers across the full ONDRI cohort, as well as in each

TABLE 2 Demographics and whole-brain volumetrics from magnetic resonance imaging comparison of ONDRI participants carrying and not carrying non-synonymous, rare variants of interest in a CVD-associated gene.

Descriptor	Total cohort	Variant carriers	Variant non-carriers	Effect size
Sample size	510	95	415	
Demographics				
Sex (male:female)	341:169	61:34	280:135	2.7e-2
Age (yr)	68.6 (7.7)	69.0 (7.2)	68.6 (7.9)	4.0e-4
Education (yr)	14.9 (2.9)	15.0 (2.8)	14.9 (3.0)	4.0e-4
MoCA	24.4 (3.4)	24.9 (3.2)	24.3 (3.4)	4.1e-3
Modified Rankin score	1.4 (0.9)	1.3 (0.8)	1.5 (0.9)	4.9e-3
Whole-brain volumetrics				
Supratentorial intracranial volume (cc)	1.3e6 (1.4e5)	1.2e6 (1.5e5)	1.3e6 (1.4e5)	3.0e-4
Normal-appearing white matter (cc)	4.1e5 (6.6e4)	4.0e5 (6.7e4)	4.1e5 (6.6e4)	2.2e-3
Normal-appearing gray matter (cc)	5.5e5 (5.5e4)	5.4e5 (6.0e4)	5.5e5 (5.4e4)	2.0e-4
Sulcal cerebrospinal fluid (cc)	2.5e5 (6.0e4)	2.5e5 (6.3e4)	2.5e5 (6.0e4)	2.0e-5
Ventricular cerebrospinal fluid (cc)	4.1e4 (2.3e4)	4.0e4 (2.2e4)	4.1e4 (2.3e4)	0.0
Periventricular WMH (mm^3)	5.6e3 (8.4e3)	7.5e3 (1.1e4)	5.2e3 (7.5e3)	1.2e-2
Deep WMH (mm^3)	6.5e2 (9.8e2)	7.3e2 (1.0e3)	6.3e2 (9.7e2)	1.4e-3
***APOE* ε2 or ε4 carriers**	222	44	178	2.7e-2

Note: Values in table represent mean (standard deviation). Effect sizes were calculated using eta squared of an ANOVA model. A large effect size is defined as >0.8, whereas a small effect size is defined as <0.2. All effect sizes reported were considered small.
Abbreviations: *APOE*, apolipoprotein E; MoCA, Montreal Cognitive Assessment; WMH, white matter hyperintensities; yr, years.

individual disease cohort. To best illustrate these comparisons, disease effects were removed.

3.2 | Associations between *ABCC6*, *COL4A1*, and *COL4A2* and SVD burden

Carrying a non-synonymous, rare variant of interest in *ABCC6* or *COL4A1/COL4A2* was not found to significantly influence ONDRI participants' PVS counts, lacune counts, or WMH volume across the full ONDRI cohort (Supplemental Tables S2 and S3). Similarly, carrier statuses of rare variants of interest in *ABCC6* or *COL4A1/COL4A2* were not significantly associated with SVD markers in either the individual neurodegenerative disease cohorts or the CVD cohort.

3.3 | Associations between *HTRA1* or *NOTCH3* and SVD burden

In contrast, carrying a non-synonymous, rare variant of interest in *NOTCH3/HTRA1* was found to significantly influence brain imaging outcomes both across the full ONDRI cohort and within specific diagnostic cohorts (Table 3). More specifically, carriers of non-synonymous, rare variants of interest in *NOTCH3/HTRA1* displayed significantly higher WMH volumes, agnostic of participant diagnosis ($p = 4.92e\text{-}03$, FDR: $p = 1.82e\text{-}02$; Figure 1A). Further, in participants diagnosed with CVD, carrying a rare variant of interest in *NOTCH3/HTRA1* was associated with significantly increased WMH volume, although the result was no longer significant following multiple testing corrections ($p = 4.94e\text{-}02$, FDR: $p = 1.30e\text{-}01$; Figure 1B). No associations of interest were observed between *NOTCH3/HTRA1* variant status and WMH volume in any of the individual neurodegenerative disease cohorts.

