COL4A2 is associated with lacunar ischemic stroke and deep ICH

Meta-analyses among 21,500 cases and 40,600 controls

Kristiina Rannikmäe, MD, PhD
Vhinoth Sivakumaran
Henry Millar
Rainer Malik, PhD
Christopher D. Anderson, MD, MMSc
Mike Chong, MSc
Tushar Dave, MS
Guido J. Falcone, MD, ScD, MPH
Israel Fernandez-Cadenas, PhD
Jordi Jimenez-Conde, MD, PhD
Arne Lindgren, MD, PhD
Joan Montaner, MD, PhD
Martin O'Donnell, MD, PhD, MRCPI, BCh, BAO
Guillaume Paré, MD, MSc, FRCPC
Farid Radmanesh, MD, MPH
Natalia S. Rost, MD, MPH
Agnieszka Slowik, MD, PhD
Martin Söderholm, MD
Matthew Traylor, PhD
Sara L. Pulit, PhD
Sudha Seshadri, MD
Brad B. Worrall, MD, MSc
Daniel Woo, MD
Hugh S. Markus, DM, FRCP
Braxton D. Mitchell, MPH, PhD
Martin Dichgans, MD, PhD
Jonathan Rosand, MD, MsC

Author list continued on next page

ABSTRACT

Objective: To determine whether common variants in familial cerebral small vessel disease (SVD) genes confer risk of sporadic cerebral SVD.

Methods: We meta-analyzed genotype data from individuals of European ancestry to determine associations of common single nucleotide polymorphisms (SNPs) in 6 familial cerebral SVD genes (*COL4A1*, *COL4A2*, *NOTCH3*, *HTRA1*, *TREX1*, and *CECR1*) with intracerebral hemorrhage (ICH) (deep, lobar, all; 1,878 cases, 2,830 controls) and ischemic stroke (IS) (lacunar, cardioembolic, large vessel disease, all; 19,569 cases, 37,853 controls). We applied data quality filters and set statistical significance thresholds accounting for linkage disequilibrium and multiple testing.

Results: A locus in *COL4A2* was associated (significance threshold $p < 3.5 \times 10^{-4}$) with both lacunar IS (lead SNP rs9515201: odds ratio [OR] 1.17, 95% confidence interval [CI] 1.11–1.24, $p = 6.62 \times 10^{-8}$) and deep ICH (lead SNP rs4771674: OR 1.28, 95% CI 1.13–1.44, $p = 5.76 \times 10^{-5}$). A SNP in *HTRA1* was associated (significance threshold $p < 5.5 \times 10^{-4}$) with lacunar IS (rs79043147: OR 1.23, 95% CI 1.10–1.37, $p = 1.90 \times 10^{-4}$) and less robustly with deep ICH. There was no clear evidence for association of common variants in either *COL4A2* or *HTRA1* with non-SVD strokes or in any of the other genes with any stroke phenotype.

Conclusions: These results provide evidence of shared genetic determinants and suggest common pathophysiologic mechanisms of distinct ischemic and hemorrhagic cerebral SVD stroke phenotypes, offering new insights into the causal mechanisms of cerebral SVD. ***Neurology*® 2017;89:1829–1839**

GLOSSARY

CI = confidence interval; **eQTL** = expression quantitative trait loci; **GWS** = genome-wide association study; **ICH** = intracerebral hemorrhage; **ISGC** = International Stroke Genetics Consortium; **LD** = linkage disequilibrium; **OR** = odds ratio; **SVD** = small vessel disease; **TOAST** = Trial of Org 10172 in Acute Stroke Treatment; **WMH** = white matter hyperintensities.

Small vessel diseases (SVDs) of the brain include a subtype that affects the small, deep, penetrating arteries and arterioles in the brain, hereafter referred to as deep cerebral SVD. This deep cerebral SVD is thought to be responsible for most symptomatic lacunar ischemic strokes and deep intracerebral hemorrhages (ICHs), as well as for substantial cognitive and physical disabilities,

From the Centre for Clinical Brain Sciences (K.R., C.L.M.S.), College of Medicine and Veterinary Medicine (V.S., H.M.), and Institute for Genetics and Molecular Medicine (C.L.M.S.), University of Edinburgh, UK; Institute for Stroke and Dementia Research (R.M., M.D.), Klinikum der Universität München, Munich, Germany; Center for Human Genetic Research (C.D.A., F.R., J.R.) and J. Philip Kistler Stroke Research Center (C.D.A., F.R., N.S.R., J.R.) and Division of Neurocritical Care and Emergency Neurology (C.D.A., F.R., J.R.), Department of Neurology, Massachusetts General Hospital, Boston; Program in Medical and Population Genetics (C.D.A., F.R., J.R.), Broad Institute, Cambridge, MA; Population Health Research Institute (M.C., M.O., G.P.), McMaster University, Hamilton Health Sciences Centre, Ontario, Canada; Department of Medicine (T.D., B.D.M.), University of Maryland School of Medicine, Baltimore; Division of Neurocritical Care and Emergency Neurology (G.J.F.), Department of Neurology, Yale University School of Medicine, New Haven, CT; Stroke Pharmacogenomics and Genetics (I.F.-C.), Fundació Docència i Recerca Mutua Terrassa, Mutua de Terrassa Hospital; Neurovascular Research Unit (J.J.-C.), Department of Neurology, and Program in Inflammation and Cardiovascular Disorders (J.J.-C.), Institut Municipal d'InvestigacioMedica–Hospital del Mar, Universitat Autonoma de Barcelona, Spain; Department of Clinical Sciences Lund (A.L.), Neurology, Lund University; Department of Neurology and Rehabilitation Medicine (A.L., M.S.), Neurology, Skåne University Hospital, Lund, Sweden; Neurovascular Research Laboratory and Neurovascular Unit (J.M.), Institut de Recerca, Hospital Vall d'Hebron, Universitat Autonoma de Barcelona, Spain; HRB Clinical Research Facility (M.O.), NUI Galway, and University Hospital Galway, Ireland; Department of Neurology (A.S.), Jagiellonian University Medical College, Krakow, Poland; Cardiovascular Epidemiology Research Group (M.S.), Department of Clinical Sciences, Lund University, Malmö, Sweden; Department of Clinical Neurosciences (M.T., H.S.M.), University of Cambridge, UK; Department of Neurology (S.L.P.), Brain Centre Rudolf Magnus, University Medical Center Utrecht, the Netherlands; Boston University Schools of Medicine and Public Health (S.S.); Framingham Heart Study (S.S.), Framingham, MA; Departments of Neurology and Public Health Sciences (B.B.W.), University of Virginia, Charlottesville; Department of Neurology (D.W.), University of Cincinnati College of Medicine, OH; Geriatrics Research and Education Clinical Center (B.D.M.), Baltimore Veterans Administration Medical Center, MD; and Munich Cluster for Systems Neurology (SyNergy) (M.D.), Germany.