Interestingly, the directions of association of non-synonymous, rare variant of interest carrier status in *NOTCH3/HTRA1* were not uniform across the disease cohorts with respect to lacune count and PVS count. Although variant status was not associated with lacune counts or PVS counts agnostic of disease cohort compared to variant non-carriers (Figures 2A and 3A, respectively), variant carriers diagnosed with CVD displayed a significantly greater number of lacunes ($p = 9.93e\text{-}03$, FDR: $p = 3.29e\text{-}02$; Figure 2B) and PVS ($p = 5.93e\text{-}04$, FDR: $p = 2.77e\text{-}03$; Figure 3B). Similarly, *NOTCH3/HTRA1* variant carriers diagnosed with AD/MCI displayed significantly greater number of lacunes than non-carriers, but the result was not significant following multiple testing corrections ($p = 4.35e\text{-}02$, FDR: $p = 1.25e\text{-}01$; Figure 2B). In contrast, *NOTCH3/HTRA1* variant carriers diagnosed with PD displayed significantly fewer lacunes than non-carriers ($p = 1.38e\text{-}02$, FDR: $p = 4.45e\text{-}02$; Figure 2B), while carriers diagnosed with FTD displayed significantly fewer PVS, but again this result was not significant following multiple testing corrections ($p = 2.35e\text{-}02$, FDR: $p = 7.04e\text{-}02$; Figure 3B). No additional significant associations were identified between non-synonymous, rare variant of interest carrier status in *NOTCH3/HTRA1* and brain volumetric outcomes in any of the individual disease cohorts.

TABLE 3 Results of multiple regression analyses used to analyze the contribution of variant carrier status in *NOTCH3/HTRA1* and disease cohort to neuroimaging metrics of PVS count, lacune count, and WMH volume.

	Lacune count				PVS count				WMH volume			
	Estimate	Standard deviation	*P* value	FDR *p* value	Estimate	Standard deviation	*P* value	FDR *p* value	Estimate	Standard deviation	*P* value	FDR *p* value
Intercept	**−3.9**	**0.9**	**8.5e-06**	**7.7e-05**	6.6e-02	0.5	8.9e-01	9.6e-01	**1.6**	**0.6**	**4.9e-03**	**1.8e-02**
NOTCH3/HTRA1 Pos	−4.1e-02	0.3	8.9e-01	9.6e-01	0.2	0.1	9.3e-02	2.1e-01	**0.5**	**0.2**	**4.3e-04**	**2.1e-03**
AD/MCI	−0.2	0.1	1.5e-01	3.2e-01	9.3e-02	6.7e-02	1.7e-01	3.5e-01	**-0.3**	**8.3e-02**	**1.2e-04**	**8.2e-04**
NOTCH3/HTRA1 Pos × AD/MCI	**1.0**	**0.5**	**4.4e-02**	1.3e-01	−0.3	0.3	3.2e-01	5.4e-01	−8.6e-02	0.3	8.0e-01	8.9e-01
CVD	**0.3**	**0.1**	**6.4e-03**	**2.4e-02**	**0.2**	**5.8e-02**	**4.4e-03**	**1.7e-02**	**0.6**	**7.2e-02**	**4.5e-14**	**9.4e-13**
NOTCH3/HTRA1 Pos × CVD	**1.0**	**0.4**	**9.9e-03**	**3.3e-02**	**0.7**	**0.2**	**5.9e-04**	**2.8e-03**	**0.5**	**0.2**	**4.9e-02**	1.3e-01
FTD	−8.6e-02	0.2	6.9e-01	8.4e-01	−3.1e-02	0.1	7.8e-01	9.1e-01	−0.1	0.1	4.3e-01	6.2e-01
NOTCH3/HTRA1 Pos × FTD	−0.9	1.2	4.5e-01	6.3e-01	**−1.2**	**0.5**	**2.4e-02**	7.0e-02	−0.6	0.6	3.2e-01	5.2e-01
PD	**−0.4**	**0.1**	**4.3e-03**	**1.7e-02**	−0.1	6.6e-02	9.4e-02	2.1e-01	**−0.2**	**8.1e-02**	**1.3e-02**	**4.0e-02**
NOTCH3/HTRA1 Pos × PD	**−1.1**	**0.4**	**1.4e-02**	**4.5e-02**	−0.2	0.2	3.0e-01	5.2e-01	0.1	0.2	6.1e-01	8.0e-01
ALS	0.9	0.2	-	-	−0.5	0.1	-	-	−0.3	0.2	-	-
NOTCH3/HTRA1 Pos × ALS	−0.5	0.9	-	-	−6.9e-02	0.5	-	-	−0.7	0.6	-	-

Note: Participant disease cohorts were transformed using weighted effect coding using the ALS cohort as the cohort that was not coded in the model. The model was rerun using the FTD cohort as the excluded model to obtain coefficient estimates for the ALS cohort, as displayed below the line. For the outcome variables that were counts, namely PVS counts and lacune counts, negative binomial generalized linear models were applied. For the continuous variable of WMH volume, Gamma log distribution generalized linear models were applied.

Significance for the multiple regression models was measured at an alpha level of 0.05, and significant results are indicated in bold.

Abbreviations: AD/MCI, Alzheimer's disease and mild cognitive impairment; ALS, amyotrophic lateral sclerosis; CVD, cerebrovascular disease with or without cognitive impairment; Dev., deviation; FTD, frontotemporal dementia; PD, Parkinson's disease; Pos, variant positive; PVS, MRI-visible perivascular spaces; WMH, white matter hyperintensities.

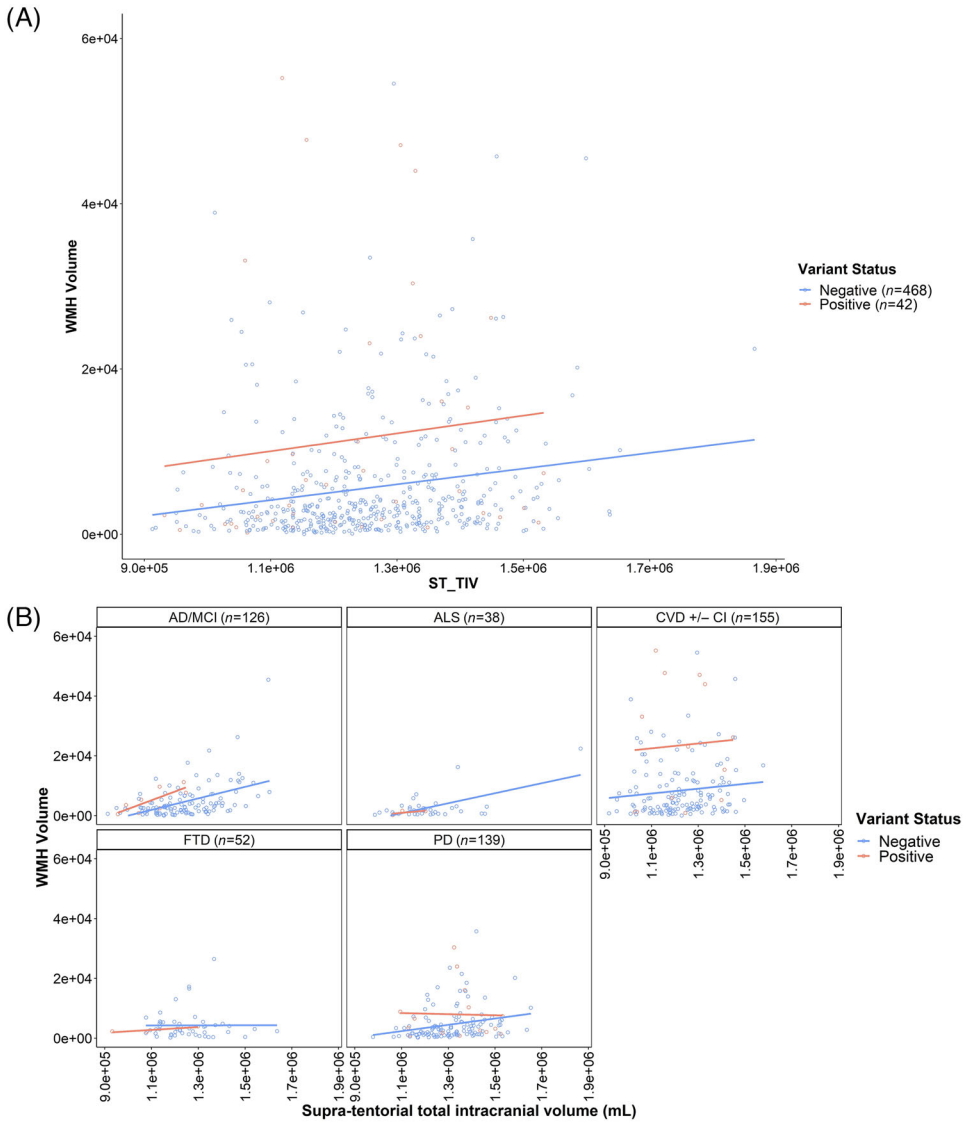

FIGURE 1 White matter hyperintensity (WMH) volume in the ONDRI participants carrying *NOTCH3*/*HTRA1* rare, non-synonymous variants compared to variant negative participants. (A) WMH volumes compared to head size volume in ONDRI participants that carried a rare, non-synonymous variant in either *NOTCH3* or *HTRA1* and those that did not. (B) WMH volumes compared to head size volume of participants within each disease cohort of ONDRI that carried a rare, non-synonymous variant in either *NOTCH3* or *HTRA1* compared to those that did not. *P* values are presented