Coinvestigators are listed at Neurology.org.

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Cathie L.M. Sudlow, BMBCh, MSc, DPhil, FRCPE
On behalf of the Stroke Genetics Network (SiGN), METASTROKE Collaboration, and International Stroke Genetics Consortium (ISGC)

Correspondence to Dr. Sudlow: cathie.sudlow@ed.ac.uk

Supplemental data at Neurology.org

and to be a major pathologic substrate for brain MRI features, including white matter hyperintensities (WMH) and brain microbleeds.[1,2] Increasing evidence supports a distinct vascular pathology for deep cerebral SVD, but our knowledge of the underlying genes and pathophysiologic mechanisms is limited, and specific treatment strategies are lacking.[1]

While the genetic determinants of common sporadic forms of cerebral SVD remain largely unknown, mutations in at least 6 genes (*COL4A1*, *COL4A2*, *HTRA1*, *CECR1*, *NOTCH3*, *TREX1*) are known to cause rare familial forms of deep cerebral SVD.[3,4] Such genes may also contain variants conferring risk for sporadic deep cerebral SVD. We previously investigated associations of common variation in the *COL4A1* and *COL4A2* genes with cerebrovascular phenotypes in a collaborative meta-analysis, demonstrating an association between an intronic *COL4A2* locus and sporadic deep ICH, and a suggestive association with other deep cerebral SVD phenotypes (lacunar ischemic stroke and WMH).[5] The same genetic locus has since been shown to be associated with WMH at genome-wide association study (GWAS) levels of significance.[6]

We aimed to extend this promising candidate gene approach to assess associations of common variants in all currently known familial deep cerebral SVD genes with stroke and its subtypes, investigating the hypothesis that associations would be specific to the 2 key sporadic deep cerebral SVD stroke phenotypes, lacunar ischemic stroke and deep ICH. We were able to take advantage of the increased sample sizes and more densely imputed genotype data now available through the International Stroke Genetics Consortium (ISGC) (http://www.strokegenetics.org/) and associated collaborative groups.

METHODS

Identification of participating studies. We identified most currently available large GWASs of stroke and stroke subtypes in individuals of European ancestry using a network of collaborations associated with the ISGC.[7–11] The entire dataset comprised 20 case-control collections including 19,569 ischemic stroke cases and 37,853 controls, with information on Trial of Org 10172 in Acute Stroke Treatment (TOAST) subtypes (lacunar ischemic stroke, large vessel disease [LVD], cardioembolic),[12] and 5 case-control collections including 1,878 ICH cases and 2,830 controls, with information on the main ICH subtypes (table 1). For the majority of case collections, population-matched controls were recruited from studies with existing genotyping data (details of case-control collections found in references 7–11).

Individual studies applied quality control measures before providing the data. All data were imputed with the 1000 Genomes Phase 1 reference dataset (or to a merged reference panel including the Genome of the Netherlands) using IMPUTE2 or MACH software[13] and provided with reference to Human Genome reference build 19.

Data collection. We collected genotype summary statistics from participating case-control collections for the *COL4A1*, *COL4A2*, *HTRA1*, *CECR1*, *NOTCH3*, and *TREX1* genes (encompassing all known familial deep cerebral SVD genes), including a 10-kbp upstream and downstream flanking region for each gene (table 2).

We focused on the lacunar ischemic stroke and deep ICH phenotypes, but we also assessed LVD, cardioembolic and all ischemic stroke for ischemic stroke case-control collections, and lobar ICH and all ICH for ICH case-control collections to show specificity of the association. For each of these phenotypes, we collected summary data from each collection for all directly genotyped or imputed SNPs in genes of interest: SNP reference number and position, allele frequencies, association effect size (β coefficient) and its standard error, association *p* value, and imputation quality measure and value.

Data analysis. ***Setting the significance threshold.*** To allow multiple testing while accounting for linkage disequilibrium (LD) between SNPs, we calculated significance *p* values for each gene using a modified version of the Nyholt method (MeffLi), which controls accurately for error rate in evaluations of real and simulated data.[14–17] We used the 1000 Genomes CEU dataset[18] SNP genotype information to calculate *p* values for 5 genomic regions, treating the *COL4A1* and *COL4A2* genes as 1 region because they are located in tandem on chromosome 13q34 and share a promoter (table 2).

Pre–meta-analysis data filtering. We further filtered the data to include only SNPs with the following attributes: (1) imputation quality ≥0.3 from MACH, IMPUTE2, or SNPTEST (because SNPs with very poor imputation quality may yield unreliable associations); (2) minor allele frequency ≥1% (because we were investigating common SNPs); (3) absolute β value <100,000 (because higher β values would generate implausible odds ratios [ORs], suggesting unreliable associations); and (4) biallelic SNPs (because the meta-analyses program could not process multiallelic SNPs).