for the interaction term of disease status and variant status from the multivariate regression analysis that modeled WMH volume as a function *NOTCH3* or *HTRA1* variant carrier status, disease cohort, and interactions between the two predictor variables, as well as adjustment for head size, age, smoking history, and *APOE* ε2 or ε4 variant status. To avoid statistical redundancy, the ALS cohort was not coded in the regression model. ST_TIV, supratentorial total intracranial volume.

4 | DISCUSSION

Here we investigated the burden of MRI-based SVD markers, including lacunes, PVS, and WMH, in neurodegenerative disease patients carrying non-synonymous, rare variants in genes previously associated with cerebrovascular neuroimaging features – *ABCC6*, *COL4A1*, *COL4A2*, *HTRA1*, and *NOTCH3*. It is well established that neurodegenerative diseases, which are characterized by neuronal cell loss, display strong genetic components likely driving disease pathology.[43,44] However, in recent years it has become increasingly more accepted that the course and manifestation of neurodegenerative diseases are also influenced by concurrent/comorbid cerebrovascular injury. In some cases, SVD may drive a neurodegenerative phenotype, as is the case with vascular dementia, whereas in other cases SVD can exist as a concurrent pathology or act as one of many contributors to disease presentation, such as in AD and PD.[45–47] In either scenario, it must be recognized that there may be unique genetic factors that can drive SVD markers, which therefore may also have relevance in neurodegenerative disease risk or features.