Meta-analyses of COL4A1, COL4A2, HTRA1, CECR1, NOTCH3, and TREX1 SNPs for each phenotype. We meta-analyzed genotype summary data from each contributing case-control collection. We assessed associations of *COL4A1*, *COL4A2*, *HTRA1*, *CECR1*, *NOTCH3*, and *TREX1* SNPs with each of the stroke phenotypes available, both those assumed to represent deep cerebral SVD specifically (lacunar ischemic stroke, deep ICH) and others (LVD ischemic stroke, cardioembolic ischemic stroke, all ischemic stroke, lobar ICH, all ICH). Our hypothesis was that associations would be specific to (or at least strongest with) deep cerebral SVD phenotypes. We used a fixed-effects inverse-variance–based model in METAL genetic meta-analysis software, weighting the β coefficients by their estimated standard errors and generating, for each SNP, the OR per additional minor allele for being a case vs a control.[19]

Post–meta-analysis data filtering. After the meta-analyses, we considered SNPs to be associated with the respective

Table 1 Participating case-control collections

Ischemic stroke								
Studies forming case-control collections[a]	Cases, n	Controls, n	CE cases, n	LVD cases, n	SVD cases, n	Mean age cases, y	Mean age controls, y	Genotyping panel
ASGC	1,162	1,244	240	421	310	73	70	Illumina 610
BRAINS	371	2,640	78	40	29	74	≥65	Illumina 610
GASROS Illumina	296	377	106	68	24	67	48	Illumina
GASROS Affy	485	3,030	198	102	59	69	51	Affymetrix
GEOS	448	498	90	37	54	41	40	Illumina H Omni
HPS	588	571	—	—	—	65	59	Illumina 610
ISGS/SWISS	1,014	1,370	235	217	187	67	65	Illumina 550/610/660
Milano	366	407	64	73	25	57	51	Illumina 610/660
WHI	306	2,170	42	31	81	59	52	Illumina 1M
VISP	1,723	1,047	—	—	—	61	63	Illumina 5M
WTCCC2 Germany	1,174	797	346	330	106	67	63	Illumina 660
WTCCC2 UK	2,374	5,175	460	498	474	72	52[b]	Illumina 660
BRAINS, ISGS, GASROS, SWISS, HABC	754	1,586	176	163	149	67	74	Illumina 650Q, 610, 1M
CIDR,[c] HRS, OAI	3,291	11,514	598	423	761	69	67	Illumina 5M, 2.5M, HumanOmni, 5Exome-4v1
Krakow	878	716	407	173	36	69	56	Illumina 5M
LSGS	459	453	157	75	55	67	56	Illumina 5M
BASICMAR, ADHD, INMA	868	1,218	408	184	276	75	—	Illumina 5M, 1M
Graz	607	815	166	85	74	69	65	Illumina 610, 5M
SAHLSIS, LSR, MDC	1,579	1,362	223	150	183	62	61	Illumina 5M, 610
Interstroke	826	863	208	188	243	68	66	Illumina Human Exome Chip, Cardiometabochip
Total[d]	19,569	37,853	4,202	3,258	3,126			—

Intracerebral hemorrhage							
Case-control collections[a]	Cases (n)	Controls (n)	Lobar ICH[f] cases (n)	Deep ICH[g] cases (n)	Mean age cases	Mean age controls	Genotyping panel
GOCHA	387	387	210	167	72	72	Illumina HumanHap 610 Quad
ISGC	528	530	181	313	72	66	Illumina HumanHap 610 Quad
GERFHS	628	573	258	370	68	67	Affymetrix 6.0
MDC	199	372	76	95	62	58	Human OmniExpress Exome Bead Chip v 1.0

Continued

Table 1 Continued

Intracerebral hemorrhage						
Case-control collections[a]	Cases (n)	Controls (n)	Lobar ICH[f] cases (n)	Deep ICH[g] cases (n)	Mean age cases	Mean age controls	Genotyping panel
Cambridge	136	968	59	77	71	60	Illumina HumanCoreExome
Total[e]	1,878	2,830	784	1,022			—

Abbreviations: ADHD = attention-deficit/hyperactivity disorder; ASGC = Australian Stroke Genetics Collaborative; BASICMAR = Base de Datos de Ictus del Hospital del Mar; BRAINS = Bio-Repository of DNA in stroke; CE = cardioembolic; CIDR = Center for Inherited Disease Research; GASROS = The MGH Genes Affecting Stroke Risk and Outcome Study; GEOS = Genetics of Early-Onset Stroke; GERFHS = Genetic and Environmental Risk Factors for Hemorrhagic Stroke; GOCHA = Genetics of Cerebral Hemorrhage with Anticoagulation; HABC = Health ABC; HPS = Heart Protection Study; HRS = Health and Retirement Study; ICH = intracerebral hemorrhage; INMA = Infancia y medio ambiente; ISGC = International Stroke Genetics Consortium; ISGS/SWISS = The Ischemic Stroke Genetics Study/Sibling With Ischaemic Stroke Study; LSGS = Leuven Stroke Genetics Study; LSR = Lund Stroke Register; LVD = large vessel disease; MDC = Malmö Diet and Cancer Study; OAI = Osteoarthritis Initiative; SAHLSIS = Sahlgrenska Academy Study of Ischemic Stroke; VISP = The Vitamin Intervention for Stroke Prevention Trial; WHI = The Women's Health Initiative; WTCCC2 = The Wellcome Trust Case-Control Consortium.

[a] Case-control collections were analyzed as individual studies and/or as groups of studies, depending on how data were provided. When the same study appears more than once in this table, nonoverlapping sets of cases were included.

[b] Approximate age at genotyping of the 2,738 controls from the 1958 Birth Cohort; age unavailable for the remaining controls.

[c] Samples genotyped in the CIDR: BRAINS, GASROS, Greater Cincinnati/Northern Kentucky Stroke Study, ISGS, Middlesex County Ischemic Stroke Study, Miami Stroke Registry and Biorepository, Nurses' Health Study, Northern Manhattan Study, Reasons for Geographic and Racial Differences in Stroke, Secondary Prevention of Small Subcortical Strokes, SWISS, WHI, and Washington University St. Louis.

[d] Further information in references 7 through 9.

[e] Further information in references 10 and 11.

[f] Lobar ICH defined as involving predominantly the cortex and underlying white matter.

[g] Deep ICH defined as ICH involving predominantly the basal ganglia, periventricular white matter, or internal capsule and infratentorial ICH.

phenotype if the relevant associations passed the relevant modified Nyholt-corrected p threshold, were based on data from ≥50% of cases contributing to the analyses, and did not demonstrate substantial heterogeneity (requiring $I^2 < 50\%$ and $p > 0.001$ from χ^2 test).

We chose our filtering thresholds from those most commonly used and accepted[6–8,20] with the aim of ensuring that any associations deemed significant would be based on SNPs with sufficient, reliable, and consistent data.

Further exploration of associated SNPs. When >1 SNP in the same gene was associated with any given phenotype, we used the 1000 Genomes project CEU population data to investigate the LD between the lead SNP (with the lowest p value) and all other associated SNPs for the relevant gene-phenotype association. SNPs in moderate or strong LD (defined respectively as r^2 and/or D′ ≥0.6 or ≥0.8) with the lead SNP were considered likely to represent a signal from the same locus.[21]

We examined associations of all lead SNPs across all case-control collections included in the respective meta-analyses and of all lead SNPs with all other phenotypes, comparing findings for deep cerebral SVD stroke phenotypes and non-SVD stroke phenotypes.

Finally, we sought functional annotation data from the Haploreg version 2 database (http://www.broadinstitute.org/mammals/haploreg/haploreg.php),[22] genotype-tissue expression portal expression quantitative trait loci (eQTL) browser (http://www.broadinstitute.org/gtex/), and the RegulomeDB database (http://regulome.stanford.edu/) for all associated SNPs.

RESULTS

Meta-analyses of *COL4A1*, *COL4A2*, *HTRA1*, *CECR1*, *NOTCH3*, and *TREX1* SNPs for each phenotype. Modified Nyholt significance thresholds for the 5 genomic regions are shown in table 2. Using our preset data filtering criteria, we found associations of 18 SNPs in *COL4A2* with lacunar ischemic stroke, 9 SNPs in *COL4A2* with deep ICH, and 1 SNP in *HTRA1* with lacunar ischemic stroke (table 3 and figure 1). Two of the SNPs in the *COL4A2* gene (rs4771674 and rs9515199) were associated with both lacunar ischemic stroke and deep ICH. There were no associations of common SNPs in *COL4A2* or *HTRA1* with any of the noncerebral SVD or combined stroke phenotypes or of common variants in *COL4A1*, *CECR1*, *NOTCH3*, or *TREX1* with any of the stroke phenotypes.

Further exploration of associated SNPs. ***LD between COL4A2 SNPs.*** The lead SNPs were rs9515201 for lacunar ischemic stroke (OR per additional A allele 1.17, 95% confidence interval [CI] 1.11–1.24, $p = 6.62 \times 10^{-8}$) and rs4771674 for deep ICH (OR per additional A allele 1.28, 95% CI 1.13–1.44, $p = 5.76 \times 10^{-5}$). These 2 lead SNPs are in strong LD with each other ($r^2 = 0.9$/D′ = 1), suggesting that this represents the same genetic signal.

We investigated the LD between the lead SNP rs9515201 (most strongly associated SNP in the locus) and all other associated SNPs in *COL4A2*. Of the other 24 SNPs in *COL4A2* associated with lacunar ischemic stroke and/or deep ICH, 19 were in

Table 2 Six genes assessed: Location, number of SNPs, and Nyholt association p values regarded as significant

Gene/genomic region	Chromosome	Coordinates[a]	SNPs		Nyholt p value
			n_{all}	n_{eff}	
COL4A1/COL4A2	13	110,791,310-111,175,373	2,555	147	3.5×10^{-4}
HTRA1	10	124,211,041-124,284,424	417	93	5.5×10^{-4}
CECR1	22	17,650,192-17,712,738	422	97	5.3×10^{-4}
NOTCH3	19	15,260,444-15,321,792	278	72	7.1×10^{-4}
TREX1	3	48,491,186-48,519,044	60	38	1.3×10^{-3}

Abbreviation: SNP = single nucleotide polymorphism.
[a] Based on the Human Genome reference build 19.

moderate to strong LD with the lead SNP, suggesting that their signal may be from the same *COL4A2* locus. The remaining 4 SNPs showed minimal LD with the lead SNP, while data for 1 SNP were not available, suggesting that there might possibly be additional relevant loci (table e-1 at Neurology.org).

Associations across individual case-control collections in the meta-analyses. The associations for *COL4A2* SNPs showed minimal to moderate heterogeneity across individual case-control collections in the lacunar ischemic stroke and deep ICH meta-analyses (I^2 = 0%–49%, heterogeneity p = 0.7–0.01; and I^2 = 0%–42%, heterogeneity p = 0.1–0.5 respectively), while the associations across individual collections for rs79043147 in *HTRA1* in the lacunar ischemic stroke meta-analysis showed only minimal heterogeneity (I^2 = 4%, heterogeneity p = 0.41), suggesting consistent results (figure e-1). All imputed SNPs showed a good imputation quality of >0.7.

Associations with other phenotypes of the lead COL4A2 SNPs. Figure 2 shows association results for the lead *COL4A2* SNPs (rs9515201 and rs4771674) associated with lacunar ischemic stroke and deep ICH across all 7 phenotypes assessed. Although rs9515201 was associated only with lacunar ischemic stroke (OR 1.17, 95% CI 1.11–1.24, $p = 6.62 \times 10^{-8}$), there was a suggestive association of similar magnitude with deep ICH (OR 1.21, 95% CI 1.07–1.37, $p = 2.15 \times 10^{-3}$). Rs4771674 was associated with both cerebral SVD phenotypes: lacunar ischemic stroke (OR 1.14, 95% CI 1.07–2.10, $p = 1.6 \times 10^{-5}$) and deep ICH (OR 1.28, 95% CI 1.13–1.44, $p = 5.76 \times 10^{-5}$).