We determined that across all neurodegenerative disease and CVD patients, non-synonymous, rare variant carrier status in *ABCC6* or *COL4A1*/*COL4A2* did not significantly influence lacune count, PVS

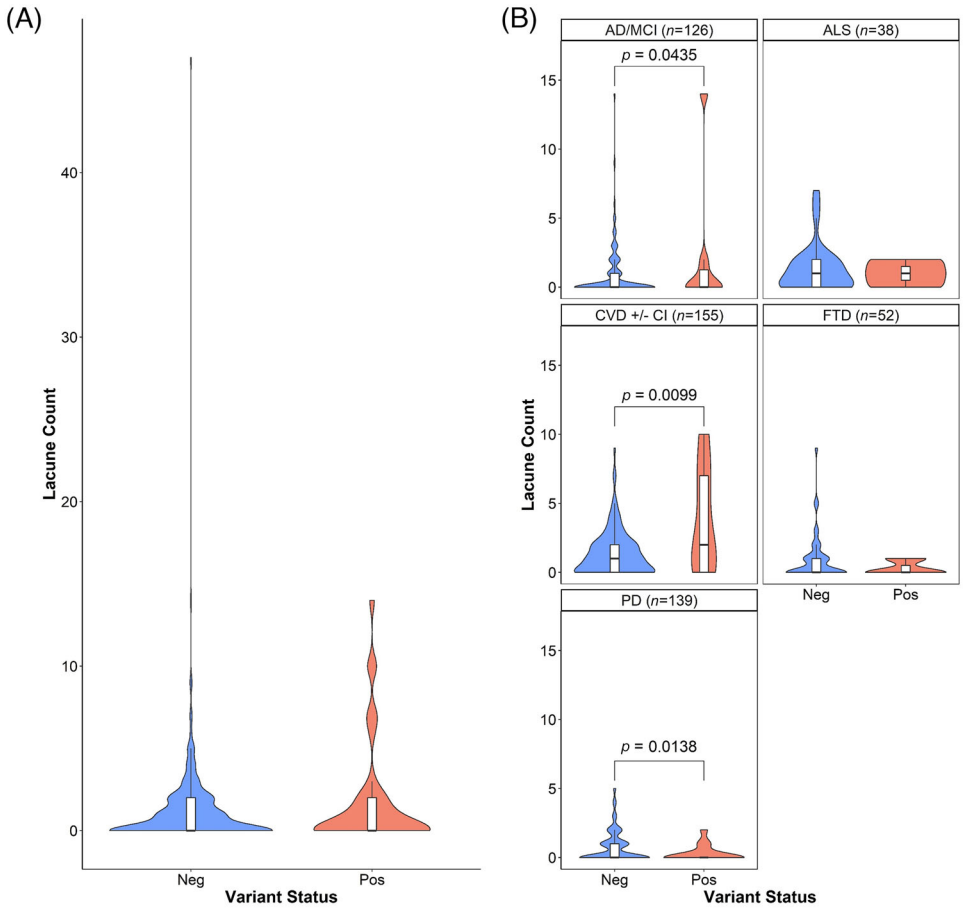

FIGURE 2 Lacune counts in ONDRI participants carrying *NOTCH3/HTRA1* rare, non-synonymous variants compared to variant negative participants. (A) Distribution of lacune counts in ONDRI participants that carried a rare, non-synonymous variant in either *NOTCH3* or *HTRA1* compared to those that did not. (B) Distribution of lacune counts in each of the participants within each disease cohort of ONDRI that carried a rare, non-synonymous variant in either *NOTCH3* or *HTRA1* compared to those that did not. *P* values are presented for the interaction term of disease status and variant status from the multivariate regression analysis that modeled lacune count as a function *NOTCH3* or *HTRA1* variant carrier status, disease cohort, and interactions between the two predictor variables, as well as adjustment for head size, age, smoking history, and *APOE* ε2 or ε4 variant status. To avoid statistical redundancy, the ALS cohort was not coded in the regression model. Indicated *P* values are uncorrected for multiple testing. Neg, variant negative; Pos, variant positive.

count, or WMH volume. In contrast, across all neurodegenerative diseases, non-synonymous, rare variant carrier status in *NOTCH3/HTRA1* was associated with higher WMH volumes. Yet the influence of variants in these genes had varying effects across the neurodegenerative diagnoses with respect to lacune and PVS counts. While carriers of *NOTCH3/HTRA1* variants diagnosed with CVD displayed a greater number of lacunes and PVS than non-carriers, carriers diagnosed with PD displayed significantly fewer lacunes than non-carriers, which is discussed further in what follows. Altogether, our results suggest that genetic influence on SVD markers in neurodegenerative disease patients is not only gene-specific but potentially disease-specific as well.