There were no associations with non-SVD stroke or combined SVD and non-SVD phenotypes. ORs for the all ischemic stroke and all ICH phenotypes were intermediate between those for SVD and those for non-SVD phenotypes, suggesting that associations with these combined phenotypes were driven by results for lacunar ischemic stroke and deep ICH.

Associations with other cerebrovascular phenotypes of the HTRA1 SNP. Rs79043147 was associated only with lacunar ischemic stroke (OR 1.23, 95% CI 1.10–1.37, $p = 1.90 \times 10^{-4}$) but also showed a suggestive association with deep ICH (OR 1.56, 95% CI 1.24–1.97, $p = 1.71 \times 10^{-4}$) (figure 2). In fact, the p value for deep ICH passed the significance threshold, but the SNP did not pass our preset heterogeneity filter and was therefore not considered associated overall. There were no associations with non-SVD stroke or combined SVD and non-SVD phenotypes.

Functional annotation. All *COL4A2* and *HTRA1* SNPs associated with lacunar ischemic stroke or deep ICH were intronic. The GTEx eQTL browser search revealed no significant eQTLs for any of these SNPs. However, the RegulomeDB database revealed that 2 *COL4A2* SNPs were in an area likely to affect binding, 2 *COL4A2* SNP were in an area less likely to affect binding, and 17 *COL4A2* SNP showed minimal binding evidence, suggesting that these SNPs are located in areas of the genome that may have regulatory functions (table e-2).

DISCUSSION Our results demonstrate an association of an intronic, possibly regulatory locus in *COL4A2* with 2 distinct deep cerebral SVD phenotypes, lacunar ischemic stroke and deep ICH. We also found an association of deep cerebral SVD with *HTRA1*, demonstrating an association with lacunar ischemic stroke and a suggestive association with deep ICH. Finding the same genetic signal associated with both ischemic and hemorrhagic sporadic stroke confirms the usefulness of a joint exploration of cerebrovascular phenotypes and the potential for genetic studies to shed light on common underlying mechanisms.

Our findings for *COL4A2* are supported by previous work showing the relevance of this genomic region in sporadic deep cerebral SVD. A sequence analysis of *COL4A1/COL4A2* found missense mutations in sporadic ICH cases.[23,24] In addition, our

Table 3 All associated SNPs passing post-meta-analysis filters

SNP	Minor allele	Major allele	Minor allele frequency	OR (95% CI)[a]	Association *p* value	Direction of effect[b]	Cases, %[c]	I^2	Heterogeneity *p* value
Lacunar ischemic stroke: *COL4A2* (Nyholt significance *p* threshold 3.5×10^{-4})									
rs9515201	A	C	0.31	1.17 (1.11-1.24)	6.62×10^{-8}	+++++++−++++++−−+	100	48	0.01
rs4771674	A	G	0.39	1.14 (1.07-1.2)	1.60×10^{-5}	+++++++−++−+?++−−+	92	49	0.01
rs113696651	T	C	0.02	1.61 (1.29-2.01)	2.25×10^{-5}	++−−+++?−+++++?+++	98	29	0.13
rs9521729	A	G	0.31	0.88 (0.83-0.94)	5.42×10^{-5}	−−−−+−−−+−−?++−−−	92	11	0.32
rs9515199	C	T	0.39	1.13 (1.06-1.19)	5.47×10^{-5}	+++++++−++−+?++−−+	92	39	0.05
rs9559771	G	A	0.12	0.84 (0.77-0.91)	6.56×10^{-5}	−−−−+−−−−−−−−−−−−	100	20	0.21
rs7319311	G	A	0.32	0.88 (0.83-0.94)	7.48×10^{-5}	−−−−+−−−−−−−?++−−−	92	0	0.45
rs4502089	C	T	0.27	0.88 (0.83-0.94)	7.49×10^{-5}	−−−−−−+−−−+−−−+++−−	100	17	0.25
rs9521768	T	G	0.27	1.13 (1.06-1.21)	9.09×10^{-5}	++++−++−+−++−++++	100	34	0.08
rs67472641	D[d]	R[d]	0.37	1.16 (1.08-1.26)	9.86×10^{-5}	?????++−+++++?????	57	24	0.24
rs9521770	G	A	0.27	1.13 (1.06-1.2)	1.21×10^{-4}	++++−++−+−++−++++	100	33	0.08
rs9583488	A	G	0.32	0.89 (0.83-0.94)	1.21×10^{-4}	−−−−+−−−−−−?+++−−	92	10	0.34
rs11619583	C	T	0.46	1.12 (1.06-1.18)	1.23×10^{-4}	+++++−++++++−++++	100	36	0.06
rs7320755	C	G	0.32	0.89 (0.84-0.94)	1.53×10^{-4}	−−−−+−−−−−−?0++−−	86	8	0.36
rs77104783	A	C	0.08	0.8 (0.71-0.9)	1.78×10^{-4}	−−−−−+−−−+−−?−−−−−	92	0	0.66
rs9515198	T	C	0.28	0.89 (0.83-0.94)	2.00×10^{-4}	−−−−+−−−−+−−?+++−−	92	13	0.30
rs7140030	A	G	0.25	1.12 (1.06-1.2)	2.36×10^{-4}	++++++++++−+++−+−+	100	36	0.06
rs9588148	T	G	0.21	0.88 (0.83-0.94)	3.19×10^{-4}	−−−−−−−−−−−−++−−−	100	3	0.41
Deep ICH: *COL4A2* (Nyholt significance *p* threshold 3.5×10^{-4})									
rs4771674	A	G	0.4	1.28 (1.13-1.44)	5.76×10^{-5}	−++++	100	27	0.24
rs9521733	C	T	0.4	1.27 (1.13-1.42)	8.10×10^{-5}	++++−	100	0	0.48
rs9521735	C	G	0.41	1.27 (1.13-1.43)	8.91×10^{-5}	++++−	100	0	0.51
rs9515200	C	G	0.39	1.27 (1.13-1.43)	9.36×10^{-5}	++++−	100	26	0.25
rs9521732	A	C	0.41	1.26 (1.12-1.42)	9.89×10^{-5}	++++−	100	0	0.45
rs9521734	T	A	0.4	1.26 (1.12-1.42)	1.01×10^{-4}	++++−	100	0	0.52
rs1999013	G	A	0.41	1.27 (1.12-1.43)	1.07×10^{-4}	−+++−	100	38	0.17

Continued

Table 3 Continued

SNP	Minor allele	Major allele	Minor allele frequency	OR (95% CI)[a]	Association *p* value	Direction of effect[b]	Cases, %[c]	I^2	Heterogeneity *p* value
rs61963197	A	G	0.37	1.28 (1.13-1.45)	1.15×10^{-4}	++++−	100	41	0.15
rs9515199	C	T	0.41	1.24 (1.11-1.4)	2.74×10^{-4}	−+++−	100	42	0.14
Lacunar ischemic stroke: *HTRA1* (Nyholt significance *p* threshold 5.5×10^{-4})									
rs79043147	T	C	0.06	1.23 (1.10-1.37)	1.91×10^{-4}	+−−−++−−+++++−++++	100	4	0.41

Abbreviations: CI = confidence interval; ICH = intracerebral hemorrhage; OR = odds ratio; SNP = single nucleotide polymorphism.
[a] OR for minor allele being the effect allele.
[b] Direction of effect shows direction of association in each case-control collection included in the meta-analyses: + (OR > 1), − (OR < 1), ? (no data), or 0 (OR = 1).
[c] Percent of overall cases contributing data to the meta-analysis.
[d] D (deletion)/R (regular) SNP (D = G, R = GGCCTGAGAAGCGACAGGGCA).

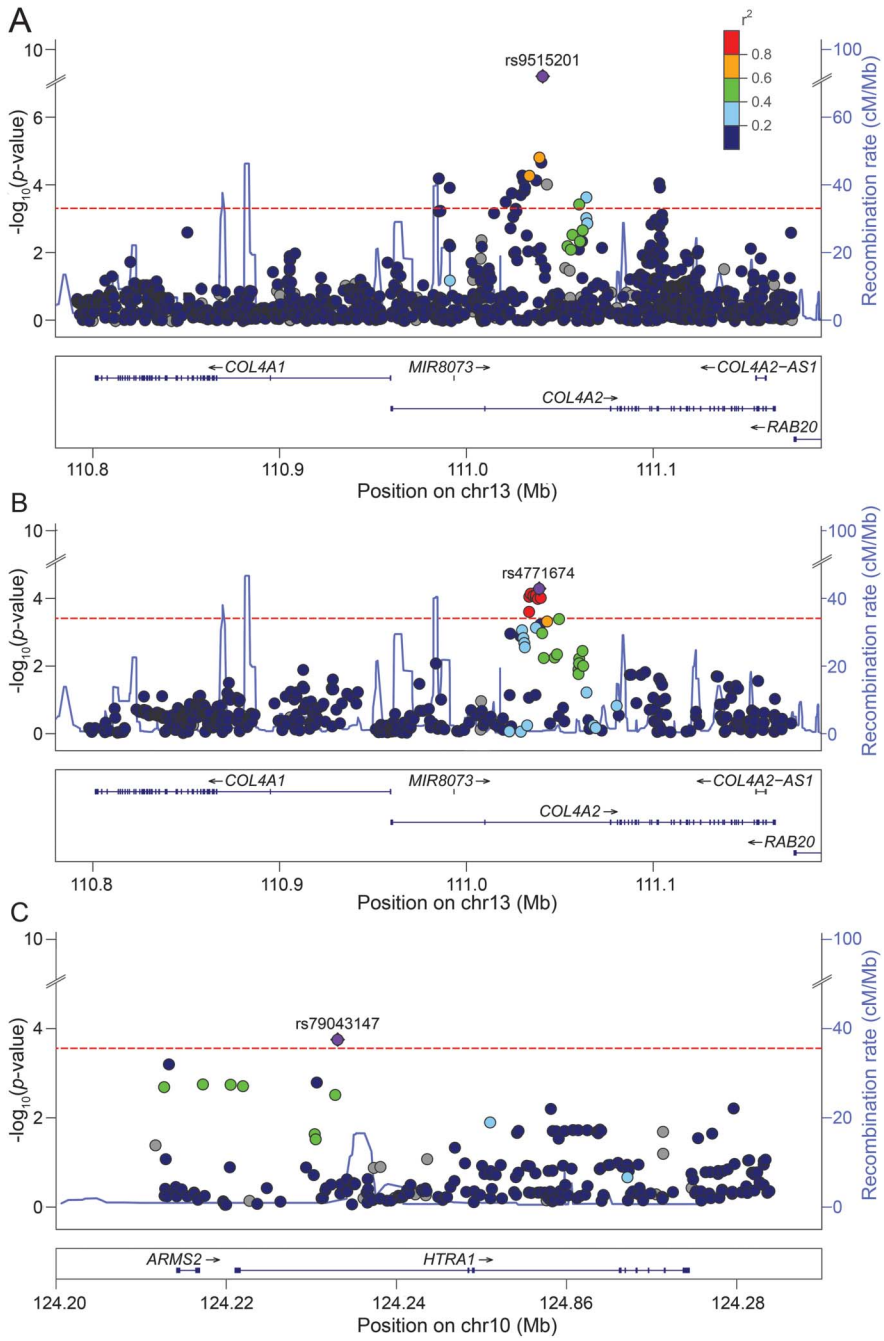
previous meta-analyses in a smaller, partly overlapping sample already demonstrated an association of this *COL4A2* locus with deep ICH and a suggestive association with other cerebral SVD phenotypes.[5] By increasing the sample size (by 40% for lacunar ischemic stroke and by 15% for deep ICH) and density of coverage in the present study, we have now established a substantial association of the same locus with lacunar ischemic stroke and confirmed the association with deep ICH. Furthermore, a recently published GWAS identified our lead SNP for lacunar ischemic stroke to be associated with another deep cerebral SVD phenotype, WMH.[6]

While the association with *COL4A2* is supported by previous data and a convincing signal for both ischemic and hemorrhagic phenotypes, the association with *HTRA1* is suggestive but less certain. On the basis of our *p* threshold, the *HTRA1* SNP was associated with both lacunar ischemic stroke and deep ICH, but there was significant heterogeneity in the deep ICH meta-analyses. We are also aware of a previous report suggesting an association of rare variation in *HTRA1* with more extreme sporadic deep cerebral SVD phenotypes.[25] Thus, this finding should be pursued in independent, large samples to replicate the association.