In our neurodegenerative disease cohort, rare genetic variants in *HTRA1* or *NOTCH3* exerted the greatest influence on markers of SVD. As previously described, heterozygous pathogenic variants in *NOTCH3* are causative for CADASIL, while homozygous pathogenic variants in *HTRA1* are causative for CARASIL, with both phenotypes sharing similar features, such as recurrent ischemic attacks, migraines with aura, cognitive decline and dementia, and hallmark WMH.[17,16,48] Within our cohort, there were more rare, non-synonymous variants reported in *NOTCH3* than in *HTRA1* ($n = 21$ and $n = 6$, respectively), and indeed, all *HTRA1* variants reported were in the heterozygous state. Yet, heterozygous variants in *HTRA1* had been previously associated with cerebral SVD but of lesser severity than CARASIL, accounting for their inclusion in our analyses.[49–51] Similarly, many of the variants in *NOTCH3* that we identified were not considered typical of CADASIL pathogenicity, as they were not cysteine-modifying or located within an epidermal growth factor-like repeat; however, previous studies suggested that these atypical *NOTCH3* variants might contribute to a CADASIL phenotype of milder severity or to more generalized SVD.[52–54] Therefore, while we are not suggesting that the *HTRA1* or *NOTCH3* rare variants identified herein are causing CARASIL or CADASIL, respectively, as confirmed by clinical assessment of our study patients, our results do suggest that the