From a biological point of view, our strategy of investigating these familial genes jointly is supported by an emerging view that the resulting familial deep cerebral SVDs have similar disease mechanisms involving disruption of the cerebrovascular matrisome. Familial mutations leading to alterations of matrisome proteins and function could be a convergent pathway driving the functional and structural alterations of small brain vessels and disease manifestations, and similar mechanisms could play a role in sporadic disease.[26]

COL4A1 and *COL4A2* genes encode the collagen protein chains, a major component of the vascular basement membrane.[27] Their dominant missense mutations are associated with basement membrane defects and endoplasmic reticulum stress and cause rare familial SVDs.[23,28–30] Recent data suggest that manipulation of endoplasmic reticulum stress (e.g., with 4-phenyl butyric acid) is a potential therapeutic option for collagen IV diseases, including hemorrhagic stroke.[28] Mutations in *HTRA1* gene are associated with cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy.[31] While the majority of *HTRA1* mutations cause this autosomal recessive cerebral SVD, heterozygous *HTRA1* mutations associated with cerebral SVD have also recently been reported.[32] *HTRA1* encodes the HTRA1 enzyme, which, through regulating transforming growth factor-β signaling, plays an important role in the formation of blood vessels.

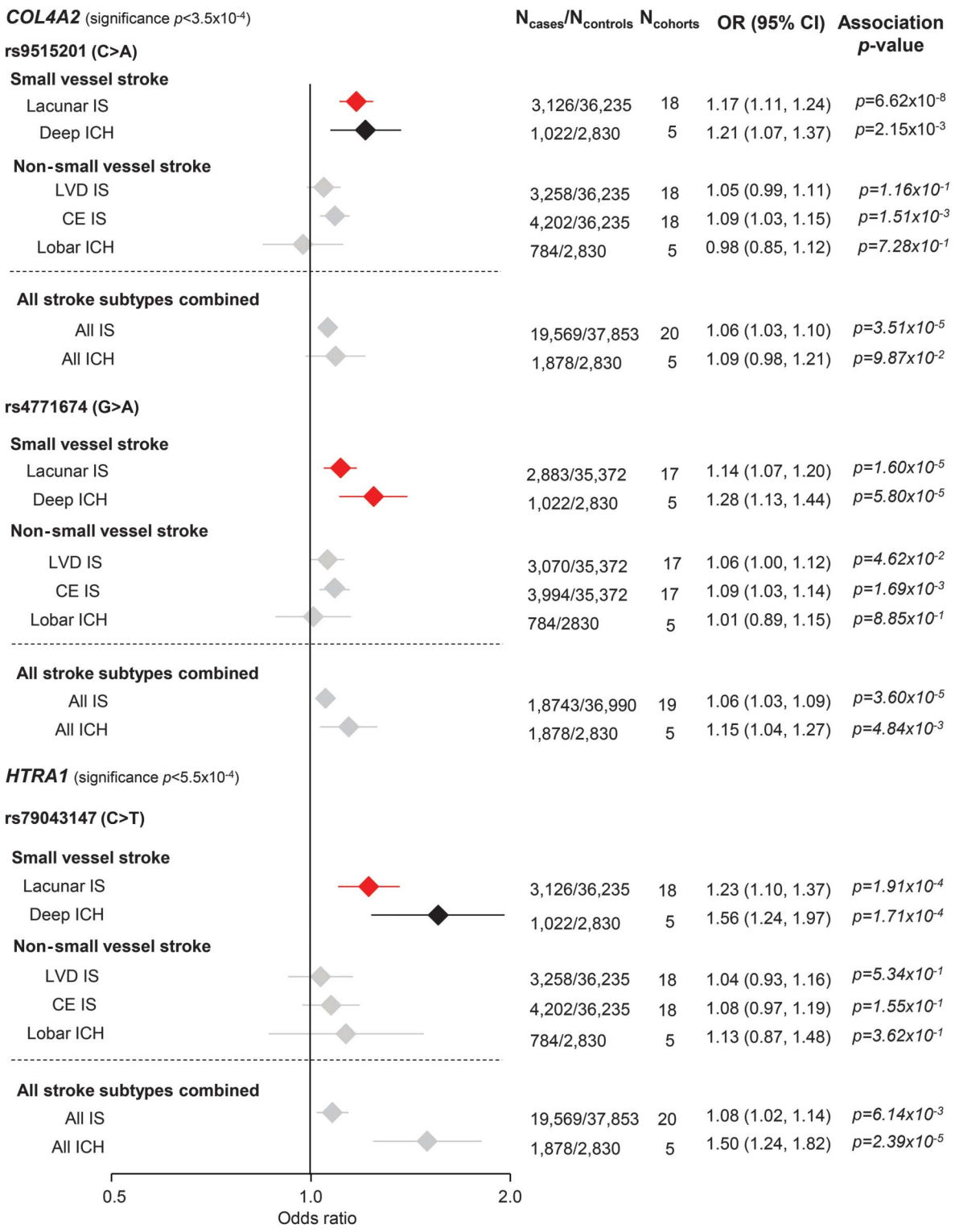
Figure 1 ***COL4A2*** **regional association plots for (A) lacunar ischemic stroke, (B) deep ICH, and (C)** ***HTRA1*** **regional association plot for lacunar ischemic stroke**



Only SNPs passing the post-meta-analysis filters (heterogeneity $I^2 < 50\%$, $p > 0.001$, ≥50% cases contributing data) are displayed. Red dashed lines mark the relevant Nyholt significance *p* thresholds. Dots mark individual SNPs with respect to their chromosomal position (x-axis) and *p* value for association between each SNP and phenotype (left y-axis). The SNP in purple is the most strongly associated (lead) SNP; linkage disequilibrium with this lead SNP determines the colors for other SNPs, as seen from the r^2 color coding on figure. Recombination rates (right y-axis), shown by the continuous blue lines, are measured as frequency of exchange per unit physical distance (centimorgan [cM]/mega base pair [Mb]). ICH = intracerebral hemorrhage; SNP = single nucleotide polymorphism.

Possible reasons for an apparent lack of an association with *COL4A1*, *CECR1*, *NOTCH3*, and *TREX1* include a genuine lack of association in our study population; weaker association not detected because of sample size; suboptimal diagnosis of SVD phenotypes in the original studies, resulting in reduced power; and variability in the density and quality of genotyping across different genes. In addition, we used a 10-kbp flanking region to cover regulatory areas for all genes; therefore, relatively more conservative *p* values may have been derived for smaller genes (*TREX1*) after adjustment for the number of

Figure 2 Associations of *COL4A2* and *HTRA1* SNPs across all phenotypes



Diamonds represent pooled ORs across all case-control collections for each phenotype, with the line through the diamond showing its 95% CI. Associations significant at relevant Nyholt threshold are shown in red; nonsignificant associations with SVD phenotypes are shown in black; and nonsignificant associations with non-SVD phenotypes are shown in gray. CE = cardioembolic; CI = confidence interval; ICH = intracerebral hemorrhage; IS = ischemic stroke; LVD = large vessel disease; OR = odds ratio; SNP = single nucleotide polymorphism.

SNPs tested. In addition, because we treated the *COL4A1/COL4A2* region as one, more conservative *p* values were derived for the *COL4A1* gene than if we had treated it as a separate region.

Our study has several strengths. We investigated a specific, prespecified hypothesis, clearly defining the phenotypes and candidate genes of interest on the basis of preexisting supporting data. Through a network of collaborative groups, we could include the majority of currently available data from stroke genetics studies of individuals of European ancestry. We used appropriate methods to correct for multiple testing.

There were some limitations. First, while we have shown that SNPs in *COL4A2* are associated with lacunar ischemic stroke and deep ICH through analyzing data for the specific candidate region, the associations did not reach GWAS significance ($p \leq 5 \times 10^{-8}$), most likely because of limited sample size. However, the lead lacunar ischemic stroke SNP (rs9515201) had a value of $p = 6.6 \times 10^{-8}$, and it has been shown that a substantial proportion of SNPs with a *p* value in this "borderline" GWAS significance range ($p > 5 \times 10^{-8}$ and $p \leq 1 \times 10^{-7}$) represent genuine, replicable associations.[33] Second, we did not adjust the statistical threshold for the number of genomic regions and phenotypes investigated, considering this overly conservative because we were investigating a series of specific related hypotheses rather than a single hypothesis. However, had we further adjusted the *COL4A1/COL4A2* region *p* value for the number of tests ($3.5 \times 10^{-4}/25 = 1.4 \times 10^{-5}$), the association for the lead SNP with lacunar ischemic stroke would have remained significant. Third, our analyses found a locus in *COL4A2* containing several SNPs associated with deep cerebral SVD, most (but not all) of which were in moderate to strong LD with the lead SNP. This suggests that the association was likely driven by the lead SNP, but the possibility remains that independently significant signals in the locus may emerge.[34] Further investigation of this would require additional analyses adjusted for the lead SNP, requiring genome-wide genetic data that were not sought for this study, given its targeted hypothesis-driven approach. Fourth, the diagnostic workup leading to TOAST subtype classification was study specific, which may introduce some heterogeneity. Fifth, not all studies controlled for age in their statistical analyses before inclusion in the meta-analyses, and this may decrease the study power. Finally, we were not able to include data for additional relevant phenotypes such as WMH and brain microbleeds in the present study.

While genetic studies of ischemic stroke and ICH have generally been pursued separately, these findings emphasize the mechanistic insights that can be gained from joint analyses of cerebrovascular phenotypes. We have shown that the same genetic signal is associated with clinically evident sporadic ischemic and hemorrhagic stroke, but the joint exploration approach is further supported by previous GWASs showing a locus on chromosome 1q22 to be associated with both deep ICH and WMH.[10,35] In addition, it has recently been shown that a locus on chromosome 6p25, near the *FOXF2* gene (also associated with familial deep SVD), is associated with all stroke (likely driven by SVD stroke phenotypes) and suggestively with WMH.[36]

Follow-up studies should further explore potential common genetic signals for deep cerebrovascular ischemic and hemorrhagic SVD phenotypes in larger sample sizes and for additional relevant phenotypes such as WMH and brain microbleeds and should include non-European ethnic groups. Future studies could also assess the potential contribution of rare variants to common cerebral SVD phenotypes in these mendelian genes. In addition, the robust findings for *COL4A2* now merit further deep sequencing of the entire genomic region among sporadic deep cerebral SVD cases, with detailed functional studies of promising variants thus identified.

AUTHOR CONTRIBUTIONS

K.R., R.M., and C.L.M.S. contributed to the conception and design of the study. K.R., V.S., and H.M. conducted the meta-analyses. K.R., V.S., H.M., R.M., and C.L.M.S. all contributed by drafting a significant portion of the manuscript. K.R., R.M., V.S., H.M., C.D.A., M.C., J.L.P., S.L.P., G.J.F., T.D., I.F.-C., J.J.-C., A.L., J.M., M.O., G.P., F.R., A.S., N.S.R., M.S., M.T., S.S., B.B.W., D.W., H.S.M., B.D.M., M.D., J.R., and C.L.M.S. contributed to the acquisition and analysis of the data. All authors reviewed and approved the final version.

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DISCLOSURE

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