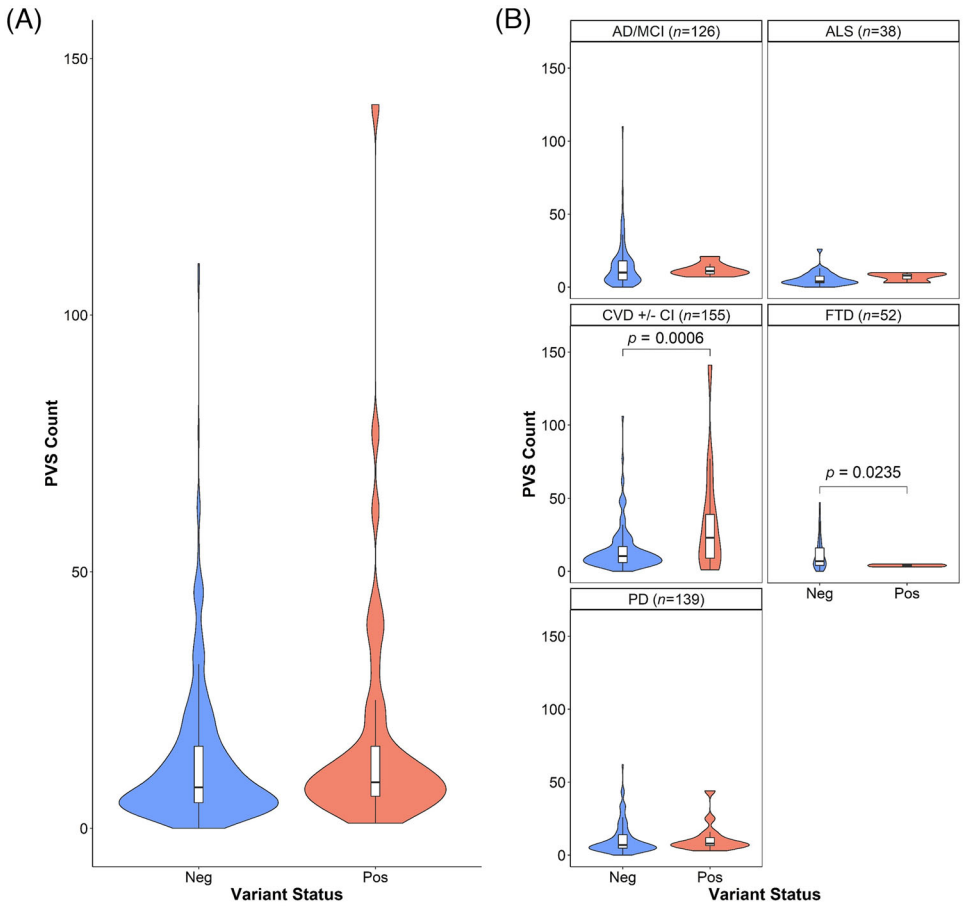

FIGURE 3 Enlarged PVS counts in ONDRI participants carrying *NOTCH3/HTRA1* rare, non-synonymous variants compared to variant negative participants. (A) Distribution of PVS counts in ONDRI participants that carried a rare, non-synonymous variant in either *NOTCH3* or *HTRA1* compared to those that did not. (B) Distribution of PVS counts in each of the participants within each disease cohort of ONDRI that carried a rare, non-synonymous variant in either *NOTCH3* or *HTRA1* compared to those that did not. *P* values are presented for the interaction term of disease status and variant status from the multivariate regression analysis that modeled PVS count as a function *NOTCH3* or *HTRA1* variant carrier status, disease cohort, and interactions between the two predictor variables, as well as adjustment for head size, age, smoking history, and *APOE* ε2 or ε4 variant status. To avoid statistical redundancy, the ALS cohort was not coded in the regression model. Indicated *P* values are uncorrected for multiple testing. Neg, variant negative; Pos, variant positive.

identified non-synonymous, rare variants identified may contribute to cerebrovascular injury in subjects, potentially acting as a contributing factor to neurodegenerative disease risk or modifying clinical presentation and progression.

Specifically, we observed an association between *NOTCH3/HTRA1* non-synonymous, rare variant carrier status and greater WMH volume across the entire neurodegenerative disease cohort. The association was unsurprising based on the striking hallmark of WMH in both CADASIL and CARASIL. Additionally, a recent analysis of >200,000 participants who self-reported as healthy from the UK Biobank identified a significant association between *NOTCH3* genetic variation and increased WMH volume, as well as ultrastructural damage to white matter.[55] Notably, an association was also observed between *HTRA1* or *NOTCH3* variants and increased WMH volume in the CVD cohort; however, this result did not pass multiple testing corrections, and therefore, we believe it is unlikely for it to fully account for the association observed across the entire cohort and that the variation is still influencing SVD markers across the neurodegenerative diseases as well. What remains to be determined is how the influence of *HTRA1* or *NOTCH3* variants on WMH volume may contribute to neurodegenerative disease risk, presentation, and progression, particularly due to the complex relationship between cerebrovascular injury and neurodegeneration.

In contrast to the WMH analysis, *NOTCH3/HTRA1* variant carrier status had variable effects on lacune and PVS count across the neurodegenerative disease cohorts. Interestingly, only the CVD cohort displayed an association between genetic variation in *NOTCH3/HTRA1* and PVS count, demonstrating a significant positive association, suggesting that the genes have an influence on PVS only in individuals with a classical CVD phenotype.

As described, the PD cohort displayed the highest proportion of *HTRA1/NOTCH3* variant carriers. In fact, a previous gene-based rare variant association analysis by our group identified a significant enrichment of *NOTCH3* rare variants in the PD cohort,[29] which led to an initial

investigation of the contribution of these variants to SVD features in only the PD patients.[56] Using a Bayesian approach, we identified a doubling of WMH volume in PD patients carrying *NOTCH3* variants compared to variant negative patients. Additionally, *NOTCH3* rare variation was associated with significantly increased lacune *volumes*. Yet, we did not observe an association between *HTRA1/NOTCH3* variant status and lacune *count* in the PD patients in the current analysis that passed multiple testing corrections. These seemingly contradictory findings highlight a current gap in the clinical-scientific literature regarding the wide variation in definition of lacunes and the differences between lacunar *counts* versus *volumes*, size criterion, etiology of infarction, and future cavitation.[57,58] Given these issues and the difficulty of assessing the precise etiological origins (eg, lipohyalinosis vs ostium/branch disease), the ONDRI study's neuroimaging group implemented a multimodal imaging-based criterion for the segmentation of subcortical lacunar infarcts, defined as regions that are hypointense on T1, hyperintense on T2, and appearing with a hypointense central core with a hyperintense rim on T2 FLAIR.[36] They are differentiated from PVS, which are isointense to gray matter on proton density, tend to be smaller in diameter, and are more linear depending on the orientation of the vasculature.[2] Together with our previous results, this suggests that, although the variant carriers do not have a greater number of lacune *counts* than non-carriers, the lacunar *volumes* that are present in the carriers tend to be much more substantial in size, potentially resulting in greater clinical consequences.

Rare genetic variants in *ABCC6*, *COL4A1*, and *COL4A2* did not account for SVD markers in neurodegenerative disease patients in our study. Of note, only heterozygous variants were observed in the studied genes across our participants. Typically, a homozygous variant is required to cause the phenotype associated with *ABCC6*, namely, pseudoxanthoma elasticum, although there have been reports of heterozygous variants in the gene causing a milder vascular disease.[59,60] Yet these cases are rather uncommon, and, due to both the subtlety of the influence of heterozygous *ABCC6* variation and our modest sample sizes, we might not have been able to detect their phenotypic influence. Further, pathogenic variants within *COL4A1* and *COL4A2* are most associated with hemorrhagic stroke[61–64]; however, individuals in the ONDRI cohort were documented to have experienced mild to moderate ischemic stroke, and those with hemorrhagic stroke were excluded. Again, this likely limited the number of variant carriers within our study and, therefore, the ability to detect associations between the collagen chain genes and markers of SVD.

In addition to the limitations imposed by our modest sample sizes described earlier, which might have increased the incidence of results that did not survive multiple testing corrections, the presented work was also inherently limited by focusing upon rare variation, which naturally resulted in fewer variant positive patients to analyze. However, it is generally accepted that variants that are less common in the general population will have a greater likelihood of higher phenotypic effects. Further, our study lacked a normative sample to determine whether the observed associations were specific to neurodegenerative and CVD cohorts, or whether similar results would have been observed in a healthy elderly population. Finally, we did not further analyze the effect of the SVD-associated genes on clinical outcomes or co-morbid vascular risk, again due to the limited power within our study. These analyses will be important for future investigations using larger sample sizes.

The mechanisms by which rare variants in SVD-associated genes – such as *HTRA1* and *NOTCH3* – may contribute to cerebrovascular injury in neurodegenerative disease patients and how that injury may influence progression of neurodegeneration remain to be determined. Yet, our findings highlight the importance of comprehensive genetic study in understanding the development of SVD markers and the potential contribution to neurodegenerative disease, particularly in light of recent literature highlighting the value of clinical genetic testing in cases of cerebral SVD and neurological phenotypes that present with features of SVD. Our results suggest that variants in these genes indeed alter the burden of MRI-based SVD markers in a variety of neurodegenerative disease patients and demonstrate that potential downstream effects on clinical correlates, pathogenesis, and disease progression must be further explored.

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CONFLICT OF INTEREST STATEMENT

The authors report no competing interests. Author disclosures are available in the supporting information.

CONSENT STATEMENT

All study participants provided written, informed consent.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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